Using DNA metabarcoding to explore spatial and demographic variation in the diet of Hawfinch (*Coccothraustes coccothraustes*) populations

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by

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Summary

Many woodland bird species within Britain have shown population declines over recent years, with unclear reasons for declines. The Hawfinch (*Coccothraustes coccothraustes*) has been declining within the UK since the 1970's, while mainland Europe populations have remained stable. This PhD thesis used DNA metabarcoding and high-throughput sequencing (HTS) of Hawfinch faecal samples to describe plant and invertebrate dietary composition across core Hawfinch population ranges within the UK and mainland Europe. I investigated the degree of dietary composition difference between distinct populations of Hawfinch as well as between demographic groups. This was to elucidate the extent that Hawfinch show dietary plasticity, which can be a determining factor allowing species to adapt to environmental changes. Given the importance of diet, it is important to understand which taxa are preferred, especially for species which are showing population declines. Hawfinch plant dietary composition was analysed to reveal if the frequency of plants detected within their diet differed from their foraging environment, indicating selective foraging.

UK Hawfinch dietary composition of plant (Chapter 2) and invertebrate (Chapter 3) taxa was found to vary spatially and between demographic groups, with mainland Europe populations showing similar patterns (Chapter 4). Analysis of the relative abundance of herbivorous taxa within UK woodlands compared with frequency of detection within Hawfinch diet (Chapter 5) indicated Hawfinch were showing selective foraging and were not consuming certain taxa relative to their availability.

This PhD thesis provides novel insights into the dietary plasticity of a declining species across a broad geographical range. I provide a clear example of how DNA metabarcoding methodologies can be applied in studies of difficult to study species, as well as how dietary composition can be driven by environmental and demographic factors. Finally, this thesis shows that Hawfinch are selective, and are consuming plants disproportionally to their availability.

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Chapter One – General Introduction

A Hawfinch nestling in the hand. All birds were captured, handled and ringed by licensed ringers endorsed by the British Trust for Ornithology (BTO). Photo credit: Andy Stanbury: Hawfinch Ringing Group.

1.1 Background

Global biodiversity is currently undergoing a rapid decline, with many avian species experiencing significant population decreases (Spiller and Dettmers 2019). It has been suggested that 13% of the world's avian species may experience extinction within 50 years, due to broad-scale declines in both bird diversity and abundance recorded across avian groups (Lindenmayer *et al.* 2018; Alderson and Sander 2022). Species which are specialised in declining habitats such as forests and wetlands are deemed more vulnerable to declines, as these habitat specialists, while able to utilise resources more efficiently within their ecological niche, are more vulnerable to the rapid declines seen across these habitat types (Correll *et al.* 2019). Global patterns of species extinctions are, however underpinned by regional and local trends in populations (Inger *et al.* 2015), with detailed studies of regional and local populations vital in order to understand broader biodiversity differences (Lindenmayer *et al.* 2018). This information is critical in order to determine which species are in need of conservation action and, subsequently can be used to implement suitable conservation actions, such as appropriate land use management (Crouzeilles *et al.* 2016) or an expansion of nature reserves (Pringle 2017).

1.1.1 Decline of woodland birds

Many British woodland bird species have shown major range contractions and decreased abundance in recent years (Hewson *et al.* 2007; Alder *et al.* 2018). Drivers behind declines are multi-factorial, and establishing the relative importance of each factor to species specific declines is challenging (Newson *et al.* 2012). Declines in woodland birds have been implicated to a number of factors, from landscape-level simplification through agricultural expansion and urban development (Gregory *et al.* 2007), changes in woodland management, including the ending of practices such as coppicing (Hewson *et al.* 2007) to fine-scale trends in habitat quality through increased pressure from deer browsing (Gill and Fuller 2007). Many declining woodland species do not have adequate ecological information associated with them, resulting in difficulties identifying species specific drivers of population decline (Amar *et al.* 2006).

1.1.2 Landscape simplification

Increasing agricultural and urban development has led to more than 40% of land on Earth being altered, with much of the undisturbed habitat fragmented due to land use changes (Foley *et al.* 2005). The largest terrestrial biomes are now crop and pastureland, and land use change directly associated with agriculture is also associated with the greatest loss of biodiversity worldwide (Tilman *et al.* 2001). Anthropogenically driven landscape transformations are becoming increasingly frequent, resulting in many semi-natural landscape features, including woodlands and hedgerows being altered either through fragmentation, removal or transformation for larger agricultural fields or urban expansion (Ikin *et al.* 2014; Lindenmayer *et al.* 2015; Burns *et al.* 2016; Neumann *et al.* 2016). Bird species dependent on woodland habitats are negatively impacted by fragmentation through factors including edge effects such as increased pesticide exposure and increased variation in micro-climatic variability (Palik and Murphy 1990; Wilkin *et al.* 2006; Bennett *et al.* 2015). In turn, the aforementioned factors can reduce suitability and quality of the remaining habitat, resulting in increased competition, disturbance and predation, as well as differences in food availability (Tew and Hesselberg 2017; Gardner *et al.* 2019; Valentine *et al.* 2019). The level of isolation is highly dependent on the land-use surrounding the habitat fragments, with agricultural environments acting as considerable movement barriers for some woodland species (Biz *et al.* 2017).

Aside from fragmentation of semi-natural habitats, changes in crop types and management can also have an important implication for species. The loss of traditional fruit orchards (Myczko *et al.* 2013; Kirby *et al.* 2015) is a major concern for conserving biodiversity, as orchards provide both grassland and broadleaved woodland supporting rich biodiversity (Herzog 1998; Horak *et al.* 2013; Myczko *et al.* 2013). Orchards are a critical refuge for arthropod and bird species which are endemic in woody habitats (Herzog 1998; Bailey *et al.*

2010). Traditional orchards are utilised by a wide range of organisms due to the combination of open-grown fruit trees, grassland, hedgerow boundaries, resembling ecologically mini-parklands, edge woodland and wood pastures (Natural England 2010). Orchards which contain a mosaic of old fruit trees and associated habitats such as fallen dead wood and ponds are ecologically vital for invertebrates and the species which feed on them (Horak 2014). Despite this, orchards have largely been neglected in favour of monocultures, mainly due to orchards low economic value (Myczko *et al.* 2013). Orchard management has shifted towards intensive management of smaller trees, heavily managed with pesticides, fertilisers and herbicides, resulting in much shorter lifespans than which would be found in natural orchard settings (Goossens *et al.* 2017).

1.1.3 Changes in woodland management

A well studied hypothesis for the decline of woodland birds is changes in woodland management (Hewson and Noble 2009). At the start of World War One, only 5% of woodland within the UK remained (Forestry Commission 2017). By 2016 UK woodland land cover had increased to 13% of the total land area, however only 1.2% is classed as ancient, semi-natural woodland (ASNW) (Forestry Commission 2017). The majority of this ancient semi-natural woodland consists of broadleaved species but also includes Scots pine (*Pinus sylvestris*) forests. Differences in tree composition and age structure over a large spatial scale can influence the bird species communities and population trends of individual species, especially if tree species composition is altered through management (Burgess *et al.* 2015). Shifts in woodland management during the 20th century have led to a decline in species such as pied flycatcher (*Ficedula hypoleuca*) and lesser spotted woodpecker (*Dendrocopos minor*) (Fuller *et al.* 2007; Alder *et al.* 2018).

By the end of the 20th century, woodland structure had changed towards heavily shaded areas, with a notable reduction in understorey complexity, predominantly due to reduced levels of coppicing (Hopkins and Kirby 2007; Mason 2007). This decrease in coppicing started between the 19th and 20th centuries, with the 20th century showing high levels of changes in both the composition and management of European woodlands (Hopkins and Kirby 2007; Bergmeier *et al.* 2010; Kirby *et al.* 2017). This change in forest composition and structure drives bird community distribution, with density and age of trees impacting on community species richness (Thompson *et al.* 2016; Barbe *et al.* 2017). Furthermore, forest composition in relation to the dominance of deciduous or coniferous tree species is an important factor for preferential habitat and resources (James and Wamer 1982; Patterson and Best 1996; Berg 1997; Amininasab *et al.* 2016).

Within eastern Europe, forest conservation has predominately been centred around protecting old forest stands, while Britain lacks the specialist tree species dependent on late forest stages

(Roberge *et al.* 2008; Alder *et al.* 2018). Fuller et al. (2007) hypothesised that the restoration of some form of woodland management would be beneficial for conservation. Woodland management has the possibility of improving habitat quality for a range of endangered vertebrate and invertebrate species associated with early successional habitats (Fuller 2013). It has been argued that heavily managed plantations may support lower levels of avian diversity compared with natural woodland due to the simplification of tree species within plantations (Jones *et al.* 2012), however structural complexity can show a positive correlation with species richness and bird abundance (Nájera and Simonetti 2010). Within the European forestry sector, there is growing support for "irregular forestry" or continuous cover forestry (CCF) systems, which maintains continuous woodland cover with mixtures of tree species via natural regeneration and the avoidance of clear-cutting (Alder *et al.* 2018). These systems are encouraged on the reasoning of having ecological, economic and ecosystem service advantages (Pukkala *et al.* 2016). Using this "irregular felling" method, canopy openings are patchy, and as a result can more closely resemble natural woodland processes, resulting in seedling regeneration, while developing a succession of tree ages (Susse *et al.* 2011). This method is expected to increase the range of ecological resources when compared to coppicing and clear-felling systems, resulting in a shift in bird community composition (Fuller *et al.* 2012). du Bus de Warnaffe and Deconchat (2008) found within beech (*Fagus sylvatica*) dominated woodlands in Belgium, bird abundances were higher in uneven stands when compared to stands which were evenly aged. Despite the success of this management system, the specific question still remains of whether the system will assist in the recovery of rapidly declining woodland biodiversity (Alder *et al.* 2018).

1.1.4 Deer browsing

Within Europe where deer (Cervidae) numbers are increasing (Suominen and Danell 2006; Dolman and Wäber 2008), they are known to have a noticeable impact on forest ecosystems. The impacts of deer browsing within woodland include reducing woody vegetative regeneration, reducing invertebrate abundance and reducing understorey density (Gill and Beardall 2001; Gill and Fuller 2007; Holt *et al.* 2010). When deer are present within woodland, understorey vegetation density decreases due to intense browsing (Joys *et al.* 2004; Gill and Fuller 2007; Holt *et al.* 2010) and during the breeding period can decrease the abundance of bird species which rely on a thick shrub layer for foraging and nesting (Allombert *et al.* 2005; Holt *et al.* 2010; Martin *et al.* 2011; Cardinal *et al.* 2012). Deer may also disturb or trample nests (Ribic and Renfrew 2003). Furthermore, deer impact the suitability of winter habitats through decreasing food resources and increased predator exposure, however it is unknown how deer browsing impacts assemblages of non-breeding woodland birds (Holt *et al.* 2013). While there is strong evidence that deer impact woodland structure within coppice studies, it

is less clear what impacts deer browsing has on mature forests (Gill and Fuller 2007). This is due to the strong shading effects which reduce understorey complexity (Fuller *et al.* 2014).

The cascade effects resulting from this change in vegetation structure and invertebrate community assemblages can severely impact woodland birds (Fuller 2001; Stewart 2001; Allombert *et al.* 2005). Gill and Fuller (2007) focused on the impacts of deer on birds within British woodlands and discovered breeding and migrant birds associated with low vegetation had a decreased overall abundance in areas which were browsed by deer compared with unbrowsed areas. While deer browsing will have little impact on canopy trees, browsing can reduce the proportion of ivy (*Hedera helix*) and honeysuckle (*Lonicera periclymenum*) present, resulting in increased nest predation as nests are more visible, resulting in lower breeding productivity (Fuller *et al.* 2014). Furthermore, the direct competition for fallen tree seed in winter when deer and seed eating bird species may both feed on these dietary items could impact over-winter survival of birds through inter-specific competition (Fuller *et al.* 2014).

1.1.5 The potential role of diet in woodland bird declines

While biodiversity loss has typically been analysed through species extinction (Valiente-Banuet *et al.* 2015), the loss of ecological interactions in which species are involved in is often overlooked (Aizen *et al.* 2012). Due to many key functions within ecosystems relying on biotic interactions, losing these may result in powerful cascade effects, potentially accelerating species loss at a local scale, causing a decay within the ecosystem (Díaz *et al.* 2013). As a result, the loss of biological interactions and the consequential loss of ecological functions associated with them may precede species extinction (Säterberg *et al.* 2013). Having knowledge of predator-prey and herbivore-plant interactions is therefore essential in order to better understand ecosystem function and to determine processes behind species interactions (Pompanon *et al.* 2012). A vital process of understanding interactions within ecological communities is to accurately clarify a species' diet profile, as this plays a pivotal role in defining species' ecological niche and determining individual fitness (Pompanon *et al.* 2012; Romano *et al.* 2020).

Diet is a central component of a bird's life cycle, ecosystem position and evolution, and therefore provides a crucial dimension within bird life history (Barnagaud *et al.* 2019). Diet determines species' energetic investment, survival, reproduction and subsequent fitness (Sibly *et al.* 2012). Evaluating avian dietary composition has been a focus of ornithologists for over a century (Slater 1892). Avian diet studies have helped to characterise ecological interactions of birds (Burin *et al.* 2016) as well as identify prey preferences as a ecological driver of the evolution across the Aves Class (Kissling *et al.* 2012; Barnagaud *et al.* 2014). Characterising the dietary niche of avian species is a vital step in identifying the role of avian species within ecosystems (Hoenig *et al.* 2022). Having base knowledge of avian prey

preferences has identified dietary shifts caused by natural and anthropogenic disturbances (Murray *et al.* 2018; Trevelline *et al.* 2018a), as well as the population and community-wide implications of these disturbances (English *et al.* 2018; Spiller and Dettmers 2019). Dietary composition studies also improve our understanding of biotic interactions, such as those from inter and intra-specific competition (McMahon and Marples 2017; Trevelline *et al.* 2018b). Bird diet studies have also highlighted the ecological services, such as crop pest predation that birds provide (Whelan *et al.* 2008). Having an in-depth understanding of birds' dietary niche allows an accurate understanding of the complex interactions which birds have within their environment, which in turn provides essential information for the conservation and management of avian species and their associated habitats (Ontiveros *et al.* 2005; O'Donnell *et al.* 2012).

Investigating spatial variation in diet is fundamental to understanding how populations locally adapt and interact with populations of certain species they are ecologically connected with (Sanford *et al.* 2003; Romano *et al.* 2020). Ecological conditions can directly affect the presence and availability of organisms, resulting in a large impact on local diversity and composition in the diet of species (Sanford *et al.* 2003). Prey consumed by animals can also influence their energy intake and fulfilment of energetic demands (Molokwu *et al.* 2011). Endotherms (i.e., birds) with wide geographical ranges are likely to experience location-dependent influences on their energetic balance, as has been previously shown in bats (Dunbar and Brigham 2010; Czenze *et al.* 2018). This is likely driven by energy expenditure (such as thermoregulatory and foraging costs) and energy intake (prey availability and dietary selection) which is likely to differ spatially between habitats (Czenze *et al.* 2018). Diet studies conducted at local scales and based on long-term research have contributed greatly to a better understanding of diet-mediated factors which can influence ecological traits, however in order to get a much better understanding of a species' feeding ecology, dietary information across the entire geographical range of a species should be obtained (Slatyer *et al.* 2013).

Interspecific niche separation in diet has been studied extensively across taxa, including bats, fish and primates (Singh *et al.* 2011; Vesterinen *et al.* 2018; Larocque *et al.* 2020), allowing the explanation of how species coexist in sympatry. Intraspecific dietary niche separation however, is also important for ecological dynamics (Cloyed and Eason 2017) and has a number of drivers. Habitat type will influence diet due to potential changes in species composition between habitat types (β-diversity). For example, plantation forests are considered poorer for biodiversity than deciduous woodland as they primarily consist of non-native tree species (Brockerhoff *et al.* 2008). Within forests, it has been found that invertebrate species richness can also differ between tree taxa (Murakami *et al.* 2008; Shutt *et al.* 2019). Climatic differences between years may also impact availability of food resources, as well as

different climatic conditions influencing the energetic balance of species (Dunbar and Brigham 2010; McClenaghan *et al.* 2019). Diet can also differ between life stages and sexes, which is likely due to differing energetic and nutritional requirements between parent and offspring (Jiguet 2002; Kerley *et al.* 2018). Dietary differences between sexes may be driven by morphological and behavioural differences, as well as differing nutritional requirements (Mata *et al.* 2016; da Silva *et al.* 2020). Environmental factors can also interact with intrinsic factors to have an effect on dietary variation between demographic groups. An example of this would be that dietary variation between sexes is only apparent during the breeding season, when males and females have differing reproductive demands (Durell *et al.* 1993; da Silva *et al.* 2020), and age differences in diet may be due to juveniles showing a reduced hunting efficiency, or being less efficient foragers than adults (Kitowski 2003; Franks and Thorogood 2018).

Food resources encompass a critical environmental factor for animal populations (Thomas 1974). The quality and quantity of food resources are known to strongly impact the overall fitness of individuals (Serrano-Davies and Sanz 2017; Tournayre *et al.* 2021), and the dynamics and viability of populations (Vickery *et al.* 2001; Johnsen *et al.* 2017). Studies have shown that certain dietary characteristics could result in an increased risk of species extinction (Tournayre *et al.* 2021). For example, species which show a narrow and specialised trophic niche (i.e. a low range of possible prey consumed) are at increased vulnerability: specialists may show greater constraints responding to environmentally driven resource availability changes than generalists (Clavel *et al.* 2011; Twining *et al.* 2019). It is important to note however, that foraging can be a flexible activity. Optimal foraging theory states that resources are exploited which maximise net energy intake while minimising energetic costs, through a trade-off between resource profitability and searching time (MacArthur and Pianka 1966). Furthermore, optimal foraging theory suggests that “specialised” predators should adopt a more generalist feeding strategy when preferred prey is in low abundance, incorporating prey which was previously ignored (Singer and Bernays 2003). Species which are qualified as generalists can also show preference towards certain prey, and be more selective when preferred prey are available in the environment (Vesterinen *et al.* 2016). Dietary plasticity is an important mechanism in order to respond to environmental changes such as seasonal or temporal fluctuations in resources, or anthropogenic pressure (Kartzinel *et al.* 2015; Smith *et al.* 2018), with a suboptimal diet being detrimental to individual fitness (Sasakawa 2009).

Despite the ecological significance of obtaining dietary and foraging information, very few avian studies have attempted to do so for generalist woodland birds, with detailed dietary knowledge of many species still unknown (Fuller *et al.* 2005; Hewson and Noble 2009). Studies to date have focused primarily on farmland birds (Holland *et al.* 2006; Pearce-Higgins

2010; Holland *et al.* 2012; Ottens *et al.* 2014), or insectivorous species (Taylor and O'Halloran 1997; McClenaghan *et al.* 2019; Møller 2019; Evens *et al.* 2020; Mitchell *et al.* 2021). There is, thus, an imperative need to explore and examine spatial and temporal variations of woodland bird diet. This will enable us to improve understanding of the influence of life stage, foraging landscape and dietary preferences on dietary plasticity, enabling the improvement and design of conservation strategies for declining species (e.g. the preservation of key landscapes) (Arrizabalaga-Escudero *et al.* 2015).

The UK woodland bird index complied by the Department for Environment, Food and Rural Affairs (DEFRA), is based upon monitoring population trend data for 37 breeding woodland bird species between 1970 and 2018. Long term monitoring (1970-2018) showed that since 1970, 19% of woodland bird species showed an increasing population, 49% showed no trend and 32% showed a population decline, while short term data (2013-2018) indicated 19% of species showed a population increase, 30% showed no trend and 51% declined (DEFRA 2018). Overall, woodland generalists showed an overall increase of 4%, while woodland specialists have showed an overall long-term decline of 48%, summarised by Figure 1.1.

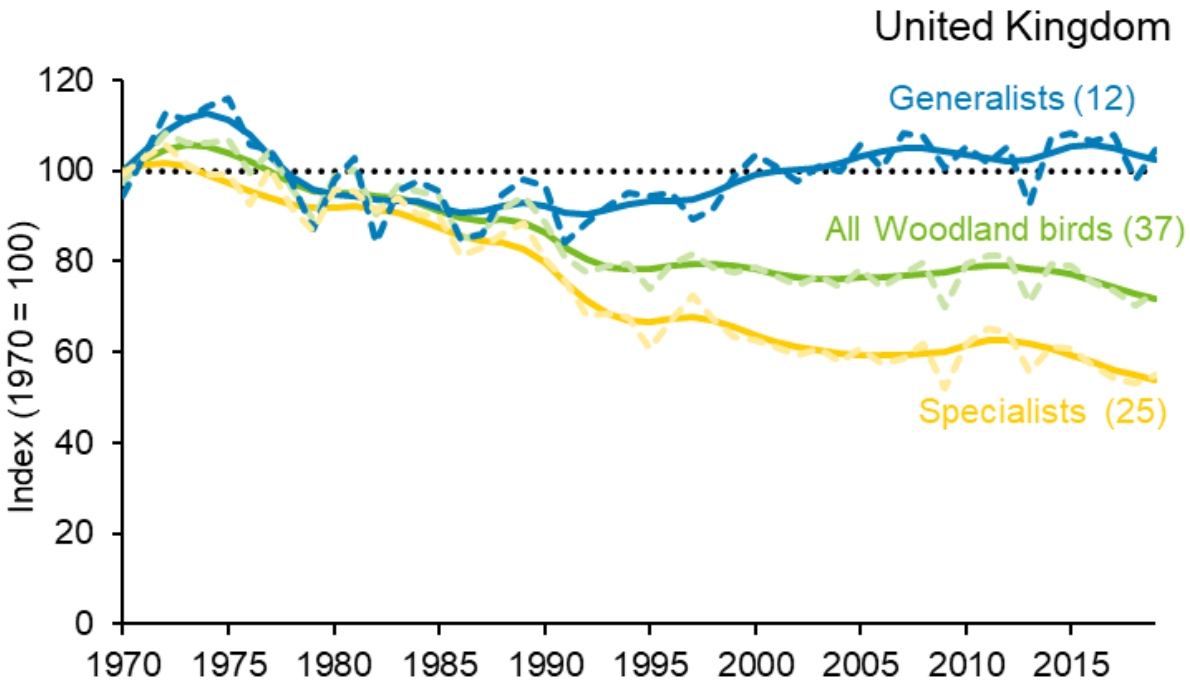


Figure 1.1. The changes in abundance of woodland bird species from 1970-2018. Figures in brackets show the number of species. The unsmoothed trends are indicated by dashed lines and smooth trends indicated by solid lines (DEFRA 2018).

1.2 The Hawfinch

1.2.1 Distribution and demographic changes

The Hawfinch (*Coccothraustes coccothraustes*) is the largest member of the Fringillidae family in the UK and is present across the temperate Palearctic, where it breeds sporadically (Mountford 1957; Newton 1967; Cramp *et al.* 1994). However, this accounts for less than half of its global breeding range, which encompasses Europe to China (Cramp *et al.* 1994; Brown and Grice 2005; Eglington and Noble 2010). The Hawfinch is one of many bird species closely associated with woodland habitats which has shown major declines over a period of a few decades (Kirby *et al.* 2018).

Information regarding Hawfinch ecology is limited, with minimal research undertaken. There is evidence that Hawfinch are loosely territorial, with nesting taking place in loose-knit colonies (Roberts and Lewis 1988). Tomialojc (2005) studied nesting Hawfinch populations within the Białowieża Forest in Poland, an ancient deciduous woodland consisting predominantly of oak (*Quercus sp.*), lime (*Tilia sp.*) and hornbeam (*Carpinus betulus*), finding the optimum breeding habitat to be within hornbeam, as well as maples (*Acer sp.*), lime and spruce (*Picea sp.*), with approximately 50% of all Hawfinch nests found in these species. Within the UK, Mountford (1957) found nests were constructed in oak or sycamore (*Acer pesudoplatanus*) at a height of 6-25m. Hawfinch populations within Britain are thought to be mainly single brooded, although anecdotal evidence has shown double brooding can occur, with re-nesting a common occurrence after initial failure (Kirby *et al.* 2019). The first eggs of the breeding season are predominantly laid between late-April to late- May (Kirby *et al.* 2018).

There is a paucity of information regarding Hawfinch population movements between breeding and non-breeding seasons. Ringing recoveries from Norway and Sweden indicate a partial level of movement between local UK and continental Europe populations (Dadam *et al.* 2013), but due to small numbers ringed there is currently insufficient evidence to distinguish any patterns (Kirby *et al.* 2015). It is likely that a varying number of continental migrants over-winter with resident species in the UK (Kirby *et al.* 2015). The distribution of Hawfinch across the UK in winter is considerably wider than the breeding season, and has increased by approximately 30% since the last estimation in 1981-84 (Lack 1986; Balmer *et al.* 2013). The lack of ringing recoveries indicate Hawfinch may show limited habitat flexibility, which is strengthened by the findings in Kirby *et al.* (2015), revealing that Hawfinch persistence was associated with greater proportion of deciduous woodland (primary habitat). This would indicate that the highest quality habitats would retain Hawfinch populations for the longest, with this “demographic hypothesis” also seen in corn buntings (*Miliaria calandra*) (Donald and Greenwood 2001).

1.2.2 Hawfinch diet

There is a paucity of information regarding Hawfinch diet, with the only detailed information found within Mountford (1957), with additional information found within Newton (1967). During the breeding season (April to August), Hawfinch were observed feeding most regularly on seeds and buds of cherry (*Prunus sp.*) and wych elm (*Ulmus glabra*) (Mountford 1957; Cramp *et al.* 1994). Other notable components of the diet include sycamore, hawthorn (*Crataegus monogyna*), blackthorn (*Prunus spinosa*), wild service tree (*Sorbus torminalis*), dogwood (*Cornus alba*), larch (*Larix decidua*) and beech (Mountford 1957). In spring, Hawfinch were observed feeding on wych elm seeds and buds as well as flowers from oak and maples (Mountford 1957; von Haartman 1978; Bijlsma 1998; Bryant 2011; Tomiałojć 2012). Species of Coleoptera, Hemiptera, Annelida, Gastropoda and Araneae were also observed to be taken during the summer (Mountford 1957). Nestling diet has been observed to be predominantly oak-roller moth (*Tortrix viridana*) and winter moth (*Operophtera brumata*) (Mountford 1957). Winter diet of Hawfinch includes, cherry, hornbeam and beech, but also damson (*Prunus instititia*), dog rose (*Rosa canina*) and wych elm (Mountford 1957; Brown and Grice 2005).

Hawfinch are known to feed mainly in the canopy, possibly due to increased foraging efficiency by reducing distances and movement between food resources, or through higher availability of seeds within the canopy (as found in Perea and Gil 2014). A decreased risk of predation may be also driving canopy feeding through higher visibility and more rapid flight response (Götmark and Post 1996). Canopy feeding may also reduce interspecific competition between Hawfinch and non-perching bird species (Perea and Gil 2014).

1.2.3 Habitat associations

Within the UK and Europe, breeding habitat includes mature trees, in the form of park/woodland, semi natural forests or mature forest plantations (Roberts and Lewis 1988; Hill *et al.* 1991; Smith *et al.* 1992; Hubálek 1999; Smart *et al.* 2007; Tomiałojć 2012). During the breeding season, Hawfinch have been shown to utilise multiple woods over large areas (Calladine and Morrison 2010). Hill *et al.* (1991) found un-managed mature oak forest was pivotal for Hawfinch populations, however the reasons behind this were unclear. Amar *et al.* (2006) revealed Hawfinch were positively associated with wet features such as standing water, indicating a steeper population decline within drier woods, due to a seed heavy diet requiring higher water intake. Smart *et al.* (2007) found Hawfinch preferred wooded, hilly landscapes and avoided areas of high disturbance such as woodland tracks. Traditional orchards provide suitable habitat for bird and arthropod species which prefer to breed within woody habitats, and are not found in extensive arable farming landscapes (Herzog 1998; Bailey *et al.* 2010). Orchards are deemed to be very valuable habitat for Hawfinch, due to their association with

traditional orchards in the breeding season and winter months (Mountford 1957; Myczko *et al.* 2013).

1.2.4 Hawfinch decline

Within Britain, Hawfinch show a very localised distribution with population strongholds exhibiting a strong westerly bias (Kirby *et al.* 2018). While the breeding population across Europe is estimated to be ~2,600,000 – 5,070,000 breeding pairs, the UK is estimated to contain only 500-1000 (Clements 2013). Due to their rarity, Hawfinch are not monitored in the UK through national or annual monitoring schemes (Kirby *et al.* 2015). Instead, population change is inferred from data compiled from periodic bird atlas surveys (Balmer *et al.* 2013). These data indicate a 76% reduction in the number of occupied 10km squares between 1968 and 2011, with the greatest decline shown during 1988-2011 (Kirby *et al.* 2018). Localised breeding extinctions across central and eastern England have been recorded, with only 4% of 10km squares in Britain now occupied (Balmer *et al.* 2013; Kirby *et al.* 2018).

Possible factors for the cause of the severe British decline are unknown. There are a number of hypotheses implicated within the wider overall decline of woodland birds including landscape modification, decreased invertebrate abundance and changes in woodland management (Fuller *et al.* 2005; Kirby *et al.* 2018). Further potential contributory factors include under-planting of ancient woodland with conifers in the 1970's and a storm in 1987 which caused the loss of a number of cherry trees, an important food resource for Hawfinch (Spencer and Kirby 1992; Kirby *et al.* 2018). To date, no studies have linked these hypotheses directly to Hawfinch declines.

In order to identify factors which may be limiting Hawfinch distribution, Kirby *et al.* (2015) studied habitat use by Hawfinch at three spatial scales, 10 km landscape, 4km local and 10x10m quadrat fine scale measurements at nest sites. They found that at both landscape and local scales breeding populations were more persistent within primary habitats of broad leaved and mixed woodland, and this primary habitat retained Hawfinch populations for the longest period of time. However, it was acknowledged that the composition of woodland tree species had changed dramatically over the Hawfinch period of decline (Hopkins and Kirby 2007). Perhaps the most well known and relevant driver behind this change was the emergence and subsequent spread of Dutch elm disease during the 1970s, which was estimated to cause the loss of an estimated 20 million trees (Gibbs *et al.* 1994). Elms are of high value to woodland birds, as they produce flowers and seeds when other food resources are low (Kirby et al. 2015). It is plausible therefore, that the decline of this resource may have directly impacted Hawfinch preparing for the upcoming breeding season by increasing foraging distances or decreasing the success of maintaining condition suitable for breeding (Kirby et al. 2015). Despite having no tangible evidence to substantiate this hypothesis,

Hawfinch have been regularly seen feeding on wych elm which has been less heavily impacted by Dutch elm disease than other elm species (Kirby et al. 2015). Further anecdotal evidence strengthening this link is supported by the pattern of Hawfinch decline, which show population declines across the areas most affected by Dutch elm disease (Kirby et al. 2015). The observed loss of Hawfinch from less-wooded areas further fits the pattern of decline, as elm was primarily a non-woodland tree within England (Kirby et al. 2015). Furthermore, Robertson and Wedge (2008) reported that the orchard area has declined by 63% in England since the 1950's, and 94% in Wales. Due to breeding Hawfinch being limited to a small core number of heavily wooded areas, this would suggest that sites which were previously deemed suitable are now unable to support viable populations, potentially due to a deteriorating wider landscape (Kirby et al. 2018).

To further understand the causes of Hawfinch decline, Kirby *et al.* (2018) examined overall nest survival rates and causes of nest failure, as well as collecting habitat data to investigate correlates of nest success. The overall aim was to further understand the components of nest success which may help identify factors causing the recent population declines. A total of 69 nests were monitored between 2013 and 2017, showing a nest success rate of 36%. Nest success showed no relationship with nest height, year, nest exposure, first egg date and study area. This was unexpected, as poor reproductive success is a common factor of declining bird populations, with long-term nest monitoring needed in order to show factors which can influence bird populations (Newton 2004; González-Braojos *et al.* 2017). The nest success rate of 36% sits between 27% reported from a stable population in Poland Tomiałojć (2012) and 39-59% estimated from a rapidly increasing population in the Netherlands (Bijlsma 1998). This figure is in line with other species with similar woodland-nesting strategies such as spotted flycatchers (*Muscicapa striata*) which have a nest success rate of 24%, and Chaffinch (*Fringilla coelebs*) with 33% (Stoate and Szczur 2001; Stevens *et al.* 2007). It is important to note however, that the study in Kirby *et al.* (2018) was undertaken in Hawfinch population strongholds, where the population is stable. It is uncertain as to whether these data represent other areas of Britain where declines have been sharper.

Contrasting with the population decline shown in UK populations, the most recent assessment from the Pan European Common Bird Monitoring Scheme (PECBMS) shows that overall, Hawfinch populations within mainland Europe have shown both long (1980-2017) and short (2005-2017) term stability (PECBMS 2019). It is important to note however, that within this assessment there is great variability, with central and eastern Europe Hawfinch populations showing moderate declines, while western Europe populations are showing moderate increases. Northern Europe meanwhile shows a stable population, while the trend is unclear in southern Europe. The factors behind the population trends seen between the UK and

Europe remain unknown, however a number of possible factors have been implicated, such as adjacent land use changes, climate change and reduction in invertebrate food supplies (Kirby *et al.* 2015).

1.3 Studying trophic interactions

Trophic interactions are crucial in setting and understanding species conservation strategies, with monitoring providing a measure of determining the success of the strategies implemented (Loch *et al.* 2020). Studying trophic interactions through dietary analysis can be done through a suite of methods, each with strengths and caveats (Pompanon *et al.* 2012). Traditionally, gut contents and faecal samples were microscopically analysed, or feeding observations were made directly (Symondson 2002). While microscopic examination has provided useful information, there are major caveats to the method. It is labour intensive and requires a high level of taxonomic expertise to accurately identify semi-digested plant and animal fragments (Pompanon *et al.* 2012). This method excludes fluid feeders and the identification of dietary items which leave no distinguishing taxonomic features (Pompanon *et al.* 2012; Garnick *et al.* 2018). Stomach content analysis enables information on dietary items which are less prone to digestion, however this can only be implemented after the subject has died, or by invasive procedures such as induced regurgitation (Alonso *et al.* 2014; Baker *et al.* 2014). Directly observing feeding behaviours also show biases towards larger, more conspicuous prey and against elusive, soil dwelling or nocturnal prey items (Rosenberg and Cooper 1990; Pompanon *et al.* 2012).

1.3.1 DNA barcoding and metabarcoding

Over the past decade, multiple studies have provided crucial information on the importance of non-invasive genetic sampling, defined as the analysis of genetic material from shed biological materials such as hair, faeces and skin as opposed to the whole organism (Beja-Pereira *et al.* 2009). Non-invasive sampling is recognised as a powerful tool within conservation genetics, due to increased efficiency of sample preparation and sequencing technology (Comtet *et al.* 2015). Molecular methods allow standardisation of methods, and can be used within complex matrices such as a faecal samples (Alda *et al.* 2007; Darling and Blum 2007; Ficetola *et al.* 2008; Bohmann *et al.* 2015; Comtet *et al.* 2015; Barnes and Turner 2016). Furthermore, molecular methods provide a whole different aspect of approaches which can generate large volumes of data extremely rapidly and more accurately than previous methodologies (Symondson 2002; King *et al.* 2008; Pompanon *et al.* 2012).

DNA barcoding is the use of short, standardised genetic markers to taxonomically identify individual species (King *et al.* 2008; Pompanon *et al.* 2012; Wallinger *et al.* 2017; Taberlet *et al.* 2018). This method has gained popularity since the start of the 21st Century when the need for universal molecular methods for species identification were first highlighted (Floyd *et al.*

2002; Hebert *et al.* 2004). Efforts in generating large databases of DNA sequences to aid in species identification led to the development of the International Barcode of Life, an international effort to centralise and make publicly available such databases including standardised protocols and methods (Comtet *et al.* 2015). In the past decade, the reduction in sequencing costs coupled with increased efforts in the generation of DNA sequences for previously genetically uncharacterised species of both animal and plants have substantially increased the number of available DNA barcode for different species (Sheth and Thaker 2017). Barcoding has been used to analyse predation and herbivory in a wide range of ecological studies, including diet analysis of seals (Deagle *et al.* 2009), bats (Zeale *et al.* 2011), birds (King *et al.* 2015) and whales (Jarman *et al.* 2004).

The “single-species” approach of DNA barcoding however, is not suitable for studies in which multiple species must be identified simultaneously from low quality DNA (Taberlet *et al.* 2018). In order to overcome this caveat and fully understand the dietary choices and food web dynamics, information from all aspects of diet need to be identified (Pompanon *et al.* 2012). DNA metabarcoding, a method in which DNA barcodes are combined with high-throughput sequencing (referred to as HTS onwards), has become one of the most commonly used molecular methods when working with environmental DNA (eDNA) and degraded samples such as faeces, gut contents and soil (Valentini *et al.* 2009; Bohmann *et al.* 2015; Taberlet *et al.* 2018). The development of HTS has largely contributed to the increased use of DNA metabarcoding, as this approach can give greater dietary specificity and sensitivity than morphological methods (Alonso *et al.* 2014; Jusino *et al.* 2019).

Metabarcoding however, is not perfect. It cannot differentiate between differing tissue states, such as a larval or adult form of the same species, or reliably inform about secondary consumption or cannibalism; while presence or absence can be detected reliably, quantitative results are still a challenge due to the large number of biases found throughout the metabarcoding process (Deagle *et al.* 2009; Piñol *et al.* 2018; Taberlet *et al.* 2018). If coverage of a specific taxonomic group is missing or incomplete due to a poor taxonomic library, DNA metabarcoding can be used to identify specimens present at a higher taxonomic level, or, based on clustering thresholds, identify Molecular Operational Taxonomic Units (MOTUs) which are clusters of similar sequences based upon a percentage match to a particular reference sequence (Floyd *et al.* 2002; Galimberti *et al.* 2012). While still providing a certain level of taxonomic resolution, if detailed (e.g. species level) resolution is needed for the study, results at a higher taxonomic resolution such as family or Order, may be problematic when attempting to make ecological conclusions from dietary analysis (Taberlet *et al.* 2007; Valentini *et al.* 2009; Deagle *et al.* 2014; Jusino *et al.* 2019). Some limitations can be minimised by careful planning of the study and experimental design. Primers should be designed around

choosing the DNA barcoding region which will maximise taxonomic resolution and taxonomic differentiation within a specific study system (Hollingsworth *et al.* 2011; Pompanon *et al.* 2012; Hollingsworth *et al.* 2016). An inclusive DNA barcoding library is also necessary in order to identify dietary items to species level (de Vere *et al.* 2012; Moorhouse-Gann *et al.* 2018; Jones *et al.* 2021). The issue of quantitative results can be overcome (at least partially) by minimising the various biases throughout the metabarcoding process, for example using mock communities of known concentrations in order to test for primer biases, as well as using final read counts to calculate relative read abundance (RRA) of each taxon (Piñol *et al.* 2018; Deagle *et al.* 2019). The use of RRA within metabarcoding studies, however, is not without controversy. Final read counts can be impacted by biases present throughout metabarcoding pipelines, including the presence of PCR inhibitors, variable DNA quantity in consumed tissues, differential DNA success rates between species consumed and different PCR amplification rates between consumed species in the diet (Pompanon *et al.* 2012; Piñol *et al.* 2018; Deagle *et al.* 2019; Lamb *et al.* 2019).

A DNA metabarcoding region should be short, with a highly variable sequence and flanked by two conserved regions (Taberlet *et al.* 2018). The metabarcoding region should allow high taxonomic resolution, coverage and high amplification rate of DNA from degraded samples (Moorhouse-Gann *et al.* 2018).

1.4 Project aims

1.4.1 Aims of this PhD project

This PhD aims to improve ecological information associated with a declining UK finch species, the Hawfinch (*Coccothraustes coccothraustes*), by using DNA metabarcoding to describe spatial patterns found within its trophic ecology, as well as revealing any dietary preferences based on relative abundances of dietary items revealed from DNA metabarcoding. Exploring spatial variation in resource use will help reveal whether variation in resource use is an important and adaptive form of dietary or nutritional flexibility under fluctuating resource availability. This thesis also seeks to establish the extent of intraspecific dietary separation among ages and sexes. The ability to understand geographical and intraspecific dietary patterns has important implications for understanding of local adaptations, and for developing effective and targeted management programmes for Hawfinch. The primary focus of this PhD was on Hawfinch population strongholds in the west of the UK and mainland European Hawfinch populations within Germany and Denmark.

Specific aims were to (i) elucidate Hawfinch diet using metabarcoding to highlight key plant and ii) invertebrate dietary elements of UK Hawfinch populations; (iii) elucidate Hawfinch diet of mainland European Hawfinch populations and to compare the results with UK populations

and finally (iv) to investigate if UK Hawfinches showed preferences for different plant species in their diet.

1.4.2 Chapter structure

Chapter 2 is the first data chapter of this thesis. Here the key plant dietary elements found within the diet of UK Hawfinch populations were revealed through DNA metabarcoding. In addition to presenting the plants consumed, intraspecific dietary differences between populations over varying temporal and spatial scales were explored. The prevalence of supplementary food, in this case defined as food specifically provided for Hawfinch within the diet was also analysed. The aim of this chapter was to examine if spatial variation in resource use, age or sex were potential drivers of niche separation in order to improve understanding of how Hawfinch from population strongholds fit into the niche of the species.

Chapter 3 used DNA metabarcoding to identify the key invertebrate dietary elements of Hawfinch populations within the UK. In addition to presenting the invertebrate species consumed, intraspecific dietary differences between populations over varying temporal and spatial scales were explored. The aim of this chapter was to investigate whether dietary composition would differ spatially, likely based upon spatial differences in energy demands, or that dietary composition would be driven by sex due to demographic differences in energy demands between males and females during the breeding season.

Chapter 4 used DNA metabarcoding to reveal plant and invertebrate dietary elements of Hawfinch populations in continental Europe for the first time. This chapter aimed to show the dietary diversity and key trophic interactions of continental European Hawfinch populations, and to reveal whether dietary composition changes spatially (between continental European countries and between continental Europe and the UK) and whether diet composition in continental European is being driven by the factors as in the UK, such as spatial differences in energetic requirements between countries, or intraspecific drivers such as sex.

Chapter 5 is the final data chapter of the PhD thesis. Here, using tree abundance data from three Hawfinch population strongholds in the UK in conjunction with dietary data obtained from Chapter 2, this chapter aimed to reveal if Hawfinch showed dietary preferences within heterogenous woodlands. This chapter aimed to test whether Hawfinch populations show population-level differences in dietary preferences, which may have implications for conservation management schemes.

Chapter 6 is the discussion section of this thesis. The findings from the PhD are discussed and the extent to which research aims were met were explored. Using the results from this thesis, suggestions on how best to implement conservation action for UK Hawfinch

populations are made through a combination of data generated from Chapters 2,3,4 and 5. Directions for future research are also explored.

Chapter Two – Exploring plant dietary elements of UK Hawfinch (*Coccothraustes coccothraustes*) populations

Male Hawfinch foraging on the forest floor in the Wye Valley. Photo credit: Andy Stanbury; Hawfinch Ringing Group.

2.1 Abstract

Investigating biogeographical and demographic patterns in the diet of species is essential in the understanding of their ecology, life-history and local adaptations. Variation in the diet of species can be affected by site-specific variables such as habitat, as well as intrinsic factors such as age or sex. This can lead to dietary niche separation, which reduces competition between individuals for resources and impacts how well species can adapt to environmental variation. Determining to what degree dietary niche separation occurs is challenging, due largely to difficulties in accurately identifying food taxa consumed. The use of molecular methodologies now makes it possible to gain a precise overview of diet and dietary plasticity in elusive species. In this chapter, the diet of a scarce woodland passerine, the Hawfinch (*Coccothraustes coccothraustes*) was revealed by utilising DNA metabarcoding to amplify plant remains in faeces. This chapter aimed to analyse and compare the dietary composition of Hawfinch populations from across five regions of the UK, as well as investigating the degree of dietary niche separation between age-classes and sexes. From 2016-2019 faecal samples were obtained from five regions across the UK with DNA extracted from 286 samples and amplified using part of the Internal Transcribed Spacer 2 region (ITS2). Diet of Hawfinch was predominantly naturally occurring taxa such as beech (*Fagus sylvatica*), hornbeam (*Carpinus*

betulus) and oak (*Quercus sp.*), however Hawfinch frequently utilised supplementary sunflower seed (*Helianthus sp.*), which is provided continuously throughout the year at artificial feed sites to enable catching of individuals. Supplementary resource use differed between sites, as well as between years, suggesting Hawfinch may respond to fluctuating environmental conditions by adapting their use of supplementary food resources. Plant taxa composition within Hawfinch diet also varied spatially, suggesting site-specific habitat factors may play a role in characterising Hawfinch diet. Diet differed between adults and juveniles, evidencing dietary differences in Hawfinch populations may be driven by this demographic parameter. The data suggest that Hawfinch feed on a much higher diversity of food resources than expected, highlighting how DNA metabarcoding can improve knowledge on trophic interactions of elusive species.

2.2 Introduction

The diet of an organism is crucial to characterising its ecological niche and is vital in determining the fitness of an individual (Romano *et al.* 2020). To gain a greater understanding of trophic interactions within the environment, it is critical to have an in-depth understanding of species' diet (Rytkönen *et al.* 2019). Estimating diet can provide crucial base knowledge for understanding the structure of ecological communities and the flow of energy and nutrients through food webs (Kartzinel *et al.* 2015; Nielsen *et al.* 2017).

Investigating spatial changes in diet is fundamental in understanding how populations are locally adapted to their environment, and how trophic interactions with populations of ecologically linked species occur (Sanford *et al.* 2003). Environmental factors such as habitat can directly impact the presence of organisms within an ecosystem (Willig *et al.* 2003) and thus has a great impact on local diversity and composition of species diet (Futuyma and Moreno 1988; Romano *et al.* 2020). Furthermore, an individual's energy balance is influenced by biotic factors such as food availability and abundance of prey (Czenze *et al.* 2018). As a result, endothermic species (such as birds) are likely to experience location-dependent factors which directly influence their energetic balance (Dunbar and Brigham 2010; Stawski and Geiser 2011). This is through expenditure (foraging costs) and energy intake (dietary composition) likely to be differing spatially (Czenze *et al.* 2018; Tournayre *et al.* 2021). As a result, it is probable that populations of the same species inhabiting different areas will differ in energetic expenditure and intake (Dunbar and Brigham 2010).

Furthermore, characterising consumers' resources can provide information about niche specialisation at both inter- and intra-specific scales (Kratina *et al.* 2012). Competition for resources can be a driver of intraspecific variation within diet (Svanbäck and Bolnick 2007). As competition for resources increases, individuals which are less competitive are driven to

choose alternative prey resources, which can lead to divergent foraging strategies and subsequent variation in diet (Svanbäck and Bolnick 2007). Intrinsic drivers of dietary niche separation can be demographic, such as age and sex (Thiemann *et al.* 2011). For example, juveniles may have a more generalist diet than adults due to naivety in food choice and being less efficient foragers compared with the more efficient adults, who therefore shower greater selectivity in food choice (Hamilton and Barclay 1998; Vander Zanden *et al.* 2013; Fayet *et al.* 2015). Distinct diets can also occur between sexes, driven by sexual dimorphism and competition, as well as differing nutritional requirements (Thiemann *et al.* 2011; da Silva *et al.* 2020). For example. differences in body size can impact which food items males or females can catch and handle (Thiemann *et al.* 2011). Furthermore, females may require different nutrients during reproduction than males (Tercel *et al.* 2022), or reproductive strategies, for example female fur seals (*Arctocephalus gazella*) must stay close to pups, resulting in them foraging in separate areas to males and consuming different prey (Jones *et al.* 2020).

Animals are often characterised by their diet, and foraging for food can impact other aspects of an animal's behaviour and survival (White 2008). Having a specialised dietary niche breadth (e.g. only consuming a small range of prey) can increase the vulnerability of dietary specialists when responding to environmentally driven changes in resource availability when compared to generalists (Twining *et al.* 2019). Herbivores, often perceived to be food limited, when subjected to a reduction in high quality food resources frequently show a decreasing population size through negative impacts on demographic parameters (Goldberg *et al.* 2020). Having detailed dietary information is important for exploring optimal foraging, where the central aim of a generalist herbivore is to maximise quality or quantity of resources while foraging, while successfully avoiding predation (Charnov 1976; Pyke *et al.* 1977). Survival and reproduction success are associated with high energy uptake (White 1983). Thus, foraging behaviours that obtain high quality food resources quickly are expected to be under high selection pressure, making dietary choices a integral part of a herbivores life history strategy (Goldberg *et al.* 2020). A reduction in high-quality food resources could result in lower reproduction and survival success, highlighting the importance of understanding dietary breadth in order to better manage species of conservation concern.

Birds have an important role within ecosystems as carnivores and herbivores, but also as seed dispersers, pollinators and ecosystem engineers (Whelan *et al.* 2016). Exploring the dietary choices of birds is a vital stepping stone towards improving our understanding of their biology and the role of birds within food webs, as well as being a useful tool for conservation management (Oehm *et al.* 2011). Previous methods of analysing diet under natural conditions have involved stomach content analysis, which necessitated killing and dissecting the bird (Scribner and Bowman 1998) and gut content analysis where study species must be killed.

This has raised many ethical issues, limiting the analyses undertaken (Oehm *et al.* 2011). To overcome these restrictions, non-lethal methods were developed involving the use of stomach or crop flushing (Hull 2006; Moorman *et al.* 2007), collecting dietary remains from faeces to use in morphological identification (Sagrario *et al.* 2007; Xavier *et al.* 2011) and visual observations of faecal samples (Taylor and O'Halloran 1997) both in the field and through the use of nest cameras (García-Salgado *et al.* 2015). Despite the latter methods being non-invasive, a complete identification of every dietary item is unachievable, due to many dietary items often becoming too digested to be accurately identified morphologically to species level (Oehm *et al.* 2011). While the above listed methods may best suited to larger birds such as penguins, subjecting passerine birds to these methods may be challenging, potentially causing stress and harm (Oehm *et al.* 2011).

Additionally, to elucidate a species' dietary composition using traditional morphology-based methods can be time consuming, and biased towards identification of distinguishable and intact undigested or semi-digested dietary items (Pompanon *et al.* 2012). The use of molecular techniques such as high-throughput sequencing (HTS) in conjunction with DNA barcoding (coined DNA metabarcoding) are being frequently utilised to assess the diet of a range of organisms (Thompson and Newmaster 2014; Lopes *et al.* 2015; Evens *et al.* 2020; Zalewski *et al.* 2021). These techniques require minimal *a priori* knowledge of the dietary composition of the study species (Valentini *et al.* 2009; Alberdi *et al.* 2017) and a wide range of taxa can be identified to fine taxonomic levels (King *et al.* 2008). For the application of these techniques to study bird diet, faecal samples are highly suitable, as they contain residual dietary DNA and can be collected with minimal disturbance to study species which may otherwise have been difficult to locate or directly observe (Pompanon *et al.* 2012; Taberlet *et al.* 2018).

Despite birds being one of the best studied animal classes, few studies have used molecular techniques to improve understanding of their trophic ecology (Alonso *et al.* 2014). In comparison with studies on mammals, in particular bats, the application of faecal metabarcoding within passerines studies is rare. However, this is an evolving field with research being undertaken on an expanding number of passerine species (Shutt *et al.* 2020; da Silva *et al.* 2020; Shutt *et al.* 2021). For example, Vo and Jedlicka (2014) used HTS to elucidate diet of the Western Bluebird (*Sialia mexicana*), Trevelline *et al.* (2016) used DNA barcoding and HTS to improve dietary knowledge of Louisiana Water thrush (*Parkesia motacilla*) chicks. Rytkönen *et al.* (2019) used DNA metabarcoding to analyse the diet of four bird species: willow tits (*Poecile montanus*), Siberian tits (*Poecile cinctus*), great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*). More recently, studies describing the diet of blue tits and wheatears (*Oenanthe oenanthe*) have been published (Shutt *et al.* 2020; da Silva *et al.* 2020). The advantage of high taxonomic resolution provided by HTS has been documented

to describe dietary changes which otherwise may be difficult to detect (Mata *et al.* 2016), however previous studies have mainly focused on bird species with specialists diets and narrow feeding niches, while HTS methodology remains lacking in studies focusing on more generalist species (but see Silva *et al.* 2020).

2.2.1 Study species

The Hawfinch (*Coccothraustes coccothraustes*) is found throughout the Palearctic, with the United Kingdom (UK) being the westerly range limit (Kirby *et al.* 2015). Due to their rarity, Hawfinch are not monitored in the UK through national or annual monitoring schemes, or by Department for Environment, Food and Rural Affairs (DEFRA) woodland bird indexes (Kirby *et al.* 2015). Instead, population change is inferred from distribution data compiled from bird atlas surveys (Balmer *et al.* 2013). These atlas data indicate a 76% reduction in the number of 10km occupied squares between 1968 and 2011, and are further evidenced by Langston *et al.* (2002), who estimated a 40% population decline between the mid 1980's to the late 1990's. (Langston *et al.* 2002; Kirby *et al.* 2015; Kirby *et al.* 2018). Localised breeding extinctions across central and eastern England have been recorded, and only 4% of 10km squares in Britain are now occupied (Balmer *et al.* 2013; Kirby *et al.* 2018). The remaining population strongholds of Hawfinch within the UK show a westerly bias, in heavily wooded landscapes defined by mature, species rich tree communities (Kirby *et al.* 2018).

The Hawfinch is predominately arboreal and is known to feed on seeds, fruits, buds and flowers, as well as invertebrates in spring (Mountford 1957). During the breeding season (typically from April to June), Hawfinch diet includes the seeds and buds of cherry (*Prunus sp.*) and elm (*Ulmus sp.*) (Mountford 1957; Cramp *et al.* 1994). Other notable components of the diet include sycamore (*Acer pseudoplatanus*), hawthorn (*Crataegus monogyna*), blackthorn (*Prunus spinosa*), wild service tree (*Sorbus torminalis*), dogwood (*Cornus alba*), larch (*Larix decidua*) and beech (*Fagus sylvatica*) (Mountford 1957). In spring, Hawfinch have also been observed feeding on elm seeds and buds as well as flowers from oak (*Quercus sp.*) and maples (*Acer sp*.) (Mountford 1957; von Haartman 1978; Bijlsma 1998; Bryant 2011; Tomiałojć 2012). Nestling diet has been observed to be predominantly larvae of the oak-roller moth (*Tortrix viridana*) and winter moth (*Operophtera brumata*) (Mountford 1957). Species of Coleoptera, Hemiptera, Annelida, Gastropoda and Araneae were observed to be taken during the summer (Mountford 1957). Winter diet of Hawfinch include, cherry, hornbeam and beech, but also includes damson (*Prunus instititia*), dog rose (*Rosa canina*) and wych elm (*Ulmus glabra*) (Mountford 1957; Brown and Grice 2005).

Hawfinch populations within north Wales have been recorded visiting supplementary feeders to access sunflower (*Helianthus sp.*) seed within garden bird feeders. The artificial feeding sites used within this study (detailed in section 2.3) use large volumes of supplementary seed

to attract and capture Hawfinch for population monitoring. It is estimated that within the UK there is one supplementary bird feeder per every nine birds which utilise garden feeders (Davies *et al.* 2009). This has resulted in enough resources provided throughout the UK to feed the entire breeding populations of the ten-most common feeder-using species, if these species consumed only supplementary food (Orros and Fellowes 2015). This large scale of resource addition is likely to have effects on both the species utilising the resource, and their natural competitors, however these effects are not well understood (Robb *et al.* 2011; Orros *et al.* 2015). There is contradictory evidence regarding the direct impacts of supplementary food on bird populations, with some studies finding advanced breeding phenology and improved reproductive success due to increased resources (Robb *et al.* 2008; Peach *et al.* 2014), while others have found contrasting results such as poor phenotypic condition and impaired reproductive investment due to an unbalanced diet (Harrison *et al.* 2010; Plummer *et al.* 2018). The high frequency of interactions between individuals at artificial feeding sites has associated health risks such as disease spread, which has resulted in large declines in some species such as the Greenfinch (*Chloris chloris*) (Lawson *et al.* 2018; Moyers *et al.* 2018). Elucidating the use of supplementary food is important due to the immediate fitness and population impacts supplementary feeding can have on wild bird populations (Shutt *et al.* 2021).

The goal of this chapter was to utilise DNA metabarcoding to investigate dietary composition in Hawfinch across the UK. Dietary composition provides a measure of available food resources, which is likely to reflect any spatial differences in the tree species communities Hawfinch are feeding on. Additionally, assessing dietary variability across Hawfinch populations will allow the assessment of whether spatial and temporal variation in resource use is an important form of dietary or nutritional flexibility, allowing Hawfinch to respond to resource fluctuation. In order to do this, I analysed the variability of the dietary composition across 11 artificial feeding sites split across five regions, sampled between 2016 and 2019. Doing so across the entire geographical range of core Hawfinch populations is important in order to reflect the most complete niche breadth (Aizpurua *et al.* 2018). This chapter also investigated intraspecific drivers of dietary composition differences, such as age and sex, hypothesising that juveniles will have differing dietary compositions than adults due to their naïve foraging strategies, and that dietary diversity will also differ between the sexes due to differing nutritional requirements between males and females pre- during and post breeding season (da Silva *et al.* 2020). Finally, this chapter aimed to determine the prevalence of supplementary food within the diet of Hawfinch, hypothesising that supplementary food prevalence may be higher in more urbanised areas where supplementary food is more readily available.

The implications of the findings for conservation management are discussed along with future research options. This chapter will focus only on the herbivorous aspect of Hawfinch diet, as plant dietary items are the main food resource utilised throughout the year (Mountford 1957). The invertebrate components of diet are discussed in Chapter 3.

2.3 Methods

2.3.1 Study sites

Fieldwork was conducted in the period March to July of 2016-2019 at 11 woodland sites in the UK. Sites selected were pre-existing Hawfinch ringing studies study areas within the Wye Valley, Dolgellau, Cardiff, the New Forest and Norfolk (Figure 2.1). The artificial feed sites used to attract Hawfinches for capture have been operational for a number of years within regions of Hawfinch population strongholds (Clements, 2013; Kirby *et al*., 2018). Study sites were broadly typical of British mixed broadleaved woodland, with sites in the Wye Valley and north Wales dominated by beech, oak and ash. The study site located in Norfolk was a mixed woodland consisting of lime (*Tilia sp*.), ash (*Fraxinus excelsior*) and maples, while the New Forest site was dominated by oak, with an understorey flora comprising of Holly (*Ilex aquifolium*) and bramble (*Rubus sp*.). All site locations are approximate for anonymity.

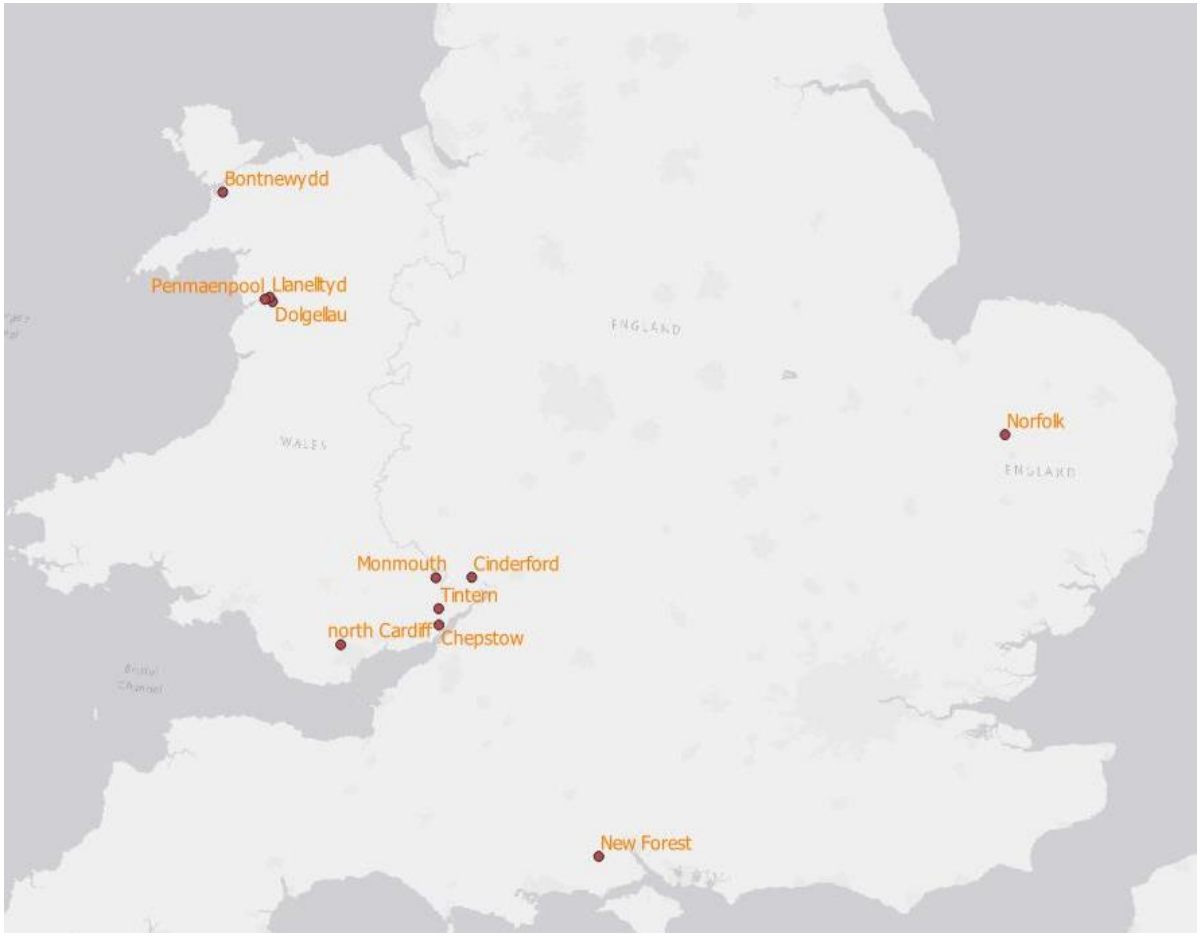


Figure 2.1. Locations of study sites where faecal samples were collected. Sites where faecal samples collected are shown as red dots. Map was constructed using QGIS (QGIS Development Team 2021).

2.3.2 Field sampling

All Hawfinches were caught using either mist or whoosh nets and where applicable, birds were fitted with a metal identification ring by professional bird ringers operating under British Trust for Ornithology (BTO) approved methodologies and ringing licences. Nets were checked frequently for Hawfinches to maximise welfare. Hawfinch were caught and individually placed within a new, clean, paper bag which was then placed inside a cloth bird bag and left for 10-20 minutes until the bird defecated. To avoid excessive stress, if birds had not defecated within this time frame they were processed (see below) and released. Faeces were removed from the paper bags using plastic toothpicks and placed in separate 2ml microcentrifuge tubes, and frozen to -20^{o}C at 1-8h after collection. To avoid contamination, a new toothpick for sample removal and new paper bag were used for each bird processed. Each sample was assigned a sample identification number based on the site and ring number of the Hawfinch. If repeated capture occurred during the same ringing session a faecal sample was not collected unless a sample was not obtained during the first capture. However, if the same individual was re-trapped during a separate session at a later date, a faecal sample was taken if provided and ring number and date recorded. For each bird captured morphometric data and time of capture were recorded, including age, maximum chord wing length, sex and body mass (Svensson 1992). Wing length was measured using a ruler to the nearest 0.5mm and body mass with a digital balance to the nearest 0.1g. Time of capture was recorded and then categorised into AM or PM. Table 2.1 summarises information regarding Hawfinch sampled within each location by age, sex and year.

2.3.3 DNA extraction, PCR amplification and high-throughput sequencing

Following the protocol for pathogen detection with modifications by Shutt *et al.* (2020) designed to improve DNA yields from avian faeces (Appendix 1.1), DNA was extracted from faecal samples in batches of 12-23 in extraction rounds using the Qiagen QIAamp DNA Stool Mini Kit (Manchester, UK). All DNA extraction sessions had one or more DNA extraction negatives included. These extraction negatives contained all the same reagents and underwent the same protocol as all other samples but contained no DNA. In order to minimise contamination, all DNA extractions were undertaken in a pre-PCR air flow fume hood, cleaned before and between uses with bleach and ethanol. Pipettes dedicated for DNA extraction only were used to minimise cross contamination from outside sources.

The second internal transcribed spacer (ITS2) gene of nuclear ribosomal DNA was targeted for amplification of plant DNA. ITS2 primers UniplantF, 5′-TGTGAATTGCARRATYCMG-3′ and UniPlantR 5′-CCCGHYTGAYYTGRGGTCDC-3′ for use within metabarcoding studies were designed by Moorhouse-Gann *et al.* (2018), and reliably amplify a 187-387 base pair fragment. A two-stage PCR process was undertaken. All PCR reactions included a PCR

negative (a reaction containing no DNA) and DNA from the plant *Zornia glochidiata* as a non-native PCR positive. Initial PCR reactions of 5µL contained 2.5 µL multiplex mix (Qiagen, Manchester UK), 0.1µL of 10 µM forward and reverse primer UniPlant primer, 1.3µL of DNase-free water, and 1µL of template DNA. DNase-free water was used instead of template DNA for negative PCR controls. Reactions were carried out in an Applied Biosystems SimpliAmp™ 96-well thermocycler. PCRs comprised 15 minutes denaturation at 95°C, followed by 40 cycles of 95°C for 30s, primer annealing at 58°C for 30s, a PCR product extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. All PCRs were set up in a DNA-free pre-PCR laboratory area before DNA was added in a separate laboratory area in order to avoid cross-contamination. PCR products were run through a 2% agarose gel, stained using SYBR®Safe (Invitrogen). To quantify band sizes, 2µL of Promega™ 100bp ladder was also included in the last well of the gel. Samples which failed to produce a band were re-tested under the same PCR conditions with 2 µL of template DNA with the volume of nuclease-free water adjusted accordingly, so overall volume remained 5 µL. If a band was not detected for a second time, the PCR was repeated using 0.5 µL of template DNA. Any samples which did not produce a band after three PCR tests were omitted.

Extracted faecal samples which showed a positive result (a DNA band on a 2% agarose gel) were taken forward for molecular identifier tagged (MID-tag) PCR. This process involved labelling the forward and reverse primers with MID-tags, following Moorhouse-Gann (2017). Samples had a unique pairing of forward and reverse tags for sample identification post-sequencing (Brown *et al.* 2014). A total of 34 unique forwards and 15 unique reverses were used (Appendix 1.2). Reactions were carried out in the same Applied Biosystems SimpliAmp™ 96-well thermocycler, with annealing temperatures optimised through temperature gradient PCRs in the same machine. PCR reactions of 25µL contained 12.5µL of multiplex PCR mix (Qiagen, Manchester, UK), 2.5µL of 2 µM forward and reverse UniPlant primer, 2.5µL of water and 5µL template DNA. PCRs comprised 15 minutes at 95°C, followed by 40 cycles of 95°C for 30s, 58°C for 90s, 72°C for 90s followed by a final extension at 72°C for 10 min.

Within each PCR 96-well plate, 12 negative (extraction and PCR) and two positive controls were included following Taberlet *et al.* (2018). Negative PCR controls consisted of DNase-free water. A negative control was included for each MID-tag to identify any contamination. All products from each individual PCR plate were categorised based on band brightness after gel electrophoresis (very faint, faint, medium, bright). The DNA concentration from a minimum of three representative PCR products per plate from each brightness category were quantified using a high sensitivity assay with a Qubit Flourometer (Thermo Fisher Scientific) to confirm whether estimating relative DNA concentration by eye from a gel photo was accurate. For

each PCR plate, samples were pooled according to concentrations determined by the Qubit Fluorometer to ensure equimolar concentration of all samples in each pool per plate. Negative controls were pooled based on the average volume pooled per plate for the Hawfinch samples.

Each pool was cleaned using SPRIselect beads (Beckman Coulter, Brea, USA) with a left-side size selection using a 1.2:1 ratio (retaining ~150-1000 bp fragments). The concentration of the pooled DNA was quantified using Qubit dsDNA High-sensitivity Assay Kits, and quality checked via TapeStation 2200 with a D1000 ScreenTape (Agilent, Santa Clara, USA). The concentration across all pools was quantified using Qubit dsDNA High-sensitivity Assay Kits, and all pools were pooled again into "combined pools". Library preparation for Illumina sequencing was undertaken on the cleaned "combined pools" via NEXTflex Rapid DNA-Seq kit (Bioo Scientific, Austin, USA), with a unique adapter added to each "combined pool". The "combined pools" were diluted to 4nM and quantified using Qubit dsDNA High-sensitivity Assay Kits. Finally, the diluted "combined pools" were pooled equimolarly into a "final pool" and sequenced on a MiSeq desktop sequencer via a v2 chip with 2 x 250bp paired-end reads (expected capacity 24-30,000,000 reads). Due to the unbalanced nature of the amplicon libraries, a 15% PhiX buffer was added to the sequencing run in order to improve cross-talk and phasing calculations.

2.3.4 Bioinformatics

The scripts used in the metabarcoding bioinformatics pipeline are available in Appendix 1.3. The Illumina run generated 6,328,388 reads. MID-tag primers were tested for truncation by calculating the percentage of reads containing less than 10bp of the MID-tag forward and reverse primer. This did not exceed 15% of the reads. Paired-end reads were quality-checked and trimmed using fastp v.0.20.0 (Chen *et al.* 2018), with a minimum quality threshold based on a Phred score with a minimum value of 33 (Mbareche *et al.* 2020) and a minimum base pair length of 180 bp. After filtering, the total number of reads was 5,958,552. The read pairs were demultiplexed using Mothur v1.39.5 (Schloss *et al.* 2009), removing the primer and MID sequences with a minimum of one mismatch. Unoise3 (Edgar 2016) was implemented within Usearch11 (Edgar 2016), removing replicates, denoising and clustering as well as removing any chimeric sequences. Any unique samples with <8 reads were discarded as they most likely represent sequencing errors.

Moorhouse-Gann *et al.* (2018) identified the ITS2 region as being unsuitable for clustering taxonomically similar sequences into molecular operational taxonomic units (MOTUs), due to multiple polymorphisms within certain plant families and the intraspecific variation at the ITS2 region not being removed by clustering, subsequently resulting in a loss of taxonomic resolution. As a result of this, a closest matching sequence approach was adopted to identify

species within the samples (Hawkins *et al.* 2015). Reads were clustered to zero-radius OTUs (hereafter zOTUs), based on a 100% clustering threshold, resulting in high taxonomic resolution and preventing incorrect clustering of variants.

For downstream analysis, the ITS2 database (Ankenbrand *et al.* 2015) was converted into a blast database following steps within the BLAST help manual (National Center for Biotechnology Information 2008). All remaining sequences were assigned a taxonomic identity from the ITS2 database using a 97% identity threshold (Ankenbrand *et al.* 2015; Banchi *et al.* 2020). Megan v6.15.2 (Huson *et al.* 2016) was used to analyse the ITS2 database output. If the top ITS2 database hit, determined by e-value, was reserved to a match with a single species then species-level identification was achieved, with the same rule applying to genus level matches. If a sequence failed to match with a plant within the ITS2 database, the BLASTn algorithm (Camacho *et al.* 2009) was used to manually search for sequence matches in GenBank. zOTUs which were not assigned to any taxonomic rank or did not correspond to an ITS2 database or BLAST sequence were considered to be erroneous, or low quality and were discarded.

To clean data prior to statistical analysis, a sequence read number methodology was implemented (Dunn *et al.* 2018) in order to remove background contamination within PCR and extraction negatives. Sequences present within samples with unused MID-tag combinations due to “tag-jumping” (Schnell *et al.* 2015) were also considered. All sequences less than the maximum read count present in unused-MID tag combinations and negative controls for each respective zOTU were removed. The matrix was then collapsed so plant species represented by multiple zOTUs were represented by a single entry. As multiple zOTUs were frequently found to correspond to the same taxonomic identity, aggregating by taxonomic identification removes distinction due to haplotypic and intra-specific variation. The final dataset was cleaned further by removing artefacts and contaminants originating from positive control samples. Taxa present within both a faecal sample and positive control sample were removed from a faecal sample if the read count of the non-positive control taxa within the faecal sample was lower than the read count detected for the non-positive control taxa within the positive control samples. All zOTUs represented by less than 10 reads were removed as these are likely to be artefacts (Schenk *et al.* 2019). Non-target taxa (e.g., fungi) and grain species suspected to be present in low-levels within supplementary food (e.g. wheat) were also removed. All taxa were converted to genus-level to standardise the taxonomic level since some zOTUs could not be resolved further. Standardising the taxonomic level also increased evenness for subsequent analysis. Finally, read counts were converted into presence-absence data.

2.3.5 Statistical analysis

For all statistical analysis, the presence/absence of each taxonomic unit within a sample was used as read count is not an accurate representation of abundance due to amplification biases (Yu *et al.* 2012; Clare, Symondson and Fenton 2014). Control samples were excluded from the analyses. All statistical analysis was carried out in R version 3.6.3 (R Core Team 2020) unless otherwise stated.

To evaluate the most prevalent taxa within Hawfinch diet, the number of samples in which a dietary zOTU occurred (frequency of occurrence, hereafter referred to as FOO), was calculated. This was expressed as a percentage (%FOO) by dividing FOO by the total number of samples and multiplying by 100. The data were categorised by sex and age (Table 2.2). In order to estimate the total dietary niche breadth, the *specpool* function in R's *vegan* package (Oksanen *et al.* 2019) was used to calculate Chao's incidence-based estimator of richness (Chao and Jost 2012; Oksanen *et al.* 2019). Observed species richness/Chao extrapolated richness gave the proportion of total dietary diversity detected. Species accumulation curves were also produced, relating the overall dietary diversity captured to the number of faecal samples analysed.

To investigate how the explanatory variables were associated with dietary composition, multivariate generalised linear models (MGLMs) were used using the function *manyglm* within the package *mvabund* (Wang *et al.* 2012). This allows for multiple species testing and implements likelihood ratio test (LRT) and re-sampled *p* values to identify significance of predictor variables. Where an individual had been sampled more than once, data was used from the first capture only in order to avoid pseudo replication and subsequent biases. Binomial regression structure was specified in the models to account for presence-absence data and subsequent mean-variance relationship of the data, with a "cloglog" link function in order to control for large numbers of zeroes in the dataset. The function *anova.manyglm* in *mvabund* was used to test the significance of each predictor variable within the model and the *p.uni = "adjusted"* argument was implemented in order to allow univariate "species by species" results to be returned (Wang *et al.* 2012). The *p*-values returned in this argument were adjusted to control for multiple testing, using a Holm's step down resampling algorithm, allowing control over family error rates (Westfall and Young 1993). Parametric bootstrap (Monte Carlo) resampling was applied to test for dietary differences, ensuring inferences took into account correlation between variables (Wang et al. 2012). This function is recommended for hypothesis testing with presence-absence data (Wang et al. 2012). When necessary, pairwise comparisons were performed using the *pairwise.comp* function of *anova.manyglm*. For all models, quantile-quantile (Q-Q) diagnostic plots were checked to ensure normality in

multivariate data and multivariate homoscedasticity was checked by plotting Dunn-Smyth residuals against fitted linear predicted values (Wang *et al.* 2012; Bates *et al.* 2015).

The predictor variables used within the *manyglm* analysis were chosen to represent environmental and biological variation across differing space and time. Regions were broadly categorised into: Wye Valley, Dolgellau, Cardiff, New Forest and Norfolk, while the artificial feeding sites were categorised at smaller spatial scales within each region; four artificial feed sites each within the Wye Valley and Dolgellau and one in Cardiff, the New Forest and Norfolk respectively.

- Region (five categories)
- Artificial feeding site (11 categories)
- Year (four years)
- Age
- Sex
- The interaction between year and site
- The interaction between sex and age

All predictor variables were categorical, and no model simplification was performed as the aim of the modelling was significance testing, rather than developing simpler predictive models. For analysis of Hawfinch diet within the Wye Valley region all birds sampled were adults and therefore adult and juvenile dietary comparisons could not be undertaken. Due to small sample sizes, samples from east Anglia and north Cardiff were excluded from intra-regional analysis (n=6 and n=7 respectively). Intra-regional analysis of New Forest Hawfinch populations was also excluded due to small sample sizes for juveniles, females and faecal samples collected within sampling year 2018 (n=2, n=5 and n=3 respectively).

In order to visualise the dietary composition across Hawfinch populations throughout the UK non-metric multidimensional scaling analysis (nMDS) via the function *metaMDS* in the *vegan* package were produced (Oksanen *et al.* 2019). The nMDS was performed with Jaccard distance in two-dimensional space (k=2) due to the presence/absence nature of the data. The plots use a distance-based metric (in this instance Jaccard index) to show how each individual (the points) differ from the mean (centroid) of their respective grouping factor. The further two individuals are apart from each other the more different their diets. Spider plots were produced using nMDS results via *ordispider* and plotted through ggplot2 (Wickham 2016) to visualise dietary composition of Hawfinch. All plots presented had a stress value of <0.2.

A binomial generalised linear mixed effect model was run in R (R Core Team 2020) to determine if the prevalence of supplementary food in the diet (sunflower seed provided at

artificial feed sites) was influenced by site or year. The model was fitted by maximum likelihood using the lme4 package (Bates *et al.* 2015). Hawfinch ring number was included as a random effect in the model to account for repeated measures of individuals. The model was validated using the function *check_model* in the package *performance* (Lüdecke *et al.* 2020), checking for multicollinearity between variables and the distribution of residuals for homoscedasticity. The final model was chosen using the function *compare_performance* within the same package (Lüdecke *et al.* 2020). Pairwise differences between factors were explored using false discovery rate corrected post-hoc tests using the *emmeans* package (Lenth 2020).

2.4 Results

In July to September 2016, 16 Hawfinch faecal samples were collected in north Wales, with an additional 38 samples collected in north Wales and the Wye Valley during April to July 2017. A total of 365 faecal samples were collected between 2016 and 2019. Upon completion of laboratory and bioinformatic pipelines, dietary data was successfully obtained from 286 individual faecal samples. A total of 138 individuals from the Wye Valley, 115 individuals from north Wales, 19 from the New Forest and seven from both Norfolk and north Cardiff (Table 2.1).

2.4.1 Hawfinch diet composition

I retrieved 6,328,388 sequences from 286 Hawfinch faecal samples. A total of 193,610 sequences were detected within negative controls. A total of 202,849 unique sequences were removed due to contamination, tag-jumping and poor-quality sequences or reads likely to be a result of degradation. After excluding 39 spurious taxa (see Appendix 1.4), 84 taxa from 51 genera were identified in the diet of Hawfinch (Table 2.2). Of the plant taxa identified, 92% were identified to species and 100% to genus. Based on Chao estimate of total richness, 75.2% of the available dietary diversity at generic level was detected across the sampling sites, where the total estimated generic diversity (Chao estimate) was 67.8 ± 12.7 (Figure 2.2). Total dietary diversity was detected at 40 genera for the Wye Valley (Chao estimate 61.0), 42 for north Wales (Chao estimate 66.3), 19 for the New Forest (Chao estimate 19.7), nine for north Cardiff (Chao estimate 15.9) and 12 for Norfolk (Chao estimate 17.4).

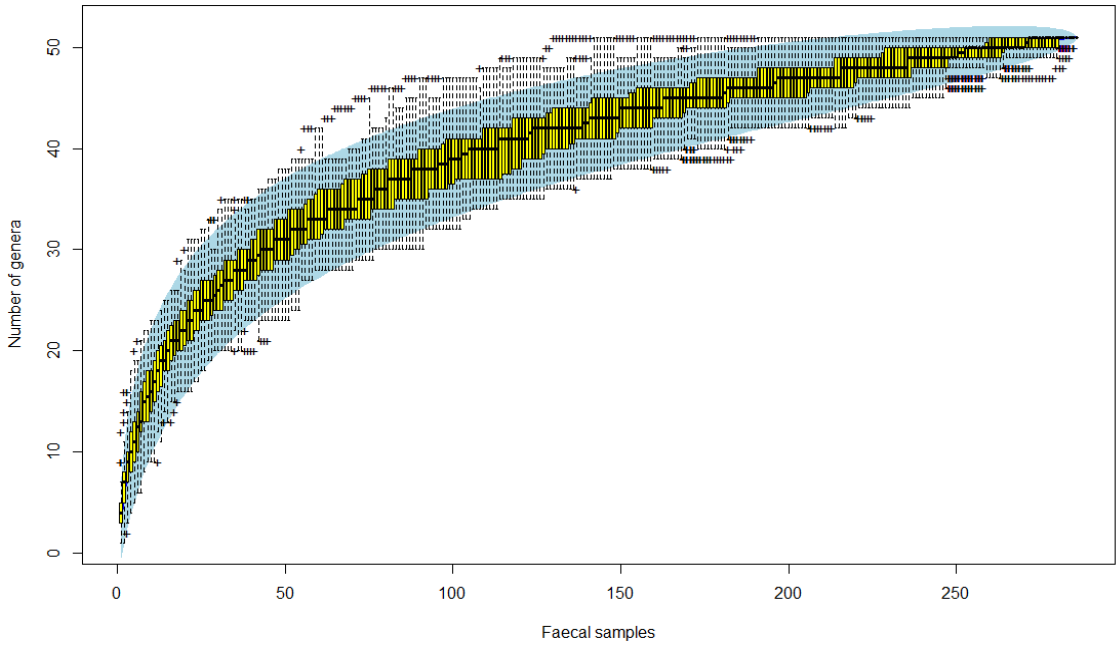


Figure 2.2. A species accumulation curve for UK sites based on the accumulation of taxonomic units (genera) detected across successive Hawfinch faecal samples. Boxplots (yellow) from raw data based on 100 permutations of samples in a random order are overlaid by confidence intervals (blue shading) and predicted points, denoted by "+".

Dietary items most frequently detected were beech, sunflower seed, and sessile oak (*Quercus petraea*) (65.4%, 44.1% and 30.1% of samples respectively, *n*= 286). Appendix 1.5 details the most frequently detected items by sampling region.

2.4.2 Hawfinch dietary variation

Hawfinch diet differed between regions (MGLM: LRT=361.0, *p*=<0.001; Figure 2.3a), age classes (LRT=122.8, *p*=<0.001; Figure 2.3b) and years (LRT=370.8, *p*=<0.001; Figure 2.3c). The interactions between age and sex (LRT=55.7, *p*=<0.001) and region and year were also significant (LRT=77.2, *p*=0.003). Pairwise comparisons indicated distinct diets were detected between all regional comparisons (Appendix 1.6). Univariate analysis revealed five taxa were associated with regional differences: *Carpinus* (LRT=26.8, *p*=0.001), *Fagus* (LRT=39.1, *p*=0.001), *Helianthus* (LRT=54.1, *p*=0.001), *Quercus* (LRT =28.3, *p*=0.001) and *Ulmus* (LRT=39.0, *p*=0.001). Specifically, *Fagus* and *Ulmus* were detected with the highest frequency in the Wye Valley (83.3% and 30.4% respectively), with birds sampled from north Wales showing the highest frequency for *Helianthus* (68.7%). *Quercus* was detected at the highest frequency from Hawfinch sampled within the New Forest (78.9%).

The distinct Hawfinch diets associated with age were found to be driven by four taxa; *Acer* (LRT=10.4, *p*=0.017), *Carpinus* (LRT=34.0, *p*=0.001), *Helianthus* (LRT=13.5, *p*=0.003) and *Salix* (LRT =10.1, *p*=0.018), all detected more frequently within juvenile samples than adult. Dietary differences between adult and juvenile Hawfinch varied between sexes (LRT=55.7,

p=<0.001), with *Betula* consumed more frequently by juvenile males (LRT=10.3, *p*=0.043). Temporal dietary differences were found to be driven by a suite of plant genera (Table 2.3), including Cashew (*Anacardium*) which was only detected in faecal samples from Hawfinch sampled in 2019. Pairwise comparisons revealed distinct diets between all sampling years (Appendix 1.6). Dietary differences between sites and sampling years were driven by *Fagus* (LRT=16.8, *p*=0.012), which had the highest prevalence in the Wye Valley in 2019. Dietary differences between sexes were not significant (LRT=62.1, *p*=0.091).

2.4.2.1 Landscape spatial variation

We were able to look at within region variation in the diet for the two regions with the most sampling effort, north Wales and the Wye Valley. Within the north Wales sampling region, there were statistical differences in Hawfinch dietary composition between artificial feeding sites (LRT=136.7, *p*=<0.001). The nMDS plot (Figure 2.4a) showed the artificial feeding site near Dolgellau was distinct, with dietary differences between the remaining artificial feeding sites showing limited separation. Pairwise comparisons revealed distinct diets between artificial feeding sites near Llanelltyd and Bontnewydd (LRT=81.2, *p*=<0.001), Penmaenpool and Llanelltyd (LRT=72.9, *p*=<0.001), Penmaenpool and Bontnewydd (LRT=58.0, *p*=<0.001) and Penmaenpool and Dolgellau (LRT=32.5, *p*=0.021). These differences were driven by *Betula* (LRT=16.5, *p*=0.012) and *Carpinus* (LRT=14.2, *p*=0.023), with *Betula* detected more frequently at the artificial feeding site near Penmaenpool and *Carpinus* detected at the highest frequency near Bontnewydd. Distinct Hawfinch diets were also associated with age (LRT=121.1, *p*=<0.001; Figure 2.4b); *Acer* (LRT=13.5, *p*=0.005) and *Carpinus* (LRT=24.5, *p*=0.001) were both detected more frequently within juvenile diet. Diet did not differ between the sexes (LRT=54.59, *p*=0.087).

Within the Wye Valley, Hawfinch diets again differed between artificial feeding sites (LRT=293.1, *p*=<0.001). Pairwise comparisons revealed distinct diets between Hawfinch sampled in artificial feeding sites near Cinderford and Chepstow (LRT=174.5, *p*=<0.001), Cinderford and Tintern (LRT=126.5, *p*=<0.001) and Tintern and Chepstow (LRT=51.8, *p*=0.05). Six taxa were identified as driving the dietary differences between artificial feeding sites: *Amelanchier* (LRT=17.12, *p*=0.005), *Anacardium* (LRT=25.8, *p*=0.001), *Fagus* (LRT=26.3, *p*=0.001), *Helianthus* (LRT=24.0, *p*=0.001), *Quercus* (LRT=18.6, *p*=0.003) and *Ulmus* (LRT=16.7, *p*=0.006). The nMDS plot (Figure 2.5) highlights the artificial feeding sites near Monmouth and Cinderford are distinct, with dietary differences between the remaining artificial feeding sites showing limited separation. Diets did not differ between the sexes (LRT=42.61, *p*=0.249).

Binomial generalised linear mixed modelling (Nakagawa R^2=0.43) revealed supplementary food prevalence within Hawfinch diet differed significantly between sampling regions and

sampling years, with supplementary food prevalence significantly higher in Hawfinch sampled in north Wales than the Wye Valley (estimate=-0.92 ±0.24, z=-3.80, *p*=0.001). Hawfinch sampled in 2016 (estimate=1.76 ±0.53, z=3.31, *p*=0.003) and 2017 (estimate=0.92 ±0.36, z=2.54, *p*=0.02) had a significantly increased prevalence of supplementary food in the diet compared with sampled birds from 2018. Hawfinch sampled in 2018 showed a significantly decreased prevalence of supplementary food when compared with birds sampled in 2019 (estimate=-0.97 ±0.25, z=-3.88, *p*=<0.001).

Table 2.1. The sampling effort of Hawfinch captured across regions of the UK, broken down by sex, age and year.

Region	Number of Hawfinch sampled (total)	Sex	Age	Year
North Wales	115	Male = 60 Female = 55	Adults = 94 Juveniles = 21	2016 = 15 2017 = 34 2018 = 9 2019 = 57
Forest Ganol	7	Male = 4 Female = 3	Adults = 7 Juveniles = 0	2016 = 0 2017 = 0 2018 = 2 2019 = 5
New Forest	19	Male = 14 Female = 5	Adults = 17 Juveniles = 2	2016 = 0 2017 = 0 2018 = 3 2019 = 16
Norfolk	7	Male = 3 Female = 4	Adults = 6 Juveniles = 1	2016 = 0 2017 = 0 2018 = 0 2019 = 7
Wye Valley	138	Male = 78 Female = 60	Adults = 134 Juveniles = 0	2016 = 1 2017 = 0 2018 = 65 2019 = 72

Table 1.2. The percentage of Hawfinch faecal samples (%FOO) testing positive for dietary items by sex and age-class. Juveniles were aged as being <1 year old.

Taxon	Common Name	All (*n*=286)	Male (*n*=159)	Female (*n*=127)	Adult (*n*=262)	Juvenile (*n*=24)
Fagus sylvatica	European Beech	65.4	67.9	62.2	66.8	50.0
Helianthus sp.	Sunflower	44.1	45.3	42.5	40.8	79.2
Quercus petraea	Sessile oak	30.1	30.2	29.9	29.4	37.5
Quercus robur	English oak	26.6	24.5	29.1	26.7	25.0
Carpinus betulus	European hornbeam	22.7	23.3	22.1	17.9	75.0
Prunus avium	Wild cherry	18.2	16.4	20.5	17.6	25.0
Ulmus glabra	Wych elm	17.1	15.1	19.7	18.3	4.2
Anacardium occidentale	Cashew	14.7	17.0	11.8	16.0	0.0
Quercus canariensis	Algerian oak	13.3	13.8	12.6	13.4	12.5
Acer pseudoplatanus	Sycamore	10.5	8.8	12.6	7.3	45.8
Betula pendula	Silver birch	9.1	7.6	11.0	8.8	12.5
Fraxinus excelsior	European Ash	8.4	7.6	9.5	7.3	20.8
Betula pubescens	Downy birch	8.0	8.2	7.9	7.3	16.7
Ilex aquifolium	Common holly	8.0	11.3	3.9	8.4	4.2
Corylus avellana	Common hazel	7.3	6.9	7.9	6.9	12.5
Hedera helix	Common ivy	7.0	4.4	10.2	6.9	8.3
Acer campestre	Field maple	6.3	4.4	8.7	6.5	4.2
Rubus sp.	Bramble	6.3	6.9	5.5	6.1	8.3
Larix decidua	European larch	5.2	5.7	4.7	5.7	0.0
Taxus baccata	English yew	5.2	6.3	3.9	4.6	12.5
Salix sp.	Willow	4.9	6.9	2.4	3.4	20.8
Picea abies	Norway spruce	3.5	5.0	1.6	3.8	0.0
Larix kaempferi	Japanese larch	3.2	3.8	2.4	3.4	0.0
Urtica dioica	Common nettle	3.2	4.4	1.6	2.3	12.5
Alnus glutinosa	Alder	2.8	2.5	3.2	2.3	8.3
Amelanchier lamarckii	Juneberry	2.8	3.8	1.6	3.1	0.0
Nothofagus obliqua	Patagonian oak	2.8	4.4	0.8	1.2	20.8

Prunus domestica	Common plum	2.8	3.8	1.6	2.7	4.2
Pinus sylvestris	Scots pine	2.5	2.5	2.4	2.3	4.2
Tilia cordata	Small-leaved lime	2.5	2.5	2.4	2.7	0.0
Acer platanoides	Norway maple	2.1	2.5	1.6	2.3	0.0
Ranunculus repens	Creeping buttercup	1.8	1.3	2.4	1.5	4.2
Sorbus aucuparia	Rowan	1.8	1.9	1.6	0.8	12.5
Taraxacum sp.	Dandelion	1.8	1.3	2.4	1.9	0.0
Abies concolor	White fir	1.4	0.6	2.4	1.2	4.2
Plantago lanceolata	Ribwort plantain	1.4	1.3	1.6	1.5	0.0
Prunus cerasifera	Cherry plum	1.4	1.9	0.8	1.2	4.2
Quercus cerris	Turkey oak	1.4	2.5	0.0	1.5	0.0
Taxus x media	Anglojap yew	1.4	1.3	1.6	1.5	0.0
Tilia platyphyllos	Large-leaved lime	1.4	0.6	2.4	1.2	4.2
Viola lactea	Pale dog-violet	1.4	1.9	0.8	1.5	0.0
Prunus serotina	Black cherry	1.1	1.3	0.8	0.0	12.5
Prunus spinosa	Blackthorn	1.1	1.9	0.0	1.2	0.0
Rhododendron ponticum	Common rhododendron	1.1	0.0	2.4	0.4	8.3
Sonchus oleraceus	Common sowthistle	1.1	0.6	1.6	0.8	4.2
Acer velutinum	Persian maple	0.7	0.6	0.8	0.8	0.0
Aucuba japonica	Japanese laurel	0.7	0.6	0.8	0.8	0.0
Castanea sativa	Sweet chestnut	0.7	0.6	0.8	0.4	4.2
Chenopodium album	Fat hen	0.7	0.0	1.6	0.8	0.0
Prunus padus	Bird cherry	0.7	0.0	1.6	0.4	4.2
Prunus persica	Peach	0.7	1.3	0.0	0.8	0.0
Quercus faginea	Portuguese oak	0.7	1.3	0.0	0.8	0.0
Rosa canina	Dog-rose	0.7	1.3	0.0	0.8	0.0
Rosa moschata	Musk rose	0.7	0.6	0.8	0.8	0.0
Salix caprea	Goat willow	0.7	1.3	0.0	0.0	8.3
Solanum sp.	Nightshade	0.7	0.0	1.6	0.8	0.0
Viola reichenbachiana	Early dog-violet	0.7	1.3	0.0	0.8	0.0

Abies delavayi	Delavay's silver-fir	0.4	0.0	0.8	0.0	4.2
Acer japonicum	Amur maple	0.4	0.0	0.8	0.4	0.0
Arctium minus	Lesser burdock	0.4	0.0	0.8	0.4	0.0
Bidens sp.	Beggarticks	0.4	0.6	0.0	0.4	0.0
Cerastium fontanum	Mouse-ear chickweed	0.4	0.0	0.8	0.0	4.2
Crataegus monogyna	Common hawthorn	0.4	0.6	0.0	0.4	0.0
Cupressus macrocarpa	Monterey cypress	0.4	0.6	0.0	0.4	0.0
Cupressus sempervirens	Mediterranean cypress	0.4	0.0	0.8	0.4	0.0
Eucalyptus sp.	Eucalyptus	0.4	0.0	0.8	0.0	4.2
Geranium robertianum	Roberts geranium	0.4	0.0	0.8	0.4	0.0
Geum urbanum	Wood avens	0.4	0.0	0.8	0.4	0.0
Heracleum sphondylium	Hogweed	0.4	0.6	0.0	0.4	0.0
Pinus luchuensis	Luchu pine	0.4	0.6	0.0	0.4	0.0
Primula veris	Cowslip	0.4	0.0	0.8	0.4	0.0
Prunus laurocerasus	Cherry laurel	0.4	0.6	0.0	0.4	0.0
Quercus rubra	Northern red oak	0.4	0.0	0.8	0.4	0.0
Rhododendron caucasicum	Rhododendron	0.4	0.0	0.8	0.0	4.2
Ribes nigrum	Blackcurrant	0.4	0.0	0.8	0.4	0.0
Rosa arvensis	Field rose	0.4	0.6	0.0	0.4	0.0
Rosa caesia	Hairy dog rose	0.4	0.6	0.0	0.4	0.0
Rubus idaeus	Red raspberry	0.4	0.0	0.8	0.4	0.0
Rubus silvaticus	Bramble	0.4	0.6	0.0	0.4	0.0
Stellaria media	Chickweed	0.4	0.0	0.8	0.4	0.0
Taraxacum officinale	Common dandelion	0.4	0.0	0.8	0.4	0.0
Tilia sp.	Lindens	0.4	0.0	0.8	0.0	4.2
Veronica chamaedrys	Bird's-eye speedwell	0.4	0.0	0.8	0.4	0.0
Vicia sepium	Bush vetch	0.4	0.6	0.0	0.4	0.0

Table 2.3. Results for the univariate *"anova"* test in the UK *manyglm* model. Significant (p<0.05) plant genera differences for the test variable "year" in the final model are shown ordered by taxonomic genera. Likelihood ratio test values (LRT) and *p*-values are given for the univariate test. Percent frequency of occurrence values (% FOO) for each plant genera across the factor level are indicated.

Predictor variable	Plant genus	LRT	*p*-value	%FOO 2016	%FOO 2017	%FOO 2018	%FOO 2019
Year	*Anacardium*	45.7	0.001	0	0	0	27.9
Year	*Betula*	43.5	0.001	6.3	48.5	1.3	7.5
Year	*Carpinus*	15.9	0.028	75	36.4	3.8	23.1
Year	*Fagus*	23.7	0.003	56.3	36.4	92.3	59.9
Year	*Helianthus*	22.7	0.009	87.5	69.7	12.8	50.3
Year	*Prunus*	17.7	0.014	50	39.4	9	23.8
Year	*Quercus*	39	0.001	25	72.7	19.2	40.1
Year	*Ulmus*	14.8	0.037	0	12.1	17.9	19

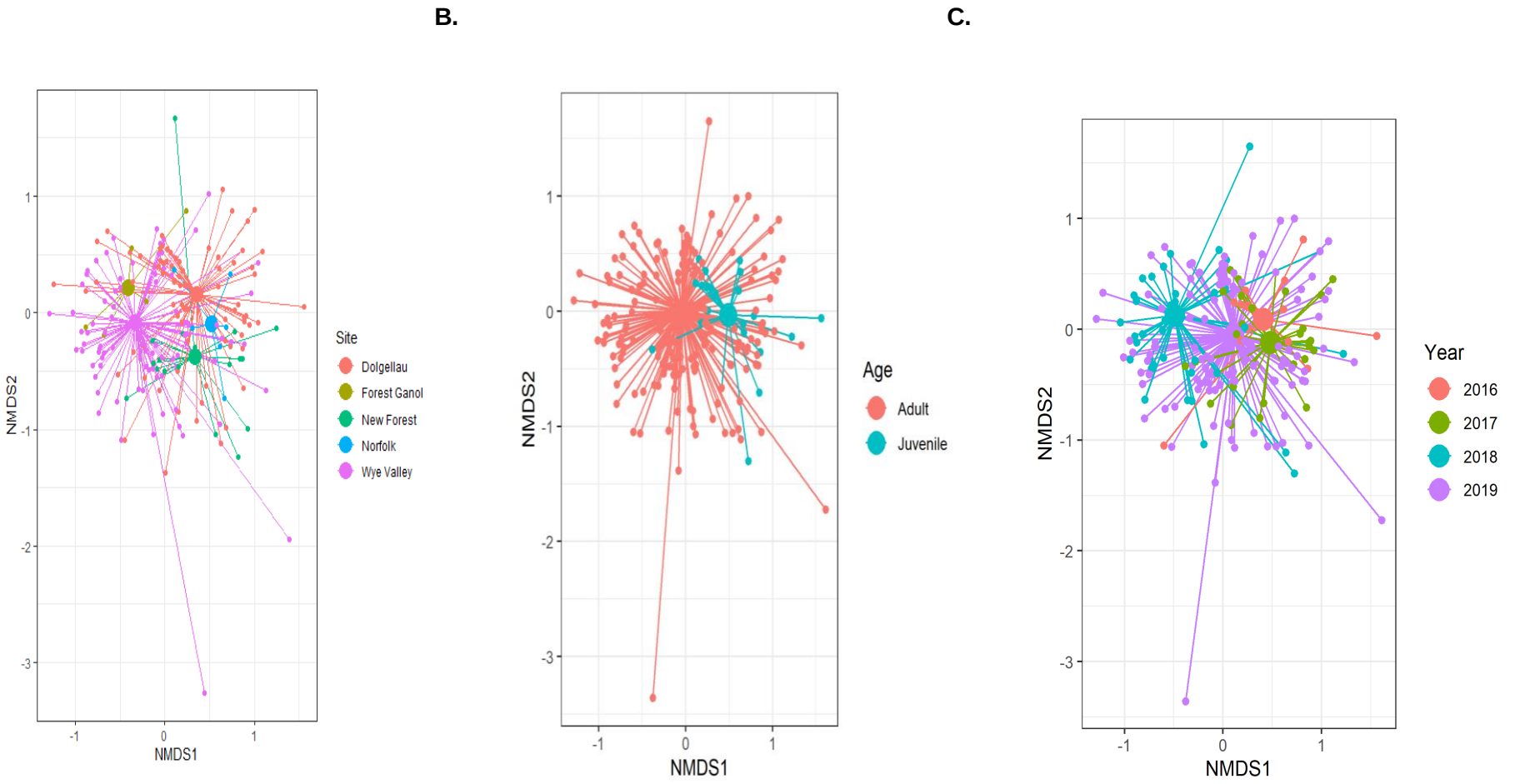
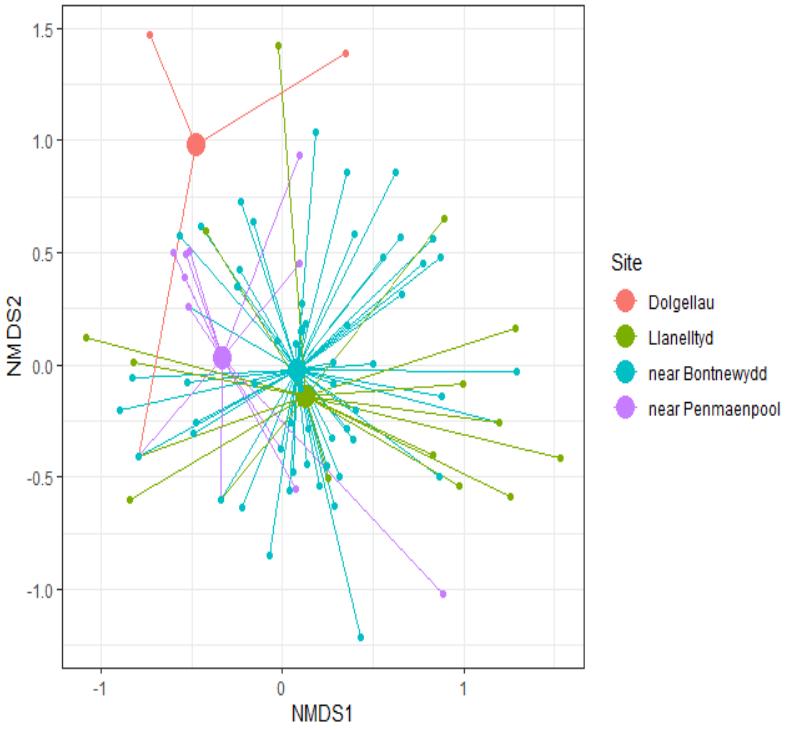


Figure 2.3. Spider plot for herbivorous taxa consumed by Hawfinch across (A) geographic regions, (B) age-classes and (C) sampling years across the UK. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its region. Stress = 0.18.

A.



B.

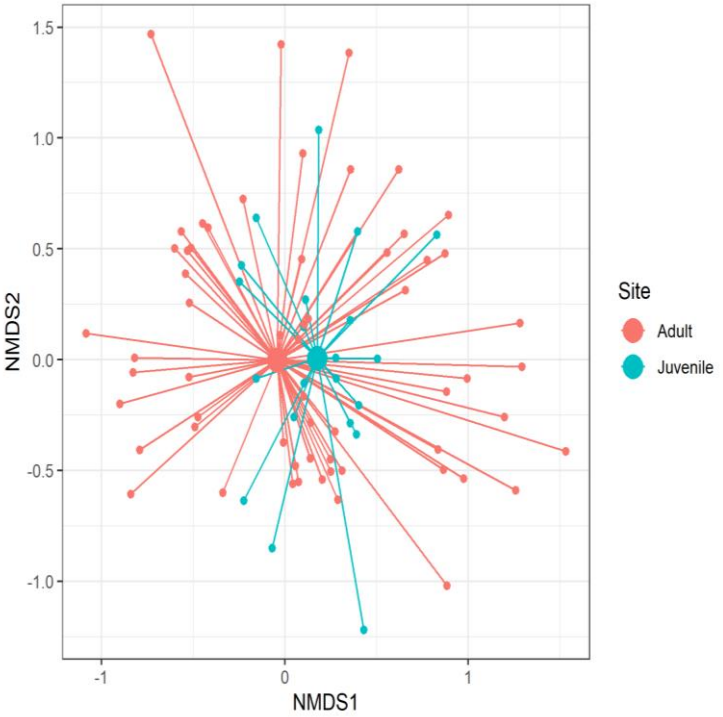


Figure 2.4. Spider plot for herbivorous taxa consumed by Hawfinch across (A) feeding sites and (B) age-class within north Wales (overleaf). Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its sample site. Stress = 0.19.

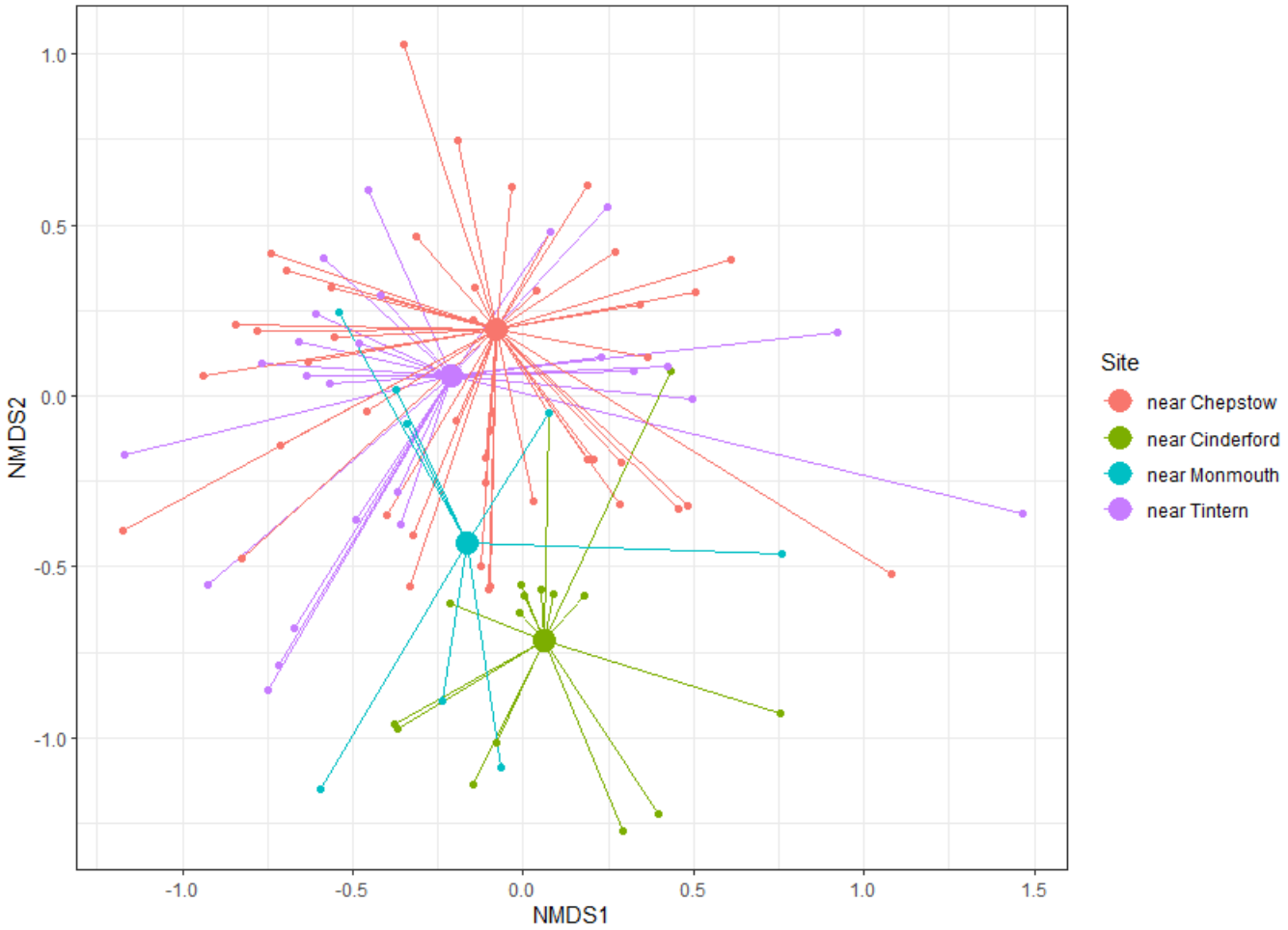


Figure 2.5. Spider plot for herbivorous taxa consumed by Hawfinch at (A) feeding sites within the Wye Valley. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its sample site. Stress = 0.16.

2.5 Discussion

Using DNA metabarcoding, this study shows Hawfinch diet is much more diversified than previously described by personal observation (Mountford 1957), with the results showing Hawfinch are able to utilise over 80 plant taxa. The results indicated strong spatial and temporal variation in diet composition, with differences between site and year potentially reflecting a response by Hawfinch to fluctuation in food availability within heterogenous environments, thus suggesting dietary plasticity may be an advantageous trait (Ribeiro *et al.* 2019). Our data suggest that regional and local differences in food availability may influence dietary composition of Hawfinch (Tournayre *et al.* 2021). There was also support for the hypothesis that diet differed due to demography. Juveniles had significantly different dietary compositions compared to adults, however there was limited support to the hypothesis that dietary composition differed between sexes.

2.5.1 Diet composition

Many taxa present in the diet were rare, as documented in previous faecal metabarcoding studies on passerines (Shutt *et al.* 2020; da Silva *et al.* 2020; Sottas *et al.* 2020). Our findings on Hawfinch diet composition agree with previous observations of this species (Mountford 1957; Newton 1967; Bijlsma 1998). Previous studies showed seeds of hornbeam, cherry and sycamore were important throughout the year (Mountford 1957; Bijlsma 1998), with buds of ash, maple and beech becoming important food resources during spring and summer (Bijlsma 1998). The importance of beech as a food resource was confirmed in this study, being the most prevalent taxon (detected in 65% of samples). It is well understood that herbivorous birds must balance food handling times with net energy intake, and a resource is deemed more profitable if it has a higher energy reward per unit handling time (Molokwu *et al.* 2011). It is known that Hawfinch feed on beech nuts during autumn and winter months (Mountford 1957) due to the moderately high fat and carbohydrate levels of the beechnuts compensating for energy losses during winter (Renner *et al.* 2013). The onset of the breeding season can drive changes in feeding preferences as nutritional needs become higher (Lima 2009). As the sampling effort in this study began during the pre-breeding season and continued to the end of summer, Hawfinch may still have been gaining a high energy reward from feeding on beech nuts, but also from the increased availability of beech buds as a food resource later in the sampling period, when bud burst occurs in April to May (Vitasse *et al.* 2009). To determine if Hawfinch are primarily feeding on a single tissue type or utilising multiple tissue types, future directions should include personal observation of Hawfinch feeding, in order to establish which tissue is being consumed. Using metabarcoding alone, it is not possible to determine which plant tissue is being eaten (seed, bud, fruit etc). Combining plant species (from

metabarcoding) in conjunction with knowing which part of a plant is being consumed, enables a better understanding of the feeding ecology of the Hawfinch.

The metabarcoding results revealed oak to be prevalent within the diet, something not reported previously. It is likely that Hawfinch were feeding on oak buds, as the phenology of bud burst for English and sessile oak coincided with the time of sampling (Wilkinson *et al.* 2017). Past research undertaken on Hawfinch diet during winter (months unspecified) and during the breeding season (April to August), broadly fitted with the sampling period of this study, and it is unusual therefore, that oak was not observed as a food resource (Mountford 1957). Hawfinch dietary studies have focused on direct observations of feeding, and while this method was widely used at the time of Mountford (1957), direct observation has known limitations such as observer bias and error, as well as results being influenced by data recorded from habitats in which a species is most observable (Matthews *et al.* 2020). The results from this research highlight the power of DNA metabarcoding to reveal previously unknown dietary items. It is important to note however, that a caveat of metabarcoding is secondary predation, which is the process of DNA being detected within the diet of a predator which has fed on a second predator, which in turn has consumed a target prey (Forin-Wiart *et al.* 2018). Secondary predation is common within natural systems, as generalist predators consume resources at multiple trophic levels (Sheppard *et al.* 2005). Secondary predation via lepidopteran taxa may have resulted in falsely inflated detection of oak (and other plant) taxa through co-amplification of plant DNA within the guts of Lepidopteran taxa consumed by Hawfinch (explored in Chapter 3). Due to metabarcoding methods being unable to determine which plant tissue is being eaten, in conjunction with Hawfinch also feeding on the same plant taxa as their prey, untangling what is “true” secondary predation is extremely challenging.

The low frequency of cherry detected may be explained by the decline of traditional cherry orchards within the UK. While cherry was present in 23.4% of samples across the UK, the genus has previously been observed to be an important food resource and frequently consumed, with Hawfinch even being described as a “pest” of cherry orchards in the 1800’s (Mountford 1957; Bijlsma 1998). However, it remains unclear as to the extent to which Hawfinch utilised cherry orchards compared with wild cherry within deciduous woodlands, and therefore this result should be treated cautiously. Traditional orchards are considered to be species-rich High Nature Value Farming Systems (HNVFS) within Europe (Cooper *et al.* 2007; Bailey *et al.* 2010), and together with woodland habitat such as semi-ancient woodland, provide an important refuge for arthropods and birds which do not occur within intensified, modern agricultural landscapes (Bailey *et al.* 2010). Orchards have declined by approximately 80% within Europe since the 1950’s, with the UK losing up to 90% in that period (Bailey *et al.* 2010). Due to the loss of these orchards and their associated cherry species, Hawfinch may

have had to demonstrate plasticity within their diet to adapt to the decreased availability of this food resource. Future studies should focus on improving macro-nutrient knowledge of this resource to improve understanding of Hawfinch nutritional requirements, as Hawfinch may feed less often on buds or flowers due to seeds offering different nutritional resources.

The reduced prevalence of elm, a commonly utilised resource in the past Mountford (1957), may be explained by the epidemic of Dutch elm disease (Thomas *et al.* 2018). The first epidemic occurred between 1920-1940 and killed ~30% of elms, while the second in the 1980s killed an estimated 28 million mature elm trees and caused the subsequent death of 20 million young elm trees throughout Europe (Thomas *et al.* 2018). Due to Dutch elm disease, the abundance and therefore decreased availability of wych elm seeds in the spring months, may have resulted in Hawfinch showing dietary plasticity in order to make use of other, more abundant food resources.

Non-native species were widespread in Hawfinch diet. Some of these may derive from bird seed and the use of garden bird feeders, such as cashew (*Anacardium occidentale*), which was found in 14.7% of samples and may result from cashew nuts being processed in the same factory as sunflower seeds used at artificial feeding sites, or cashew nuts being used as a filler in the sunflower seed mix. Other non-native species may be a result of Hawfinch utilising arboretums within their foraging areas, with Blackwater and Bolderwood arboretums situated within the New Forest, and the Cyril Hart Arboretum located close to the Forest of Dean. It is known that Hawfinch utilise forest habitat at a landscape scale (Kirby *et al.* 2015), and therefore may be utilising non-native taxa found within these arboretums. Furthermore, gardens visited by Hawfinch may contain mature non-native tree species, as well as Hawfinch accessing non-native tree species introduced for forestry purposes, such as eucalyptus (Brundu and Richardson 2016; Pötzelsberger *et al.* 2020).

2.5.2 Variation in Hawfinch diet

The results from this study revealed that taxa within Hawfinch diet vary spatially and temporally. This spatial variation is consistent with similar metabarcoding studies of birds, as well as studies on insectivorous bats (McClenaghan *et al.* 2019; Alberdi *et al.* 2020; Shutt *et al.* 2020). This could indicate local dietary specialisation; however it is more probable that Hawfinch show dietary plasticity and these spatial differences in diet composition arise from changing availability of food resources. This may be a result of habitat differences within each study region. The Wye Valley and north Cardiff regions occur within heterogeneous woodlands consisting of predominately beech and elm, while the north Wales region consisted of woodland supporting hornbeam and cherry. This differentiation in resource use can be seen in the nMDS plot (Figure 2.3). While there is a degree of overlap between all regions, the Wye

Valley and north Cardiff sampling regions are situated closer together, indicating dietary taxa detected from Hawfinch sampled within these regions show higher levels of similarity than dietary taxa from Hawfinch sampled in north Wales. Future work should analyse the dietary richness of Hawfinch at landscape and local scales, in order to examine the breadth of Hawfinch diet and to investigate whether Hawfinch show more specialised or generalist diets. This could also be compared across sites, to determine whether site-specific differences in tree species and, therefore, assumed food availability was a factor behind dietary differences.

To the best of my knowledge, this is the first study to explore intraspecific age-class dietary differences within a passerine species. The significant variation in diet between adult and juvenile Hawfinch may be due to juvenile birds being inexperienced at associating cues with food resources, with younger birds having had fewer opportunities to gain foraging experience (Franks and Thorogood 2018). Within sea birds, age-related dietary provisioning differences have been attributed to differing foraging behaviours during peak resource demands (Mott *et al.* 2016). Juvenile birds do not forage as effectively as adults, and therefore will make use of abundant and easy to obtain food resources (Franks and Thorogood 2018). It is important to note, however that juvenile sample size was small (n=24) and therefore the results presented in this study should be viewed accordingly. Morphological differences between adults and juveniles, such bill size or shape may impact on juveniles ability to process similar food resources to adult birds (Temeles *et al.* 2017). Bill length and size were not measured in this study, but should be included in future work, to investigate and explore any morphological differences between juvenile and adult birds.

Previous studies exploring sexual resource partitioning have focused on species with considerable sexual dimorphism in body size, bill size, or shape, while dietary differences within monomorphic species remain underrepresented within the literature (Bravo *et al.* 2016; Temeles *et al.* 2017; Thalinger *et al.* 2018; da Silva *et al.* 2020). This study did not detect sexual differences in diet composition, possibly related to Hawfinch showing minimal sexual dimorphism, which in principle allows both sexes to utilise similar plant taxa and may offer a plausible explanation as to why dietary differences were not detected.

2.5.3 The use of supplementary food within Hawfinch populations

Supplementary food was frequently detected within Hawfinch faecal samples (44.1%). This was expected as supplementary food is provided *ad libitum* from December-July, with all faecal samples for this study collected at artificial feeding sites within this time period. An interesting result was the spatial variation in supplementary food prevalence. Hawfinch sampled in north Wales showed a far higher prevalence of supplementary food within the diet (FOO 68.7%) compared with individuals sampled from the Wye Valley (FOO 23.9%). It has

been noted from previous observations that Hawfinch populations in north Wales visit supplementary feeding sites within woodland and garden environments more frequently than in other areas of the UK. This spatial differences in Hawfinch diet, and the change in prevalence of supplementary food, may be due to a shortage of preferred natural food items within the north Wales sites at the time of sampling. The Hawfinch population within north Wales may have developed a widespread use of garden feeders, as Hawfinch visiting garden feeders has been consistently observed over many years (Paul Bellamy, pers. comms). Furthermore, the frequency of occurrence of supplementary food was greater within juveniles than adults (FOO 79.2% and 40.8% respectively). This increased prevalence within juvenile diet was expected, as young birds are likely to be inefficient foragers and will make use of easily accessible food resources (Franks and Thorogood 2018). An additional reason behind the differences found in supplementary food consumption between age classes may be competition. Adult birds may be more dominant than juveniles, and if juveniles need to avoid more dominant adults, they may visit differing foraging locations and exploit sub-optimal food types (van den Hout *et al.* 2014).

Binomial mixed modelling showed a significant difference in supplementary food prevalence between Hawfinch populations in north Wales and the Wye Valley. The reduced prevalence for supplementary food in Hawfinch populations within sites in the Wye Valley could indicate a preference towards natural food resources known to be more profitable than supplementary food, either energetically or nutritionally, with many seed eating birds selecting diets to meet both these requirements (Molokwu *et al.* 2011). The foraging environment within the Wye Valley may also contain an increased abundance of natural food resources when compared with foraging areas within north Wales. The temporal variation of supplementary food may have been driven by climatic conditions. Under conditions of high rainfall and colder temperatures, natural food availability is decreased (Southwood *et al.* 2004; Shutt *et al.* 2020) and as a result, net benefits from supplementary food are increased as a result of nutritional limitation (Nager *et al.* 1997). The increased prevalence of supplementary food detected in Hawfinch sampled in 2016 compared with 2018 may have been driven by the higher average rainfall measured in 2016 (1,380.5mm) compared with 1,320.8mm in 2018 (Met Office 2018), resulting in an increased benefit from supplementary food if foraging effectiveness was limited by rainfall. Furthermore, the sampling months in 2016 were July and September with Hawfinch faecal samples collected in January, April, May and July of 2018. Temporal changes in supplementary food prevalence may be driven by natural food availability as the spring “peak” of invertebrates (Southwood *et al.* 2004) would have already occurred by July and September when the faecal samples were collected. The decreased prevalence of supplementary food

from samples collected in 2018 compared with 2019 may also be attributed to a change in natural food availability.

With metabarcoding data providing information on the prevalence of natural food items, these data can be used in combination with plant phenology information to highlight periods where natural food may be scarce. This information can be used to guide management of Hawfinch conservation in limiting the provision of supplementary food only to selected times. The energetic and nutritional requirements for Hawfinch have not been quantified, and little is known to what extent these requirements are met by both wild and supplementary food. To increase understanding of the requirement for supplementary food and to, ultimately, reduce the volume used, these knowledge gaps require further information.

It is important to monitor the availability of anthropogenic food resources as this can lead to dietary changes, changes in body condition (Auman *et al.* 2008), productivity (Plummer *et al.* 2013) and population size (Duhem *et al.* 2008). Some of these impacts can be beneficial, such as reduced energy expenditure, increased body condition and increased breeding performance (Auman *et al.* 2008; Flack *et al.* 2016). However, if the diet shifts towards food resources of poorer quality, this can cause nutritional stress (Will *et al.* 2015), reduce both adult and fledgling body mass (Österblom *et al.* 2006) and may be linked to population declines (Kitaysky *et al.* 2006). For this study, artificial feeding sites were unavoidable for sample collection. Encouraging Hawfinch to feed in flocks on the ground is the only viable method which enables mist net capture and study of Hawfinches. While supplementary food can enhance bird survival, and is predominantly used by wild birds when natural food supplies are reduced, research has shown there is an increased risk that wild birds may become reliant on artificial food sources (Lawson *et al.* 2018; Støstad *et al.* 2019). Furthermore, supplementary feeding may increase the risk of disease transmission such as trichomoniasis (Lawson *et al.* 2018). There is evidence that high congregation densities for a prolonged period of time and poor hygiene can result in pathogen contamination of feeding stations (Murray *et al.* 2016; Lawson *et al.* 2018). Additionally, Støstad *et al.* (2019) found evidence that a high intake of sunflower seed can negatively impact sperm quality of finches. This is due to sunflower seeds containing high levels of linoleic acid, which in high levels can damage the cell membrane of sperm cells (Støstad *et al.* 2019). It is therefore vital to manage the volume and type of supplemental feed provided.

2.5.4 Conclusions and recommendations for future research and conservation management

Dietary analysis by metabarcoding indicates that Hawfinch show broad dietary niche breadth. However, there is evidence to suggest that subpopulations of Hawfinch may be reliant on supplementary food, and future research must take this into consideration. A consequence of

such reliance could be unintended negative effects such as disease transmission, decreased breeding success and reliance on artificial feeding sites (Lawson *et al.* 2018; Støstad *et al.* 2019). Combining the metabarcoding data with nutritional and phenological information would help to identify gaps in natural food availability as well as improving understanding of the importance of each dietary item for the consumer. Conservation management of planting specific taxa in order to fill these gaps can then be undertaken. However, only supplying supplementary food during the gaps in natural food availability may reduce dependency and disease transmission.

Hawfinch are known to ground feed on fallen seed during winter months (Mountford 1957), however the winter diet of Hawfinch has not been explored in detail. This study has focused on Hawfinch diet between March-June, therefore, to improve knowledge of Hawfinch diet throughout the year, a wider sampling season should be undertaken. This will generate an insight in order to establish whether the “hunger gap” can be identified as a factor in Hawfinch decline, as identified in other seed-eating species (Siriwardena *et al.* 2008).

The results of this study were only possible due to the high taxonomic resolution available through metabarcoding methods. As metabarcoding is becoming more prevalent within ecological research, it becomes increasingly important to understand how taxonomic resolution can impact ecological studies, although species-level identification may not always be necessary, depending on the question (Brown *et al.* 2014; Renaud *et al.* 2020). The study presented is an example of how the utilisation of DNA metabarcoding can improve ecological understandings and to improve insights into fine scale ecological patterns. Finally, determining the nutritional composition of tissue type consumed for each species is recommended to understand the importance of each dietary item for consumer fitness.

2.6 Acknowledgements

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Chapter Three – Exploring invertebrate taxa in the diet of UK Hawfinch (*Coccothraustes coccothraustes*) populations

A Hawfinch foraging on the forest floor. Photo credit: Andy Stanbury; Hawfinch Ringing Group.

3.1 Abstract

Dietary niche separation can reduce resource use competition between individuals within populations and can impact how individuals respond to environmental variation. While environmental factors may impact the diet of individuals within a population, dietary differences may occur within, for example, the same habitat. Individual dietary differences can also be driven by intrinsic factors such as age and sex. There is limited knowledge of the diets of woodland bird species, due primarily to difficulties in accurately identifying taxa consumed. The Hawfinch (*Coccothraustes coccothraustes*) is a primarily herbivorous bird (Chapter 2), however it is known to take invertebrates during the breeding season. To analyse the dietary composition of invertebrate taxa in Hawfinch diet, DNA metabarcoding of invertebrate remains in faeces was undertaken. Faecal samples were obtained from 2016-2019 from Hawfinches across five regions the UK. DNA was extracted from 120 individuals and invertebrate DNA was amplified using the Cytochrome Oxidase I (COI) barcoding region. Winter moth (*Operophtera brumata*), St Mark's Fly (*Bibio marci*) and tree slug (*Lehmannia marginata*) were the most frequently detected species. Invertebrate dietary composition was distinct between

regions of the UK, as well as between years, indicating Hawfinch show spatial and temporal variation in their diet. Males were found to have significantly fewer prey items in their diet than females during the breeding season, indicating that males may be more selective foragers, focus on plants compared to invertebrates, or that females are limited in their foraging due to being unable to forage far from nesting sites.

3.2 Introduction

Understanding how the composition of diet varies within bird populations can be an important step into understanding how a population or species will be able to adapt in response to anthropogenic changes in their foraging environment (Mitchell *et al.* 2021). Environmental change is likely to impact certain guilds of species to a greater extent than others, for example omnivorous species, which increase their dependence on insect prey during the breeding season (Stone *et al.* 2019). The dietary composition of insectivorous birds may reflect local spatial and temporal variation in prey abundance related to habitat type (Mills *et al.* 2020), as well as reflecting the nutritional content of prey or habitat quality (Razeng and Watson 2015). Within forests, invertebrate richness can vary between tree taxa and within the UK, willow (*Salix sp.*), oak (*Quercus sp.*) and birch (*Betula sp.*) have been found to contain the highest invertebrate species richness (Shutt *et al.* 2019). Thus, invertebrate richness and community composition can in turn impact reproductive success, as birds have differing nutritional requirements at different life cycle events, for example many woodland bird species feed chicks a higher proportion of spiders during early chick development in order to provide amino acids necessary for growth (Ramsay and Houston 2003). Declines in insect abundance have been widely reported in Europe and other parts of the world (Hallmann *et al.* 2017; Hallmann *et al.* 2020; Sánchez-Bayo and Wyckhuys 2021). If widespread declines in insect abundance continue, this could impact avian responses to environmental change, by reducing the ability to meet energetic requirements during the breeding season (Bowler *et al.* 2019). Research has shown that insects, many of which have a short generation time, may be able to respond to environmental changes more rapidly than other organisms (Thomas *et al.* 2004) such as insectivorous avian species (Jedlicka *et al.* 2017). This can result in invertebrate food availability being too depleted to support avian populations during the breeding season (Visser *et al.* 2012; Ramakers *et al.* 2019).

Dietary variation can also be driven by intrinsic factors such as sexual partitioning of food resources (Svanbäck and Bolnick 2007; Jones *et al.* 2020). Sexual differentiation in resource use is commonly observed in vertebrates (Mata *et al.* 2016). Segregation is often associated with behavioural or morphological differences between sexes which subsequently impacts life-history traits such as diet (Mata *et al.* 2016; da Silva *et al.* 2020). Sexual differences in prey

choice may occur through different nutritional requirements required for egg production in females (da Silva *et al.* 2020), or through reduced foraging distances, as the female cannot leave the nest for long periods (Amininasab *et al.* 2017). This may result in females foraging closer to their offspring, and subsequently feeding on more abundant or predictable prey items, while more mobile males may be able to exploit a wider prey range (da Silva *et al.* 2020). While sexual partitioning of food resources is known to occur between bird species exhibiting sexual dimorphism (Bravo *et al.* 2016; Thalinger *et al.* 2018), the hypothesis that differences in prey choice also occurs in monomorphic species remains poorly explored (Cleasby *et al.* 2015; da Silva *et al.* 2020).

Identifying drivers of dietary variation in woodland birds is challenging. This is due to woodland birds often foraging high within trees on small prey items, resulting in relatively little being known about their diet. It is difficult to accurately identify dietary items from observational data which typically only record information on foraging location within trees and attempt to infer food availability (Mackenzie *et al.* 2014). Within temperate environments, insectivorous passerine birds often demonstrate broad dietary ranges, feeding on a wide range of invertebrate taxa (Cholewa and Wesołowski 2011; Shutt *et al.* 2020). However, dietary variability within generalist woodland species is a poorly understood topic. It has been proposed that the diet of generalists could vary spatiotemporally, based upon invertebrate resource availability and prey preferences of the consumer (Shutt *et al.* 2020). Spatial variation of invertebrates consumed may result in geographical patterns in avian population density, breeding productivity differences and local adaptation to resource use (Shutt *et al.* 2020).

3.2.1 Study species

The Hawfinch is found extensively throughout the Palearctic, with the United Kingdom (UK) at the westerly range limit (Kirby *et al.* 2015). Although Hawfinch are not globally threatened, they showed a 76% reduction in the number of 10km squares occupied in Britain between 1968 and 2011 (Balmer *et al.* 2013), further evidenced by Langston *et al.* (2002), who estimated a 40% population decline between the mid 1980's and late 1990's (Langston *et al.* 2002; Kirby *et al.* 2015; Kirby *et al.* 2018). Localised breeding extinctions across central and eastern England have been recorded, and now Hawfinch only occupy 4% of 10km squares in the UK (Balmer *et al.* 2013; Kirby *et al.* 2018). The population strongholds within the UK show a westerly bias, in heavily wooded landscapes defined by mature, species rich tree communities (Kirby *et al.* 2018). Hawfinch are thought to be predominantly single-brooded, although a study by Kirby *et al.* (2019) showed double brooding can occur, with the main egg laying period being late-April to late-May. Hawfinch have been shown to be primarily herbivorous (Chapter 2), however they are known to feed on invertebrates during the breeding season (Mountford 1957). Previous observations have suggested that nestling diet comprises

primarily the larvae of the oak roller moth (*Tortrix viridana*) and winter moth (*Operophtera brumtata*), with adult Hawfinches feeding primarily on moths (Lepidoptera), beetles (Coleoptera), bugs (Hemiptera), earthworms (Annelida), snails (Gastropoda) and spiders (Araneae) (Mountford 1957).

Previous dietary studies have used traditional dietary determination methods such as direct observation of feeding, or microscopic examination of gut or faecal samples (Pompanon *et al.* 2012). However, these methods have major limitations. Direct observation of feeding is difficult when observing woodland birds feeding on small invertebrates within the canopy. Microscopic identification of taxa is labour intensive and demands a high level of taxonomic knowledge in order to correctly identify semi-digested fragments of plants or animals (Pompanon *et al.* 2012; Shutt *et al.* 2020). Furthermore, this problem is intensified when inferring dietary composition of insectivorous birds, as many of the dietary components will be missed due to no hard remains or diagnostic taxonomic features remaining (Pompanon *et al.* 2012; Rytkönen *et al.* 2019). Consequently, this has resulted in dietary components identified to a low taxonomic resolution (Rytkönen *et al.* 2019).

The combination of high-throughput sequencing (HTS) with DNA barcoding, referred to as "DNA metabarcoding" has provided a platform to obtain dietary information from food remains within faecal samples or stomach contents at a high taxonomic resolution, while being non-invasive (Symondson 2002; Pompanon *et al.* 2012; Deagle *et al.* 2019). Due to previous methodologies recording species at a coarse taxonomic resolution, subtle differences in prey consumption may not be detected, resulting in fine scale inferences relating to species' ecology not being identified (Mata *et al.* 2016). This is evidenced in dietary analysis methods used within avian ecology, including direct observation of foraging (Matthews *et al.* 2020), morphological identification of semi-digested food remains (Bravo *et al.* 2016), fatty acids and alcohol analysis (Owen *et al.* 2013) and stable isotope analysis (Ruhl *et al.* 2019). In a diet which contains a wide range of invertebrate taxa, DNA barcodes from regions of the COI mitochondrial gene region have become the standard and used in many species-level identification studies (Kress *et al.* 2015). This is due to the expansive taxonomic coverage and depth within the USA, Canada, UK and European taxonomic COI reference sequence databases (Porter and Hajibabaei 2018). Therefore, the possibility of false taxonomic assignment is reduced, and improved higher taxonomic resolution is possible (Somervuo *et al.* 2017; Andújar *et al.* 2018; Porter and Hajibabaei 2018).

The application of metabarcoding within avian studies has been minimal, in part due to the challenging process of extracting and amplifying dietary DNA from avian faeces (Vo and Jedlicka 2014; Jedlicka *et al.* 2017; Shutt *et al.* 2020). Consequently, avian faecal

metabarcoding studies have been limited to small sample sizes and/or locations (King *et al.* 2015; Jedlicka *et al.* 2017; Rytkönen *et al.* 2019). Crisol-Martínez *et al.* (2016) and Jedlicka *et al.* (2017) successfully demonstrated that insectivorous birds consumed herbivorous insects found within agricultural landscapes, providing a pest-reduction service within agro-ecosystems. Trevelline *et al.* (2016) demonstrated nestlings of the Louisiana waterthrush (*Parkesia motacilla*), a stream-dependent species, were consuming terrestrial Lepidoptera which may have escaped detection in previous dietary studies which used traditional morphological approaches. Shutt *et al.* (2020) successfully studied the diet of blue tits (*Cyanistes caeruleus*) during the breeding season to far higher taxonomic resolution than previous work. These previous studies illustrate some of the new insights that DNA metabarcoding can afford.

This chapter aimed to use a DNA metabarcoding methodology to investigate the prey dietary composition of Hawfinch populations across five population strongholds in the UK. This chapter aimed to show the dietary diversity and key trophic interactions of Hawfinch, examine spatial and temporal variation in diet, as well as examining dietary sexual segregation between males and females. More specifically, I hypothesised:

i) Hawfinch dietary composition would vary spatially and temporally, as prey consumption will differ between regions due to spatial and temporal differences in prey availability.
ii) Dietary richness would differ between sexes (da Silva *et al.* 2020), as females are spatially limited to where they can forage due to increased parental care.
iii) Dietary composition would differ between sexes due to differing energetic requirements of males and females.

3.3 Methods

3.3.1 Study area

All study areas and field sampling methods are as described in Chapter 2.

3.3.2 DNA extraction, PCR amplification and high-throughput sequencing

DNA extraction was carried out as described in Chapter 2, following modifications by Shutt *et al.* (2020). Primers used were mlCOIintF, 5'–GGWACWGGWTGAACWGTWTAYCCYCC-3' (Leray *et al.* 2013), with Nancy 5'-ACTAGCAGTACCCGGTAAAATTAAAATATAAACTTC-3', (Simon *et al.* 1992), following selection and modification by Stockdale (2018) for amplification of a 306 base pair Cytochrome Oxidase I (COI) region. Primers were validated to ensure DNA amplification from the expected range of invertebrate taxa (seven insect orders, one arachnid order and one mollusc order).

A two-stage PCR process was undertaken as documented in Chapter Two. Initial Polymerase Chain Reactions (PCRs) of 5µL contained 2.5 µL multiplex mix (Qiagen, Manchester UK), 0.1µL of 10 µM MICOIintF and Nancy primer pair, 1.3µL of DNase-free water, and 1µL of template DNA. 1 µL of DNase-free water was used instead of template DNA for negative PCR controls. Reactions were carried out in an Applied Biosystems SimpliAmp™ 96-well thermocycler. PCRs comprised 15 minutes denaturation at 95°C, followed by 35 cycles of 95°C for 30s, primer annealing 55°C for 90s, a PCR product extension at 72°C for 90s followed by a final extension at 72°C for 10 min. PCR products were run through a 2% agarose gel, stained using SYBR®Safe (Invitrogen). To quantify band sizes, 2µL of Promega™ 100bp ladder was included in the final well of the gel. If a band was not detected for a second time, the PCR was repeated using 0.5 µL of template DNA. Any samples which did not produce a ban after three PCR tests were omitted.

Samples which showed a positive result (a DNA band on a 2% agarose gel) were taken forward for molecular identifier tagged (MID-tag) PCR as detailed in Chapter 2. This process involved labelling the forward and reverse primers with MID-tags, following (Moorhouse-Gann 2017). Samples had a unique pairing of forward and reverse tags for sample identification post-sequencing (Brown *et al.* 2014). A total of 25 unique forwards and 12 unique reverses were used (Appendix 2.1). Reactions were carried out in the same Applied Biosystems SimpliAmp™ 96-well thermocycler, with annealing temperatures optimised through temperature gradient PCRs in the same machine. MID-tagged PCR reactions of 25µL contained 12.5µL of multiplex PCR mix (Qiagen, Manchester, UK), 2.5µL of 2 µM forward MICOIintF and reverse Nancy primer, 2.5µL of water and 5µL template DNA were undertaken, comprised of 15 minutes at 95°C, followed by 35 cycles of 95°C for 30s, 55°C for 90s, 72°C for 90s followed by a final extension at 72°C for 10 min.

Within each PCR 96-well plate, 12 negative (extraction and PCR) and two positive controls were included following Taberlet *et al.* (2018). Negative PCR controls consisted of DNase-free water. A negative control was included for each MID-tag to identify any contamination within primers. All products from each individual PCR plate were categorised based on band brightness after gel electrophoresis, categorised as very faint, faint, medium and bright. The DNA concentration from a minimum of two representative PCR products per plate from each brightness category were quantified using a high sensitivity assay with a Qubit Flourometer (Thermo Fisher Scientific) to confirm accuracy of estimating relative DNA concentration by eye from a gel photo. Each PCR plate was pooled according to concentrations determined by a Qubit Fluorometer to ensure approximate equimolar concentration of all samples in each pool.

Each pool was cleaned using SPRIselect beads (Beckman Coulter, Brea, USA) with a left-side size selection using a 0.9:1 ratio (retaining ~250-1000 bp fragments). The concentration of the pooled DNA was quantified using Qubit dsDNA High-sensitivity Assay Kits, and quality checked via TapeStation 2200 with a D1000 ScreenTape (Agilent, Santa Clara, USA). The concentration across all pools was quantified using Qubit dsDNA High-sensitivity Assay Kits, and all pools were combined again into combined pools. Library preparation for Illumina sequencing was undertaken on the cleaned combined pools via NEXTflex Rapid DNA-Seq kit (Bioo Scientific, Austin, USA), with a unique adapter ligated to each combined pool. Combined pools were diluted to 4nM and quantified using Qubit dsDNA High-sensitivity Assay Kits. Finally, the diluted combined pools were pooled equimolarly into a final pool combining all Hawfinch faecal samples and sequenced on a MiSeq desktop sequencer via a v2 chip with 2 x 250bp paired-end reads (expected capacity 24-30,000,000 reads).

3.3.3 Bioinformatics

The scripts used in the metabarcoding bioinformatics pipeline are available in Appendix 2.2. The results of the Illumina sequencing generated 12,307,560 reads. MID-tag primers were tested for truncation by calculating the percentage of reads containing less than 10bp of the MID-tag forward and reverse primer. This did not exceed 15% of the reads. All reads were quality-checked and trimmed using fastp v.0.20.0 (Chen *et al.* 2018), with a minimum quality threshold based on a Phred score with a minimum value of 33 (Mbareche *et al.* 2020) and a minimum base pair length of 280 bp. After filtering, the total number of reads was 9,652,972. The read pairs were demultiplexed using Mothur v1.39.5 (Schloss *et al.* 2009), removing the primer and MID sequences. Unoise3 was implemented within Usearch11 (Edgar 2016), removing replicates, denoising and clustering as well as removing any chimeric sequences. Any unique samples with <8 reads were discarded as they most likely represent sequencing errors. A closest matching sequence approach was adopted to identify species within the samples (Hawkins *et al.* 2015). Reads were clustered to zero-radius Operational Taxonomic Units (hereafter zOTUs), based on a 100% clustering threshold. The blast algorithm (Camacho *et al.* 2009) was used to query the NCBI nucleotide database and classify all zOTUs using a cut off of 97% sequence identity, the standard approach for metabarcoding studies (Alberdi *et al.* 2017). Megan v6.15.2 (Huson *et al.* 2016) was used to analyse the output. If the top BLAST hit, determined by the lowest e-value was reserved to a match with a single species then species-level identification was achieved, with the same rule applying to genus level matches. zOTUs which were not assigned to any taxonomic rank or did not correspond to any BLAST sequence were considered to be erroneous, or low quality and were discarded.

To clean data prior to statistical analysis, a sequence read number methodology was implemented (Dunn *et al.* 2018) in order to remove background contamination within PCR and extraction negatives. Sequences present within samples with unused MID-tag combinations due to “tag-jumping” (Schnell *et al.* 2015) were also considered. All sequences less than the maximum read count present in unused-MID tag combinations and negative controls for each respective zOTU were removed. The matrix was then collapsed so invertebrate species detections represented by multiple zOTUs were represented by a single entry. As multiple zOTUs were found to correspond to the same taxonomic identity, aggregating by taxonomic identification removes distinction due to haplotypic and intra-specific variation (Moorhouse-Gann *et al.* 2018). The final dataset was cleaned further by removing artefacts and contaminants originating from positive control samples. Taxa present within both a faecal sample and positive control sample were removed from a faecal sample if the read count of the non-positive control taxa within the faecal sample was lower than the read count detected for the non-positive control taxa within the positive control samples. All zOTUs represented by less then 10 reads were removed as these are likely to be artefacts (Schenk *et al.* 2019). No Hawfinch DNA was amplified, and any zOTUs matching bacteria, gastrotrichs, fungi or algae were removed. Each taxon was checked for its occurrence within the UK, with all taxa identified occurring within the UK. All taxa were converted to genus level to standardise the taxonomic level, since some zOTUs could not be resolved further. Standardising the taxonomic level also increased evenness for subsequent analysis. Finally, read counts were converted into presence-absence of each invertebrate taxon.

3.3.4 Statistical analysis

For all statistical analysis, the presence/absence of each taxonomic unit within a faecal sample was used. Control samples were excluded from the analyses. All statistical analysis were carried out in R version 3.6.3 (R Core Team 2020) unless otherwise stated. To evaluate the most prevalent taxa within Hawfinch diet, the number of samples in which a dietary zOTU occurred (frequency of occurrence, hereafter referred to as FOO), was calculated. This was expressed as a percentage (%FOO) by dividing FOO by the total number of samples and multiplying by 100.

3.3.5 Hawfinch dietary variation

To determine whether plant or invertebrate taxonomic richness in Hawfinch diet was greater, the total number of plant (obtained from Chapter 2) and invertebrate genera consumed by individual Hawfinch were calculated. Only Hawfinch samples which provided results for both invertebrate and plant taxa within the diet were included. No individual Hawfinch which had tested positive for both plant and invertebrate DNA was sampled more than once. The data were not normally distributed and could not be transformed through data transformations

therefore a Wilcoxon matched-pairs test was undertaken to test for a significant difference in the median species richness of plant and invertebrate taxa in the diet. The boxplot was created using the *ggboxplot* function within the *ggpubr* package (Kassambara 2020).

To investigate how the explanatory variables were associated with invertebrate dietary composition, multivariate generalised linear models (MGLMs) were used using the function *manyglm* within the package *mvabund* (Wang *et al.* 2012). This allows for multiple species testing and implements a likelihood ratio test (LRT) and re-sampled *p* values to identify significance. Binomial regression was specified in the models to account for presence-absence data and subsequent mean-variance relationships of the data. The function *anova.manyglm* in *mvabund* was used to test the significance of each term within the model and the *p.uni = adjusted* argument was implemented in order to allow univariate "species by species" results to be returned (Wang *et al.* 2012). The *p*-values returned in this argument were adjusted to control for multiple testing, using a Holm's step down resampling algorithm, allowing control over family error rates (Westfall and Young 1993). Parametric bootstrap (Monte Carlo) resampling was undertaken to ensure inferences took into account correlation between variables (Wang *et al.* 2012). This function is also recommended for hypothesis testing with presence-absence data (Wang et al. 2012). When necessary, pairwise comparisons were performed using the *pairwise.comp* function of *anova.manyglm*. The independent variables were chosen to represent environmental and biological variation across space and time:

- Region (five categories)
- Year (four years)
- Sex
- The interaction between year and region

All variables were categorical and no model simplification was performed as the aim of the modelling was significance testing, rather than developing simpler predictive models. For all models, quantile-quantile (Q-Q) diagnostic plots were checked to ensure normality in multivariate data and multivariate homoscedasticity was checked by plotting Dunn-Smyth residuals against fitted linear predicted values (Wang *et al.* 2012; Bates *et al.* 2015). The relationship between age classes was not investigated due to a small sample size for juveniles (*n*=8). Furthermore, community composition differences within Hawfinch diet were analysed at a smaller spatial scale. Analysis of diets between artificial feeding sites within north Wales were not included due to small sample sizes collected from certain artificial feed sites (Dolgellau *n*=3 and Llanelltyd *n*=4), and sampling years (2016 *n*=3 and 2019 *n*=7). Analysis between artificial feeding sites within the Wye Valley and the New Forest respectively were

not included due to small sample sizes collected from the Wye Valley (Monmouth *n*=5), females in the New Forest (*n*=3) and faecal samples collected in the New Forest during sampling year 2018 (*n*=2). To portray dietary overlap between geographic regions, bipartite food webs were constructed using the *bipartite* package (Dormann *et al.* 2008). For clarity due to the number of taxonomic units found in Hawfinch diet, invertebrate zOTUs were analysed at the order level.

Dietary composition was visualised using non-metric multidimensional scaling (nMDS) via the function *metaMDS* in the *vegan* package (Oksanen *et al.* 2019). The nMDS was performed with Jaccard dissimilarities run in three dimensions (*k*=3), due to the presence/absence nature of the data and high stress statistic values (>2) when analysed using (*k*=2). Spider plots were produced using nMDS results via *ordispider* and plotted in two dimensions through ggplot2 (Wickham 2016).

To test for differences in the number of prey items detected in Hawfinch diet, a negative binomial GLM with a log link function was fitted with region, year of sampling and sex as independent variables. Significance was tested using the *Anova* function in the package car (Fox and Weisburg 2011). Models were validated using the function *check_model* in the package *performance* (Lüdecke *et al.* 2020), checking for assumptions of normality and homogeneity of model residuals, to test for multicollinearity, autocorrelation, influential observations and overdispersion. The final model was chosen using backwards stepwise model refinement based upon AIC value using the *step* function within the base R package as a simpler predicted model was preferred, rather than simply significance testing as in the *manyglm* models.

3.4 Results

3.4.1 Hawfinch diet composition

DNA was successfully amplified from 120 individuals. Invertebrate DNA was first detected within faecal samples from Hawfinch captured in late March 2017 (three samples), with the remaining 117 samples containing invertebrate DNA from Hawfinch captured across two fieldwork seasons from 26/4/17- 4/7/18 and 11/4/19 - 28/6/19.

I retrieved 12,307,560 sequences from 120 Hawfinch faecal samples. A total of 119,241 sequences were detected within negative controls. A total of 555,017 unique sequences were removed due to contamination, tag-jumping and poor quality sequences or reads likely to be a result of degradation. After excluding 21 non-prey taxa and contamination (see Appendix 2.3), 118 invertebrate dietary taxa were detected within Hawfinch diet. Of the taxa identified, 96% were identified to species level, and 100% to genus. The most frequently detected prey

taxa were winter moth, St Mark's fly (*Bibio marci*) and tree slug (*Lehmannia marginata*), found in 35.8%, 31.7% and 30.8% of samples respectively (*n*=120). There were eight invertebrate orders present in at least one sample, with zOTUs matched to the order Lepidoptera most taxon rich (73 taxa), and commonly recorded (present in 61.9% of samples). Other recorded orders were Araneae, Diptera and Hymenoptera (Figure 3.1). A comprehensive breakdown of the invertebrate prey taxa is provided in Table 3.1.

3.4.2 Hawfinch dietary variation

A Wilcoxon matched-pairs test revealed a significant difference between plant and invertebrate taxonomic richness within the diet of Hawfinch (V=3650, *p*=<0.001) (Figure 3.2). The mean number of plant taxa detected in Hawfinch faecal samples was 4.1(σ=1.94**)**, while the mean number of invertebrate taxa was 5.9 (σ=4.57).

3.4.2.1 Spatial and sex variation

Distinct Hawfinch diets were detected between regions within the UK (MGLM: LRT=443.6, *p*=<0.001; Figure 3.3a). Pairwise comparisons indicated distinct diets between all possible regional comparisons (Appendix 2.4). Univariate analysis revealed dietary differences between regions were driven by five taxa: dipteran *Bibio* detected more frequently within the Wye Valley (LRT=22.6, *p*=<0.001), hymenopteran *Cimbex* detected more frequently in north Wales (LRT=18.9, *p*=0.019), lepidopteran *Erannis, Eupsilia* which were detected more frequently within north Wales, and lepidopteran *Operophtera* detected more frequently in the Wye Valley (LRT=34.4, *p*=<0.001, LRT=22.8, *p*=<0.001 and LRT=42.1, *p*=<0.001 respectively). Distinct diets were not detected between sexes (LRT=109.0, *p*=0.106).

3.4.2.2 Temporal variation

Hawfinch diets differed between year (LRT= 602.6, *p*=<0.001; Figure 3.3b). Temporal dietary differences were found to be driven by 13 invertebrate genera (Table 3.2). Pairwise comparisons indicated distinct diets were detected between all temporal comparisons except 2016 v 2018 (Appendix 2.4). The interaction between region and year was also significant (LRT=38.5, *p*=0.004). Dietary differences found between sites and sampling years were found to be driven by lepidopteran *Agrochola* (LRT=12.1, *p*=0.028). A limitation however, was inconsistent sampling across sampling years, with the New Forest sampled in 2018-2019 and Norfolk only in 2019, thus the diet contributions for earlier years are absent.

3.4.2.3 Landscape spatial scale and sex variation

At a smaller spatial scale, Hawfinch faecal samples collected from north Wales showed distinct diets between sexes (LRT=77.0, *p*=0.037; Figure 3.4a) and year (LRT=216.7, *p*=<0.001; Figure 3.4b). While no specific genera were associated with the differences detected between the sexes, three genera differed temporally; *Cimbex* (LRT=15.0, *p*=0.042), *Lehmannia*

(LRT=20.7, *p*=0.002) and *Operophtera* (LRT=17.8, *p*=0.012). *Cimbex* were only detected in Hawfinch sampled in 2017, *Lehmannia* were detected more frequently in the diet of Hawfinch sampled in 2018 (%FOO =100%), with *Operophtera* detected only within the diet of Hawfinch sampled in 2017. Within the Wye Valley, dietary differences were detected between the sexes (LRT=86.4, *p*=0.002; Figure 3.5a) and between sampling years (LRT=313.9, *p*=<0.001; Figure 3.5b). Again, while no specific genera were associated with the differences between sexes, 12 genera differed through time (Table 3.3).

Negative binomial GLM analysis (pseudo R^2=0.7) revealed significant differences between sexes (LR Chisq=4.4, *df*=1, *p*=0.035) and sampling years (LR Chisq=70.6, *df*=3, *p*=<0.001) in the number of prey taxa per faecal sample, with males having fewer taxonomic units per faecal sample than females (5.6 and 6.8 respectively). No significant differences in number of prey taxa within Hawfinch diet were found between geographic regions (LR Chisq=6.0, *df*=4, *p*=0.139).

Table 3.1. The percentage of Hawfinch faecal samples testing positive for dietary items broken down by sex and age-class.

		Percentage of samples testing positive for a dietary item				
Taxon	Common name	All (*n*=120)	Males (*n*=65)	Females (*n*=55)	Adults (*n*=112)	Juveniles (*n*=8)
Operophtera brumata	Winter moth	35.8	30.8	41.8	37.5	12.5
Bibio marci	St Mark's fly	31.7	29.2	34.5	33.9	0.0
Lehmannia marginata	Tree slug	30.8	30.8	30.9	29.5	50.0
Erannis defoliaria	Mottled umber	26.7	21.5	32.7	27.7	12.5
Cosmia trapezina	Dun-bar	23.3	24.6	21.8	24.1	12.5
Conistra vaccinii	Chestnut	21.7	23.1	20	22.3	12.5
Tortricodes alternella	Winter shade moth	21.7	15.4	29.1	23.2	0.0
Eupsilia transversa	The satellite moth	20.8	16.9	25.5	22.3	0.0
Orthosia cerasi	Common quaker	19.2	13.8	25.5	19.6	12.5
Agriopis marginaria	Dotted border	15.8	16.9	14.5	16.1	12.5
Amphipyra pyramidea	Copper underwing	15.8	16.9	14.5	16.1	12.5
Colotois pennaria	Feathered thorn	15.8	16.9	14.5	16.1	12.5
Epirrita christyi	Pale november	15	12.3	18.2	15.2	12.5
Agrochola circellaris	The brick	14.2	15.4	12.7	14.3	12.5
Eudemis profundana	Diamond-back marble	14.2	15.4	12.7	15.2	0.0
Epirrita dilutata	November moth	11.7	10.8	12.7	12.5	0.0
Agriopis aurantiaria	Scarce umber	10.8	7.7	14.5	11.6	0.0
Ptycholoma lecheana	Leche's twist moth	10.8	6.2	16.4	11.6	0.0
Pandemis cerasana	Barred fruit-tree tortrix	10	7.7	12.7	10.7	0.0
Syrphus torvus	Hairy-eyed syrphus	10	4.6	16.4	10.7	0.0
Orthosia cruda	Small quaker	9.2	9.2	9.1	9.8	0.0
Anorthoa munda	Twin-spotted quaker	7.5	7.7	7.3	7.1	12.5
Archips crataeganus	Brown oak tortrix	7.5	4.6	10.9	8.0	0.0

Cimbex femoratus	Birch sawfly	7.5	10.8	3.6	6.3	25.0
Quercusia quercus	Purple hairstreak	7.5	6.2	9.1	8.0	0.0
Epirrita autumnata	Autumnal moth	6.7	3.1	10.9	6.3	12.5
Archips xylosteana	Variegated golden tortrix	5.8	10.8	0	6.3	0.0
Cepaea nemoralis	Grove snail	5.8	4.6	7.3	6.3	0.0
Syrphus ribesii	Humming syrphus	5.8	4.6	7.3	6.3	0.0
Apethymus serotinus	Sawfly	5	6.2	3.6	5.4	0.0
Coleophora flavipennella	Tipped oak case-bearer	5	3.1	7.3	5.4	0.0
Ectropis crepuscularia	Engrailed moth	5	1.5	9.1	4.5	12.5
Gypsonoma dealbana	Common cloaked shoot	5	4.6	5.5	5.4	0.0
Satyrium w album	White-letter hairstreak	5	7.7	1.8	5.4	0.0
Vitrina pellucida	Land snail	5	4.6	5.5	5.4	0.0
Agrochola macilenta	Yellow-line quaker	4.2	4.6	3.6	3.6	12.5
Anyphaena accentuata	Buzzing spider	4.2	0	9.1	3.6	12.5
Hemerobius micans	Lacewing	4.2	3.1	5.5	4.5	0.0
Hydriomena furcata	July highflyer	4.2	6.2	1.8	4.5	0.0
Philodromus albidus	Crab spider	4.2	3.1	5.5	4.5	0.0
Acleris rhombana	Rhomboid tortrix	3.3	3.1	3.6	3.6	0.0
Adela reaumurella	Green longhorn moth	3.3	3.1	3.6	3.6	0.0
Amphipyra berbera	Svensson's copper underwing	3.3	3.1	3.6	3.6	0.0
Apocheima pilosaria	Pale brindled beauty	3.3	3.1	3.6	3.6	0.0
Blastobasis adustella	Furness dowd	3.3	1.5	5.5	3.6	0.0
Coleophora lutipennella	Common oak case-bearer	3.3	1.5	5.5	3.6	0.0

Operophtera fagata	Northern winter moth	3.3	3.1	3.6	3.6	0.0
Orthosia incerta	Clouded drab	3.3	1.5	5.5	1.8	25.0
Tetragnatha obtusa	Long-jawed orb-weaver	3.3	3.1	3.6	3.6	0.0
Agelastica alni	Alder leaf beetle	2.5	1.5	3.6	1.8	12.5
Anelosimus vittatus	Cobweb spider	2.5	4.6	0	2.7	0.0
Clubiona brevipes	Sac spider	2.5	1.5	3.6	2.7	0.0
Coleophora laricella	Larch casebearer moth	2.5	1.5	3.6	2.7	0.0
Culicoides impunctatus	Highland midge	2.5	3.1	1.8	1.8	12.5
Egle groenlandica	Willow catkin fly	2.5	1.5	3.6	2.7	0.0
Paradarisa consonaria	Brindled square spot	2.5	1.5	3.6	0.9	25.0
Philodromus aureolus	Wandering crab spider	2.5	3.1	1.8	1.8	12.5
Ypsolopha alpella	Barred smudge	2.5	3.1	1.8	2.7	0.0
Ypsolopha ustella	Variable smudge	2.5	3.1	1.8	2.7	0.0
Aethalura punctulata	Grey birch moth	1.7	0	3.6	0.0	25.0
Agriopis leucophaearia	Spring usher	1.7	1.5	1.8	1.8	0.0
Alsophila aescularia	March moth	1.7	1.5	1.8	1.8	0.0
Apocheima hispidaria	Small brindled beauty	1.7	0	3.6	1.8	0.0
Araneus triguttatus	Orb weaver	1.7	1.5	1.8	1.8	0.0
Argyresthia pruniella	Cherry fruit moth	1.7	1.5	1.8	1.8	0.0
Campaea margaritaria	Light emerald	1.7	1.5	1.8	1.8	0.0
Carcina quercana	Oak skeletonizer moth	1.7	1.5	1.8	1.8	0.0
Clausilia bidentata	Two-toothed door snail	1.7	1.5	1.8	1.8	0.0
Craniophora ligustri	The coronet	1.7	3.1	0	1.8	0.0

Drepana falcataria	Pebble hook-tip	1.7	1.5	1.8	0.0	25.0
Epinotia abbreviana	Brown elm bell	1.7	1.5	1.8	1.8	0.0
Eupithecia abbreviata	Brindled pug	1.7	3.1	0	1.8	0.0
Hedya nubiferana	Marbled orchard tortrix	1.7	1.5	1.8	1.8	0.0
Limax cinereoniger	Ash-grey slug	1.7	3.1	0	1.8	0.0
Lypha dubia	Tachinid fly	1.7	1.5	1.8	1.8	0.0
Philodromus dispar	Crab spider	1.7	1.5	1.8	1.8	0.0
Philodromus praedatus	Crab spider	1.7	1.5	1.8	1.8	0.0
Phycita roborella	Dotted oak knot-horn	1.7	3.1	0	1.8	0.0
Phyllobius pyri	Common leaf weevil	1.7	1.5	1.8	1.8	0.0
Polydrusus undatus	Weevil	1.7	3.1	0	0.9	12.5
Scathophaga stercoraria	Yellow dung fly	1.7	0	3.6	1.8	0.0
Spilonota laricana	Larch - bud moth	1.7	1.5	1.8	1.8	0.0
Syrphus vitripennis	Hoverfly	1.7	1.5	1.8	1.8	0.0
Thera britannica	Spruce carpet	1.7	1.5	1.8	1.8	0.0
Zeiraphera isertana	Cock's-head bell	1.7	3.1	0	1.8	0.0
Aleimma loeflingiana	Yellow oak button	0.8	1.5	0	0.9	0.0
Anatis ocellata	Eyed ladybird	0.8	1.5	0	0.0	12.5
Apotomis sp.	Tortrix moth	0.8	0	1.8	0.9	0.0
Apotomis turbidana	White-shouldered marble	0.8	0	1.8	0.9	0.0
Coleophora ibipenella	Forest case-bearer	0.8	1.5	0	0.9	0.0
Ditula angustiorana	Fruit-tree tortrix	0.8	0	1.8	0.9	0.0
Diurnea fagella	March dagger	0.8	1.5	0	0.9	0.0
Epinotia immundana	Common birch bell	0.8	1.5	0	0.9	0.0
Eudonia mercurella	Small Grey	0.8	0	1.8	0.9	0.0

Formica pratensis	Black-backed meadow ant	0.8	1.5	0	0.0	12.5
Lithophane ornitopus	Grey shoulder-knot	0.8	0	1.8	0.9	0.0
Lucilia sp.	Green bottle flies	0.8	0	1.8	0.9	0.0
Lymantria dispar	Gypsy moth	0.8	1.5	0	0.0	12.5
Nematus alniastri	Sawfly	0.8	0	1.8	0.0	12.5
Neomyia cornicina	Green bottle flies	0.8	0	1.8	0.9	0.0
Neriene peltata	Platform hammock spider	0.8	1.5	0	0.9	0.0
Oncopsis speciosa	Leafhoppers	0.8	0	1.8	0.9	0.0
Orchesia minor	False darkling beetle	0.8	0	1.8	0.9	0.0
Oswaldia muscaria	Tachinid fly	0.8	0	1.8	0.9	0.0
Pandemis cinnamomeana	White-faced tortrix	0.8	1.5	0	0.9	0.0
Philodromus collinus	Running crab spider	0.8	1.5	0	0.0	12.5
Phorocera obscura	Tachinid fly	0.8	1.5	0	0.9	0.0
Phyllobius maculicornis	Green leaf weevil	0.8	0	1.8	0.9	0.0
Polydrusus tereticollis	Weevil	0.8	1.5	0	0.9	0.0
Pseudargyrotoza conwagana	Yellow-spot twist	0.8	1.5	0	0.9	0.0
Psoricoptera gibbosella	Humped crest	0.8	1.5	0	0.9	0.0
Saaristoa abnormis	Sheet weaver spider	0.8	0	1.8	0.9	0.0
Selenia tetralunaria	Purple thorn	0.8	0	1.8	0.0	12.5
Stenolechia gemmella	Black-dotted groundling	0.8	0	1.8	0.9	0.0
Stomoxys calcitrans	Stable fly	0.8	0	1.8	0.9	0.0
Syrrhizus sp.	Braconid wasp	0.8	0	1.8	0.9	0.0
Tipula paludosa	Crane fly	0.8	1.5	0	0.9	0.0
Xyleninae sp.	Owlet moth	0.8	0	1.8	0.9	0.0

Table 3.2. Results for the univariate *"anova"* test in the national *manyglm* model. Significant (p<0.05) prey genera differences for the test variable "year" are shown. Likelihood ratio test values (LRT) and p-values are given. Percent frequency of occurrence values (% FOO) for each prey genus are indicated.

Predictor variable	Prey genus	LRT	*p*-value	%FOO 2016	%FOO 2017	%FOO 2018	%FOO 2019
Year	*Agriopis*	25.6	0.002	0	55.6	0	24.6
Year	*Cimbex*	20.6	0.003	0	44.4	2.6	0
Year	*Coleophora*	21.1	0.003	0	0	0	18
Year	*Colotois*	20.2	0.003	0	38.9	2.6	18
Year	*Cosmia*	28.8	0.001	0	11.1	0	42.6
Year	*Erannis*	23.7	0.002	0	61.1	10.5	27.9
Year	*Eudemis*	15.8	0.022	0	5.6	0	26.2
Year	*Eupsilia*	21.3	0.002	0	0	2.6	39.3
Year	*Lehmannia*	19.8	0.004	66.7	22.2	52.6	18
Year	*Operophtera*	42.1	0.001	0	55.6	5.3	52.5
Year	*Orthosia*	26.4	0.001	0	33.3	2.6	37.7
Year	*Ptycholoma*	19.5	0.004	0	0	0	21.3
Year	*Tortricodes*	21.4	0.002	0	5.6	2.6	39.3

Table 3.3. Results for the univariate *"anova"* test in the Wye Valley *manyglm* model. Significant (p<0.05) prey genera differences for the test variable "year" are shown. Likelihood ratio test values (LRT) and *p*-values are given. Percent frequency of occurrence values (% FOO) for each prey genus are indicated.

Predictor variable	Prey genus	LRT	*p*-value	%FOO 2018	%FOO 2019
Year	*Agriopis*	9.8	0.03	0	22.2
Year	*Agrochola*	14.1	0.003	0	30.6
Year	*Cepaea*	13	0.008	25.9	0
Year	*Cosmia*	23.7	0.002	0	47.2
Year	*Epirrita*	17.1	0.002	0	36.1
Year	*Erannis*	10	0.022	0	22.2
Year	*Eupsilia*	19.9	0.002	3.7	50
Year	*Operophtera*	20.8	0.002	3.7	52.8
Year	*Orthosia*	22.6	0.002	0	44.4
Year	*Pandemis*	11.5	0.015	0	25
Year	*Ptycholoma*	16.6	0.002	0	33.3
Year	*Tortricodes*	23.2	0.002	0	44.4

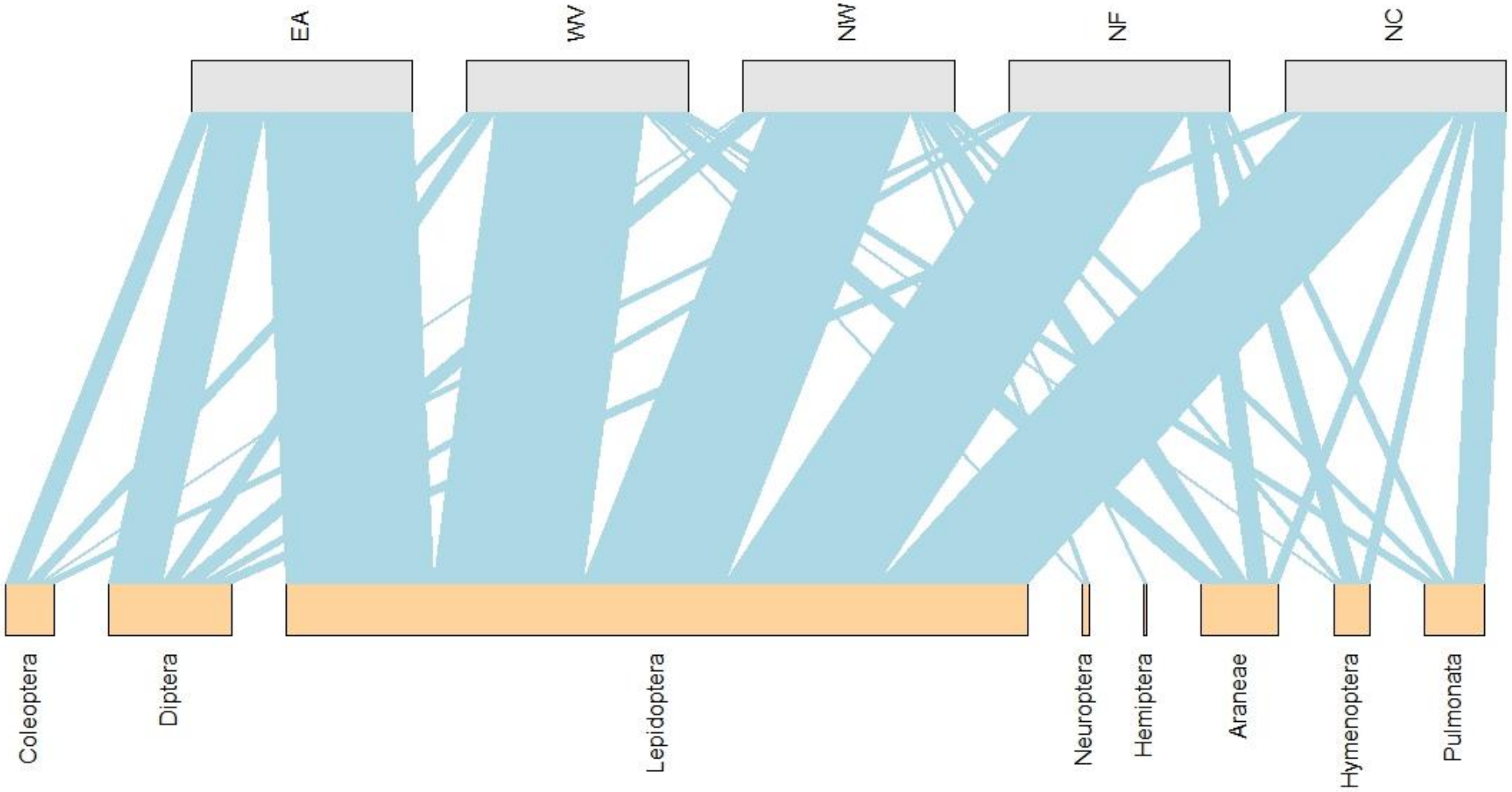


Figure 3.1. Bipartite food web showing dietary overlap between Hawfinch populations within the UK. The upper bars represent the geographic region in which Hawfinch were sampled and the lower bar represents invertebrate taxonomic units grouped at order level. The width of the bar represents the number of samples from (upper bar) containing (lower bar) that species or taxonomic unit. Interactions between geographic regions are shown by lines between bars; thicker lines represent more frequent interactions. EA=East Anglia, WV= Wye Valley, NW=north Wales, NF=New Forest and NC=north Cardiff.

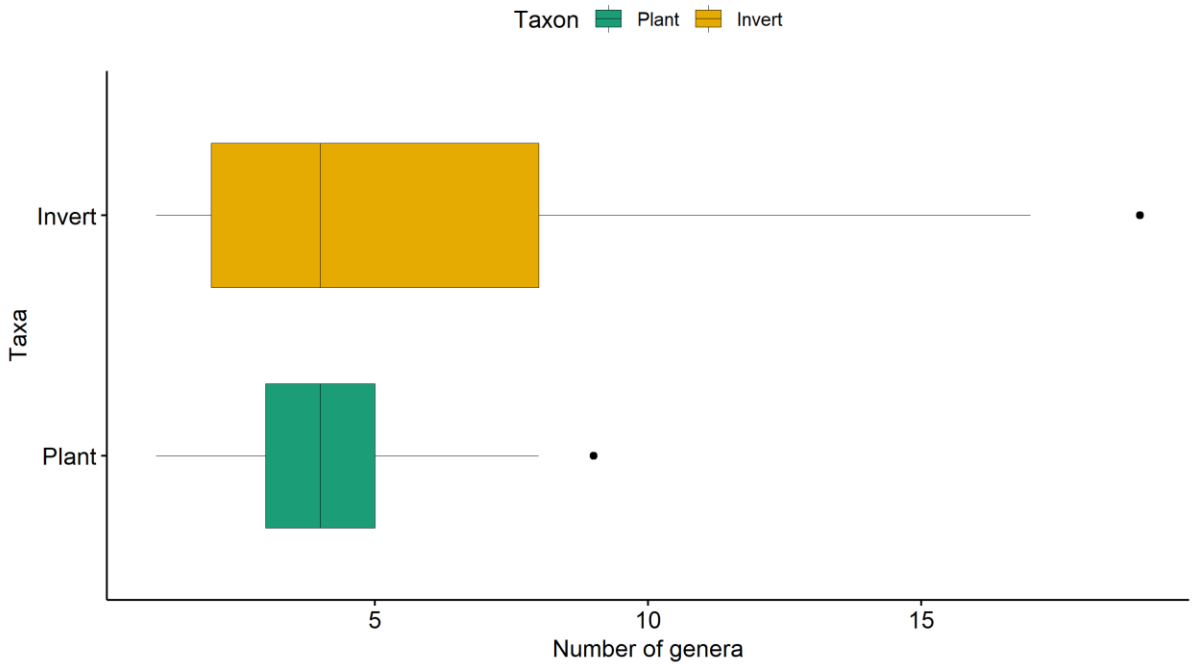


Figure 3.2. The taxonomic richness of plant and invertebrate genera in the diet of Hawfinch. The width of the box indicates the interquartile range. Whiskers show the highest and lowest values (excluding outliers indicated black circles). The vertical lines within the boxes indicate median number of genera detected.

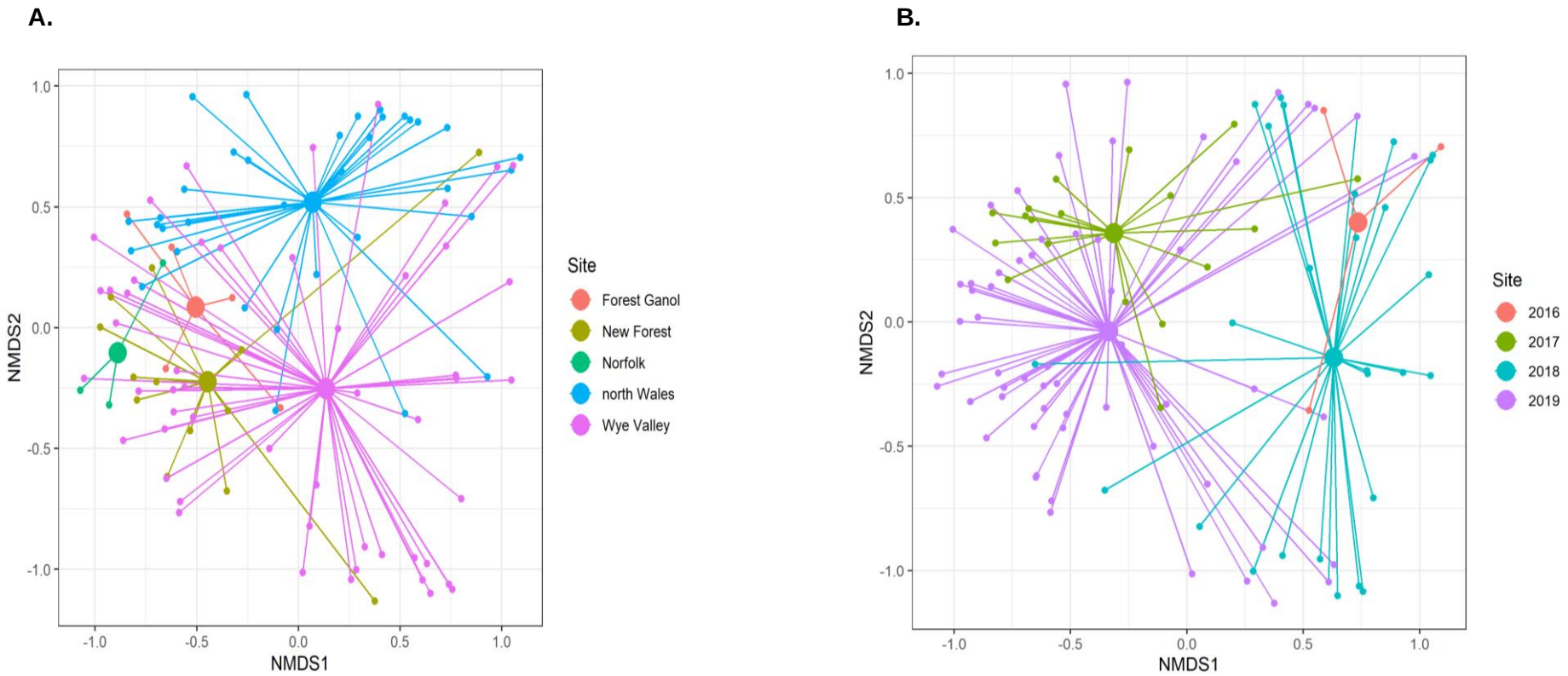
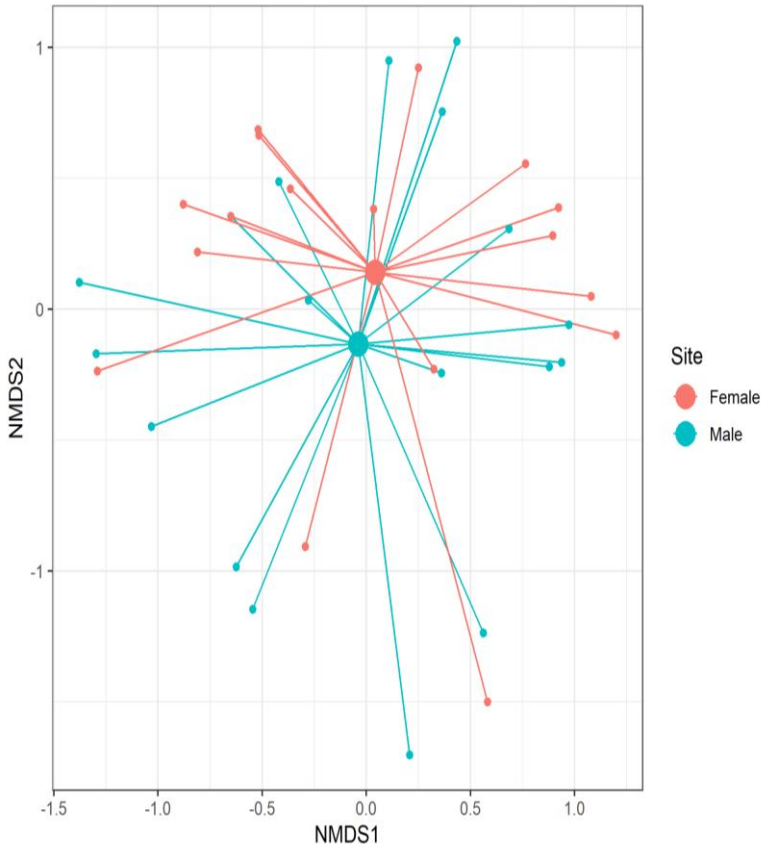


Figure 3.3. Spider plot for invertebrate taxa consumed by Hawfinch across (A) geographic regions and (B) year across the UK. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its region. Stress = 0.15.

A.



B.

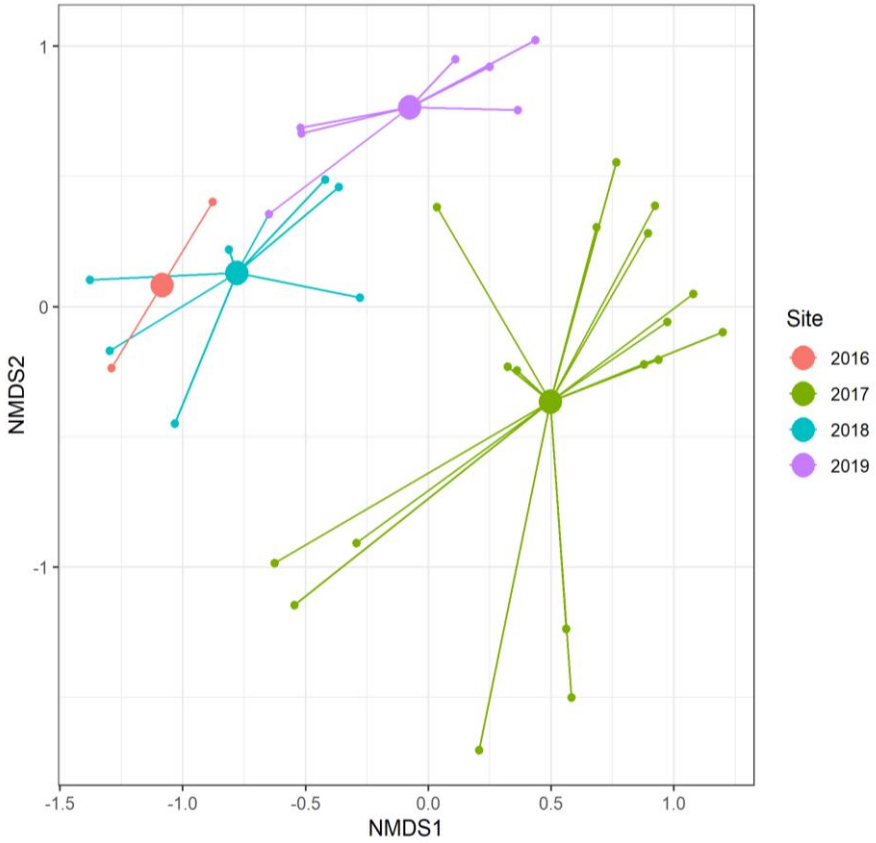
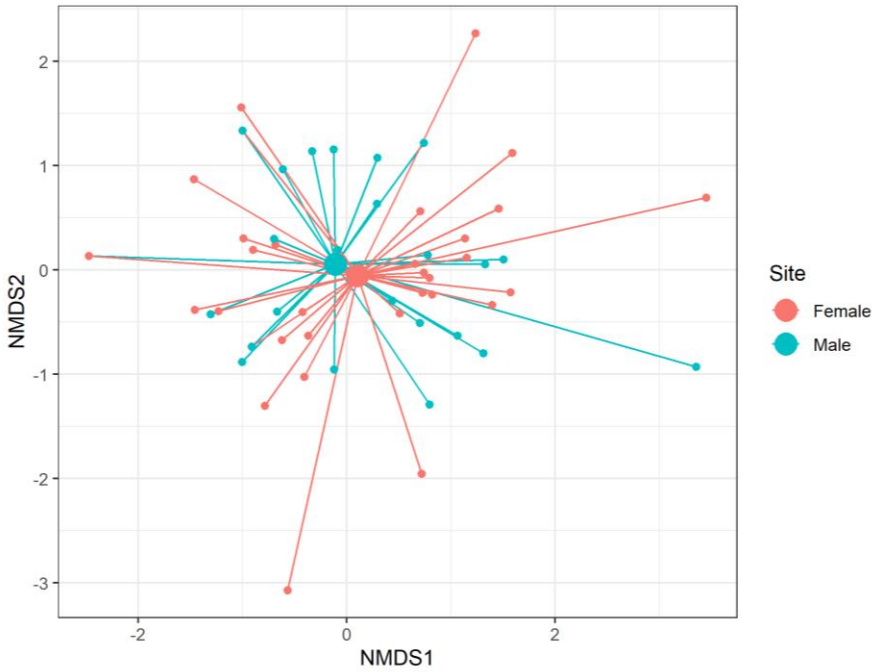


Figure 3.4. Spider plot for invertebrate taxa consumed by Hawfinch grouped by (A) sex and (B) year within the north Wales sampling region. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its sex. Stress = 0.14.

A.



B.

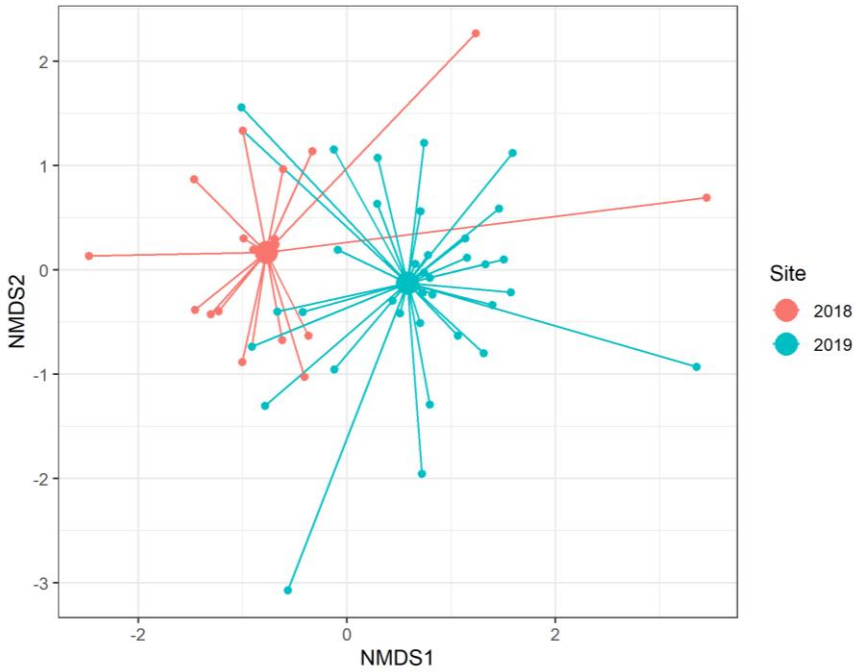


Figure 3.5. Spider plot for invertebrate taxa consumed by Hawfinch grouped by (A) sex and (B) year within the Wye Valley sampling region. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its sex. Stress = 0.10.

3.5 Discussion

The results of this study demonstrate that faecal metabarcoding can provide detailed insights into the diet of a woodland bird with a broad dietary niche. Furthermore, it provides the first comprehensive analysis of invertebrate prey taxa within Hawfinch diet, indicating Hawfinch are omnivorous and able to utilise at least 118 prey taxa. The results show evidence that at a local scale, Hawfinch are showing dietary composition differences between males and females, and that at a regional scale, males show a significantly lower dietary richness than females.

3.5.1 Dietary composition

Lepidoptera, Coleoptera, Hemiptera, Annelida, Gastropoda and Araneae have all been observed as prey at the order level (Mountford 1957) and all (excluding Annelida) were detected within this study. As with previous metabarcoding of generalist insectivores, most taxa were detected infrequently within the diet (Brown *et al.* 2014; Aizpurua *et al.* 2018; Alberdi *et al.* 2020; Evens *et al.* 2020; Mitchell *et al.* 2021).

An important aspect to consider within any DNA metabarcoding study is prey detection biases, which can impact the results and subsequent ecological interpretation of metabarcoding studies (Forsman *et al.* 2022). The choice of primers is considered to be one of, if not the most important steps for reducing biases (Hoenig *et al.* 2022). The choice of primers can impact amplification efficiency and taxonomic classification of subsequent amplicon sequences (Brandon-Mong *et al.* 2015). As a result, the primer choice will subsequently influence the understanding of prey composition within the diet and thus the interpretations of foraging ecology based from these results (Alberdi *et al.* 2018; Forsman *et al.* 2022). Primer biases are considered particularly problematic when undetected taxa are ones which contribute substantially to the foraging ecology of the study species (Forsman *et al.* 2022). Hawfinch have previously been observed to feed mainly on Lepidoptera (Mountford 1957), which was well represented at high frequency of occurrences across sites. The primer pair used in this study were originally used to characterise the diet of blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*), and subsequently were designed to amplify a broad range of invertebrate taxa, including Annelida, which constitutes a large part of the aforementioned species diet (Stockdale 2018). A wide range of invertebrate taxa were detected in Hawfinch diet, while Annelida was not detected, indicating Hawfinch are not utilising Annelida as a food resource, as opposed to primer biases leading to a false negative (type II error) and incorrect conclusions of Hawfinch foraging ecology. It is important to note however that no primer pair can provide a completely unbiased and comprehensive account of species' diet due to highly degraded DNA failing to amplify in PCR reactions, primer biases and differences in

mitochondrial copy number per cell (reviewed in Clare 2014). A one-locus-several-primer approach should be used more readily within DNA metabarcoding studies in order to maximise taxonomic coverage and minimise false negatives (Corse *et al.* 2019).

The high prevalence of winter moth within Hawfinch diet is not unexpected, as this larva is an important food resource for other woodland passerine species, such as nestling tits (Perrins 1991). The earliest date that winter moth was detected within the diet was mid-April, with prevalence increasing throughout April and May. Kirby *et al.* (2019) found Hawfinch egg laying commonly started during the third week of April and peaked in mid May. This temporal increase in the number of nests coincides with the increased incidence of winter moth within the diet, and most likely corresponds to a change in the availability of winter moth larvae. This finding raises the possibility that Hawfinch may be using the availability of winter moth as a breeding cue, as has been suggested in other passerine species (Shutt et al., 2020). The lower prevalence in juvenile diet may be a result of sampling timing. Sampling of juvenile birds was undertaken in early to late summer, when the availability of winter moth may be decreased, as larvae begin to pupate in early June (Hittenbeck *et al.* 2019), therefore reducing the availability of this food resource to juvenile birds. This result should be interpreted with caution however, as this is based upon a low sample size.

In contrast, the high prevalence of tree slug within the diet was unexpected, as it was previously thought that only snails were consumed (Mountford 1957). This may be explained by the availability of algae and lichens within woodland, which are the main components of tree slug diet (Kappes 2006). During wet weather, tree slugs feed on algae growing on tree trunks, but remain under the bark of dead timber during unsuitable weather (Kappes 2006). Thus, tree slugs may be taken during periods of high rainfall when foraging efficiency for defoliating Lepidoptera is reduced (Ortega-Jimenez and Dudley 2012; Morganti *et al.* 2017). Furthermore, Pulmonata were not detected in the diet of Hawfinch sampled from east Anglian sites, possibly due to the east of the UK typically being drier with less rainfall days (Simpson and Jones 2014), reducing the availability of Pulmonata as a food resource.

A further unexpected result was the prevalence of St Mark's fly. Previous dietary studies on Hawfinch have not found any Diptera, yet the results from this study show St Mark's fly to be present within 31.7% of samples. St Mark's fly is so called because the adults emerge on approximately the 25th of April (St Mark's Day). Analysis of the frequency of occurrence data and date of faecal sample collection revealed that St Mark's fly was not detected within Hawfinch diet until mid to late April. While metabarcoding cannot distinguish tissue type of prey taxa, it can be hypothesised that due to St Mark's Fly larvae developing within the soil

(Frouz *et al.* 2019), Hawfinch are feeding on adult individuals during peak availability in late April/early May. Therefore, the increased incidence of St Mark's fly in the diet may correspond with a change in availability of this species. Additionally, St Mark's fly was not present within the juvenile dietary data. Sampling of juvenile birds was undertaken in early to late summer, when the availability of St Mark's fly would be lower, due to adult flies living for only 1-2 weeks.

All three of the commonest dietary lepidopteran taxa detected in this study are polyphagous, associated with deciduous woodland tree species including beech and oak, with winter moth and mottled umber known to use oak as host plants and frequently occurring together (Hittenbeck *et al.* 2019). All the aforementioned tree species were present within Hawfinch diet, therefore it can be hypothesised that Hawfinch may be showing local dietary specialisation, as found in studies of insectivorous bats (Salinas-Ramos *et al.* 2015). It is more likely however, that this apparent specialisation arises from prey availability fluctuations (Moran and Southwood 1982; Shutt *et al.* 2020), and that prey availability may be an important factor influencing the dietary composition and spatial variation of Hawfinch diet, as has been found in insectivorous bat species (Czenze *et al.* 2018; Tournayre *et al.* 2021).

It is important to acknowledge however, the possibility of secondary predation within DNA metabarcoding studies (Tercel *et al.* 2021). Secondary predation is the detection of dietary items within the digestive systems of Hawfinch prey. Hawfinch feed primarily within the canopy (Mountford 1957), and will only come to the ground to feed on fallen seed in late winter. This would suggest that most invertebrate taxa were predated from the vegetation or bark within the tree canopy, resulting in possible accidental ingestion of plant taxa when gleaning prey items from trees. Conversely, secondary predation may be present through the accidental ingestion of lepidopteran eggs within tree buds, however the temporal period in which Lepidoptera were present within the diet does not support accidental ingestion. This is due to samples showing dietary presence in April-May, when many lepidopteran taxa have active juvenile stages (Blažek *et al.* 2021). While secondary predation is an issue for foraging behaviour studies, even if not directly hunted, consumed species which are indirectly ingested will still contribute towards the nutritional intake, therefore are valid within dietary categorisation of the consumer (Bowser *et al.* 2013; Nielsen *et al.* 2017).

3.5.2 Variation in Hawfinch diet

Diet is likely to reflect a mixture of prey availability, abundance and preference, with Hawfinch consuming a broader range of invertebrate taxa in comparison to plant taxa, reflecting what may be naturally available within the environment. Food preference, rather than availability or abundance has been found to contribute towards dietary shifts from invertebrates to fruit,

potentially enabling birds to seasonally balance nutrient and energy intake (Marshall *et al.* 2016). Invertebrates are typically a high protein to calorie ratio food resource, with certain species providing specific nutritional value, for example spiders provide high levels of the amino acid cysteine (Ramsay and Houston 2003; Marshall *et al.* 2016). Hawfinch egg laying begins around mid April (Kirby *et al.* 2019), and the presence of invertebrates within the diet during the breeding season has been recorded in other passerine dietary studies conducted over similar temporal periods (Newton, 1967; Shutt et al., 2020). This may help to provide specific nutrients beneficial to breeding physiology, such as egg production in females, as well as providing high protein food for chicks (Marshall *et al.* 2016). These dietary patterns are commonly observed in other passerine species such as chaffinch (*Fringilla coelebs*) (Holland *et al.* 2006).

The results from this study revealed that Hawfinch diet varied between geographical regions within the UK, and amongst sampling years. This spatial variation is consistent with similar metabarcoding studies of birds, as well as studies focussing on insectivorous bats (Clare, Symondson, Broders, *et al.* 2014; McClenaghan *et al.* 2019; Shutt *et al.* 2020). The variation in diet shown between sampling years may be explained by seasonal environments, which are characterised by short term peaks in resource availability (Hinks *et al.* 2015). Within woodland environments, caterpillars' peak availability is a few weeks during spring, as caterpillars hatch in synchrony with bud burst on host tree species (Hinks *et al.* 2015). Year by year, bud burst can vary by up to three weeks, and it has been shown that individual trees within populations can demonstrate bud burst variation (Hinks *et al.* 2015). In a temporally heterogeneous environment such as woodland, Hawfinch may be utilising differing taxa dependant on abundance, with bud burst variation a possible driver of this. It can be tentatively suggested that the temporal variation shown in this study may be, in part, due to variation in bud burst, and therefore variation in the availability of lepidopteran taxa at the time of sampling. Climatic factors may have been partly driving the results found within this study, as climatic factors such as drought and high temperature decrease activity periods for gastropods (Nicolai and Ansart 2017), therefore reducing foraging time and subsequent availability as a food resource to Hawfinch.

Males consumed a lower mean number of prey taxa than females, which may be linked to behavioural differences between sexes such as the differences in reproductive roles (Freeman 2014; da Silva *et al.* 2020). This may be a result of differing requirements for reproduction and growth during the breeding season, for example egg-laying, with females facing a trade-off between self maintenance and reproduction (García-Campa *et al.* 2020). Female Hawfinch may also be restricted in foraging due to nesting activities, while the higher mobility of males may enable them to forage for more nutritious prey taxa that are less abundant in the

immediate environment around the nest site (da Silva *et al.* 2020). Sexual dietary differentiation reported at a local scale in this study may be a result of increased intraspecific competition during the breeding period, which can be crucial in the fragmented landscapes where Hawfinch occur.

3.5.3 Conclusions and recommendations for future research

The previous assessment of Hawfinch diet undertaken by Mountford (1957), coupled with the extensive COI barcode reference library which exists for UK arthropods gives the opportunity to validate metabarcoding methods, and determine if biologically plausible inferences regarding diet can be validated. This study highlights subtle temporal, spatial and biological differences within Hawfinch diet, and to the best of my knowledge, is the first study to explore the invertebrate element of Hawfinch diet in depth.

Dietary analysis by metabarcoding indicates that Hawfinch are generalists with broad dietary niches. The variation in the diet shows spatiotemporal patterns, which is common within other metabarcoding studies of passerines (Shutt *et al.* 2020; Sottas *et al.* 2020). Inter-regional variation in diet may be due to the differing species of host plants required by the differing lepidopteran taxa within each region. In order to maximise the power of dietary analysis, increasing the temporal scale of sampling would be beneficial for future work. Invertebrate abundances should be recorded and deviations from random foraging should be explored in order to increase ecological understanding of Hawfinch feeding ecology within woodlands.

3.6 Acknowledgements

Thank you to all the RSPB field staff, Will Kirby and the members of the Hawfinch ringing group for your assistance with collecting faecal samples. Thank you to Angela Marchbank and Trudy Workman at the Genomics Hub at Cardiff University for their assistance in the Illumina library preparation and sequencing. Thank you to Lorna Drake and Sarah Davies for training and advice in bioinformatics. Thanks to Jen Stockdale and Sarah Davies for primer testing. Thank you to the Welsh Ornithological Society for generously providing funding allowing fieldwork to be undertaken.

Chapter Four - Comparison of the diet of Hawfinch (*Coccothraustes coccothraustes*) between stable mainland European and declining UK populations

Hawfinch in the hand. All birds were captured, handled and ringed by licensed ringers endorsed by the British Trust for Ornithology (BTO). Photo credit: Andy Stanbury: Hawfinch Ringing Group.

4.1 Abstract

The investigation of biogeographical patterns in the diet of widely distributed species is essential in the understanding of their ecology and local adaptations, as well as species' long-term conservation. This can be particularly challenging due to their wide distribution and high ecological plasticity. Dietary richness and variation are under-studied in woodland bird species, due primarily to challenges in accurately identifying plant and invertebrate taxa consumed. The Hawfinch (*Coccothraustes coccothraustes*) has been declining in the UK

since the 1970's, however over the same time period populations within mainland Europe have remained stable. Ecological drivers behind this differing trend are still unknown; one possibility is differences in diet, yet little research has been carried out into Hawfinch diet in mainland Europe or elsewhere. This study aimed to identify the key trophic interactions of Hawfinch populations within Europe, and to explore spatial variation in diet at a large biogeographical scale between two European countries, as well as between UK and European Hawfinch populations, thus providing essential information for future management and conservation of Hawfinch and their habitats. Faecal samples were collected between January and July of 2019 from Hawfinch caught at six artificial feed sites; two in Denmark and four in Germany. DNA was extracted from 91 samples and plant Internal Transcribed Spacer 2 (ITS2) and invertebrate Cytochrome Oxidase Subunit 1 (COI) barcodes were amplified. A total of 55 and 56 plant and invertebrate taxa were identified within the diet, with plant and insect orders Fagales and Lepidoptera respectively the most frequently detected. Hawfinch dietary composition differed significantly between continental sites as well as between continental European and UK populations, suggesting that Hawfinch show dietary plasticity, making use of available food resources which are likely to differ spatially.

4.2 Introduction

The current loss of vertebrate species is estimated to be approximately 1000 times faster than background rates of extinction from fossil records, with Earth having entered into a sixth mass extinction event (Ceballos *et al.* 2017; Brodie *et al.* 2021). Organisms interact with a number of species around them, as well as interacting with their environment, resulting in each species affecting the functioning of its ecosystem (Boast *et al.* 2018; Brodie *et al.* 2018; Brodie *et al.* 2021). Over the last 500 years an extinction wave has occurred through habitat loss, pollution, invasive species and anthropogenic exploitation, as well as interactions among these factors which has led to the decline in population of a wide range of vertebrate species (Ceballos and Ehrlich 2002; Petchey and Gaston 2002; Gaston and Fuller 2008; Rodolfo *et al.* 2014; Ceballos *et al.* 2017).

While conservation efforts have focused on slowing the rate of decline of less abundant species, there is considerably less targeted management towards more common species (Inger *et al.* 2015). Species which are abundant within an ecosystem can often define ecosystem dynamics and structure (Gaston 2010). A minor decrease in the abundance of common species within ecosystems can result in the loss of a large number of individuals and biomass, which can have far-reaching impacts (Ellison *et al.* 2005; Gaston 2010). Birds have been the subject of some of the longest and most comprehensive ecological monitoring schemes, and are frequently used as indicators of environmental change (Jørgensen *et al.* 2016; Bowler *et al.* 2019). Large numbers of studies investigating the population dynamics of

woodland birds have been undertaken across Europe, however these have been focused across a comparatively small spatial scale and rarely across countries (Gregory *et al.* 2007). While these studies have provided important insights into population trends and interactions within a local environment, applying findings beyond the scale of these studies has been challenging (Kouki and Väänänen 2000; Gregory *et al.* 2007). While population studies across multiple countries have occurred, assessments and comparisons have been at a coarse scale (Angelstam and Mikusiński 1994; Angelstam *et al.* 2004; Gregory *et al.* 2007).

Gregory *et al*. (2007) analysed breeding bird data from 18 European countries and found a 13% decline in common forest birds across Europe, while common forest specialists (a subset of common forest birds) declined by 18% from 1980 to 2003. The pattern of decline shown within forest specialists contrasted with generalist species (those able to occupy a range of habitats), which remained stable. The main drivers of European bird decline have been linked to the impacts of land-use changes, such as intensive management of agricultural land and forest management, with many woodland species highly sensitive to habitat alteration (Roberge and Angelstam 2006; Burns *et al.* 2016; Jørgensen *et al.* 2016; Bowler *et al.* 2019). Habitat alteration can result in the fragmentation of suitable habitat, resulting in woodland birds living in sub-optimal environments (Hinsley *et al.* 2008). These reduced patches of habitat may lack the necessary abundance of food resources necessary to sustain a population through reduced foraging opportunities (Stauss *et al.* 2005; Hinsley *et al.* 2008). Inger *et al.* (2015) discovered European birds are declining rapidly, with much of this decline driven by farmland intensification. There have been well publicised population declines of common birds across Europe, including the House Sparrow (*Passer domesticus*) and European Starling (*Sturnus vulgaris*), while some rare species have shown an increase in population, likely a result of direct conservation actions (De Laet and Summers-Smith 2007; Smith *et al.* 2012; Inger *et al.* 2015).

Detailed dietary information is therefore crucial for improving ecological understanding of a species and may provide insight into species' declines. Dietary niche breadth – the variety of taxa that a species consumes (Roughgarden 1972), influences the geographical distribution of species (Slatyer *et al.* 2013), ecological network structure (Layman *et al.* 2015) and sensitivity to environmental change (Colles *et al.* 2009). Diet may affect species' responses to environmental change through insect population declines and range shifts which may result in birds having a reduced ability to meet energetic requirements (Bowler *et al.* 2019). Food supplementation experiments have revealed food availability is a driver behind bird demographic rates and population abundances (Seward *et al.* 2013). Insects have shorter generation times, allowing a quicker response to environmental change (Thomas *et al.* 2004), rendering insectivorous birds more sensitive to environmental change than other bird species

such as generalists. Indirect effects of diet may result from covariation between diet and other factors such as habitat or temperature preferences (Barnagaud *et al.* 2012).

Investigating how diet differs spatially is fundamental in understanding how populations are locally adapted to the populations of species on which they feed (Romano *et al.* 2020). Ecological and climatic conditions directly affect the presence and availability of organisms, resulting in substantial impacts on species composition within the diet (Willig *et al.* 2003; Romano *et al.* 2020). Variation in the distribution of prey species across large spatial gradients has been shown to impact food consumption and predation strategies (Terraube and Arroyo 2011; Romano *et al.* 2020). Additionally, spatial adjustments in dietary composition are likely to be of high significance to individuals for life-history characteristics such as reproduction, and therefore the question of where and how differently populations exploit resources has practical implications for conservation management (Terraube and Arroyo 2011). Despite the importance of acquiring information regarding intraspecific dietary variation, particularly across large spatial scales, this information is currently lacking for many woodland bird species.

Dietary variation can also be driven by intrinsic factors such as sexual partitioning of food resources (Svanbäck and Bolnick 2007; Jones *et al.* 2020). Sexual differentiation in resource use is commonly observed in vertebrates (Mata *et al.* 2016). Segregation is often associated with behavioural or morphological differences between sexes which subsequently impacts life-history traits such as diet (Mata *et al.* 2016; da Silva *et al.* 2020). Sexual differences in food choice may occur through different nutritional requirements required, such as in birds via egg production in females (da Silva *et al.* 2020), or through reduced foraging distances, as the female cannot leave the nest for long periods (Amininasab *et al.* 2017). This may result in females foraging closer to their offspring, and subsequently feeding on more abundant or predictable prey items, while more mobile males may be able to exploit a wider prey range (da Silva *et al.* 2020). While sexual partitioning of food resources is known to occur between bird species exhibiting sexual dimorphism (Bravo *et al.* 2016; Thalinger *et al.* 2018), the hypothesis that differences in prey choice also occurs in monomorphic species remains poorly explored (Cleasby *et al.* 2015; da Silva *et al.* 2020).

One of the main difficulties when conducting dietary studies is related to limitations of dietary analysis methods. For example, microscopic analysis of faecal samples rarely provides the depth of taxonomic resolution required to detect species-level dietary differences (da Silva *et al.* 2020). Recent developments in genetic analysis of diet have enabled the use of molecular barcodes amplified from faecal DNA and analysed using high-throughput sequencing (HTS), coined “metabarcoding” (Taberlet *et al.* 2018). This has resulted in higher taxonomic resolution

of species identified within dietary studies and improved taxonomic accuracy (Ando *et al.* 2013; Galimberti *et al.* 2016; Dunn *et al.* 2018).

Within metabarcoding studies, detection of plant species have traditionally used sections of plant genes *rbcL* and *matK*, which have the power to provide up to 75% species-level discrimination when combined (de Vere *et al.* 2012). Limitations on amplicon length in HTS (maximum of 2 x 300 base pair reads on an Illumina Miseq), as well as primers designed to amplify short barcodes in order to detect DNA in degraded samples (Pompanon *et al.* 2012; Ando *et al.* 2013; Dunn *et al.* 2018) has resulted in these gene regions providing reduced taxonomic resolution in analysis of faecal samples (Pompanon *et al.* 2012). The Internal Transcribed Spacer 2 (ITS2) nuclear gene has been proposed as a suitable barcode for dietary analysis (Moorhouse-Gann *et al.* 2018). Universal plant primers targeting the ITS2 region have been developed, producing amplicons of 187-380 base pairs (Dunn *et al.* 2018; Moorhouse-Gann *et al.* 2018). This has enabled the most variable region within the gene to be targeted, with the amplicon length suitable for use within DNA metabarcoding studies (Moorhouse-Gann *et al.* 2018). ITS2 primers have been successfully used in dietary studies of the Turtle dove (*Streptopelia turtur*), Pink pigeon (*Nesoenas mayeri*) and Telfair's skink (*Leiolopisma telfairii*) (Moorhouse-Gann 2017; Dunn *et al.* 2018; Moorhouse-Gann *et al.* 2018). In a diet which contains a wide range of invertebrate taxa, DNA barcodes from the cytochrome c oxidase subunit I (COI) mitochondrial gene region have become the standard and are used in many species-level identification studies (Kress *et al.* 2015). This is due to the extensive taxonomic coverage and depth within the Canadian, European, UK and USA taxonomic COI reference sequence databases (Porter and Hajibabaei 2018). Such large databases reduce the possibility of false taxonomic assignment and improve higher taxonomic resolution (Somervuo *et al.* 2017; Andújar *et al.* 2018; Porter and Hajibabaei 2018).

4.2.1 Study species

The Hawfinch (*Coccothraustes coccothraustes*) is widespread throughout mainland Europe and has shown to be resident, a short-distance migrant or summer visitor (Tomialojc 2005). While the Hawfinch is declining within the UK, mainland European populations have remained stable, based upon data from the Pan European Common Bird Monitoring Scheme (PECBMS) (Kirby *et al.* 2018; PECBMS 2019). The largest breeding populations are found in Germany (160-350,000 birds) and Poland (200-400,000 birds) (PECBMS 2019). Within Europe, Hawfinch are found throughout flood plain, mature and semi-natural forests containing beech (*Fagus sylvatica*), lime (*Tilia sp.*), oak (*Quercus sp.*) and hornbeam (*Carpinus betulus*) (Bijlsma 1998; Tomialojc 2005). In a high proportion of western Europe, Hawfinch breed within deciduous, broadleaved woodland, however towards eastern Europe conifer-dominated stands are utilised (Tomialojc 2005). It has been established that within the UK Hawfinch are

predominately arboreal and known to feed on seeds, fruits, buds and flowers, as well as invertebrates in spring (Mountford 1957). Molecular analysis of herbivorous dietary items (Chapter 2) found Hawfinch in the UK were frequently consuming beech, cherry (*Prunus sp.*), hornbeam and oak. Nestling diet has been observed to be predominantly oak-roller moth (*Tortrix viridana*) and winter moth (*Operophtera brumata*) (Mountford 1957). Species of Coleoptera, Hemiptera, Annelida, Gastropoda and Araneae have been observed to be taken during the spring and summer (Mountford 1957; Newton 1967). Within the UK, molecular dietary analysis of prey (Chapter 3) detected 118 invertebrate taxa in Hawfinch diet, with Lepidoptera being the most diverse and prevalent Order (73 taxa and present in 61.86% of samples). Winter moth, St Mark's fly (*Bibio marci*) and tree slug (*Lehmannia marginata*) were the taxa most frequently detected the diet. However, to date, there is no detailed research within the literature describing the range and composition of the diet of Hawfinch within mainland Europe.

In this chapter, the main aim was to describe the biogeographical dietary composition of Hawfinch and to explore dietary differences across differing regions where they reside. Spatial variation in diet was explored, as Hawfinch have been previously shown to be generalist feeders across differing heterogeneous woodlands (Chapters Two and Three), with presumed access to differing plant and invertebrate communities. I examined spatial variation in resource use in order to examine if dietary flexibility was adaptive when resources differ spatially. Specifically, I hypothesised that Hawfinch diet composition will differ between mainland European countries and between mainland Europe and the UK due to differing habitat types, as foraging may be constrained by local food availability. Secondly, I hypothesised that dietary richness would differ between the two European countries due to spatial differences in food availability. My second research question was to determine whether dietary composition would differ due to demographic differences in nutritional or energetic requirements between males and females due to reproduction demands.

4.3 Methods

4.3.1 Study sites and field sampling

Fieldwork was conducted between January and July 2019 at six artificial feeding sites, two in Denmark and four in Germany (Figure 4.1a and Figure 4.1b respectively). The Danish artificial feeding sites were located within urban environments in central Jutland, while German sites were located within heterogeneous woodland situated near the town of Hilden, Velbert and Bad Homburg. Feeding sites were primarily mixed broadleaved woodland with beech, oak and birch (*Betula sp.)* being the dominant species. Sites were selected where pre-existing Hawfinch ringing studies are undertaken. All field sampling methods are as described in Chapter Two.

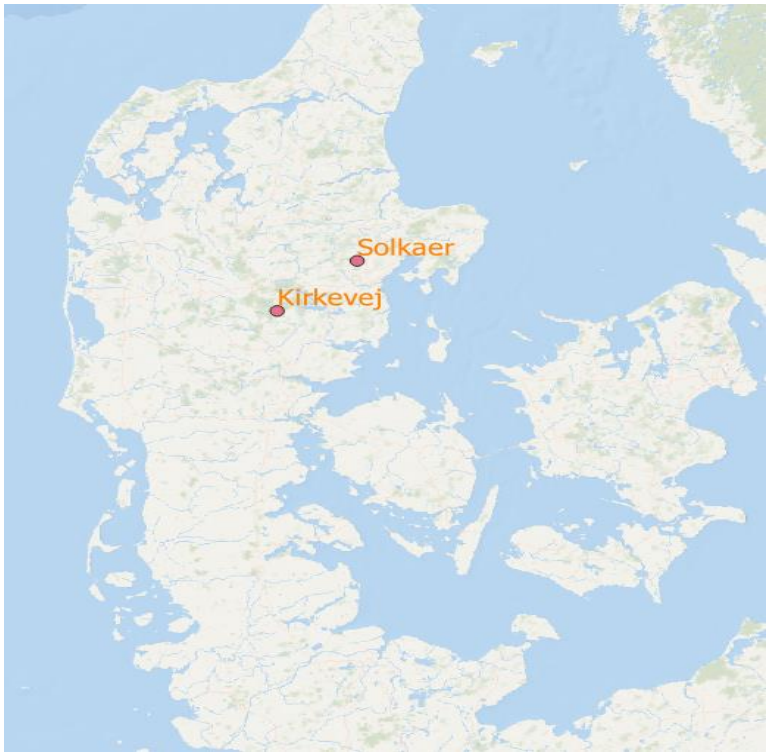


Figure 4.1a. Location of Danish fieldwork sites. Map was constructed using QGIS (QGIS Development Team 2021).

Figure 4.1b. Location of German fieldwork sites. Map was constructed using QGIS (QGIS Development Team 2021)

4.3.2 DNA extraction, PCR amplification and high-throughput sequencing

DNA extraction, PCR amplification using primers UniPlantF and UniplantR for amplification of the ITS2 region of the plant nuclear gene (Moorhouse-Gann *et al.* 2018) and MICOIintF (Leray

et al. 2013) and Nancy (Simon *et al.* 1992) primers for amplification of the invertebrate COI region, library preparation and bioinformatics were undertaken as described in Chapters 2 and 3.

4.3.3 Statistical analysis

For all statistical analysis, the presence/absence of each taxonomic unit within a faecal sample was used as read count is not an accurate representation of abundance due to amplification biases (Yu *et al.* 2012). Control samples were excluded from the analyses. Unless otherwise stated, all statistical analysis were undertaken in R version 3.6.3 (R Core Team 2020).

To identify the most prevalent taxa within Hawfinch diet, the number of samples in which a dietary taxon occurred (frequency of occurrence), was calculated. To test for differences in plant and invertebrate richness in the diet of Hawfinch populations, an initial Poisson and quasi-Poisson GLM was undertaken which revealed an overdispersion statistic value of 1.6. Therefore, the standard errors were corrected using a negative binomial model with a log link function where the variance was $mu+mu^2/theta$ where mu was the mean of the dependent variable distribution and theta was the dispersion parameter of the negative binomial model (theta=8.1). The model was validated using the function *check_model* in the package *performance* (Lüdecke *et al.* 2020), checking for multicollinearity between variables and the distribution of residuals for homoscedasticity.

To investigate how explanatory variables were associated with spatial dietary composition between Danish and German sites and between mainland European and UK sites, separate plant and invertebrate multivariate generalised linear models (MGLMs) were fitted using the function *manyglm* within the package *mvabund* (Wang *et al.* 2012). This allows for multiple species testing and implements a likelihood ratio test (LRT) and re-sampled *p*-values to determine significance. Where an individual had been sampled more than once, data were used from the first capture only to avoid pseudo replication and subsequent biases. Binomial regression structure was specified in the models to account for presence-absence data and a "cloglog" link function was specified to control for large numbers of zeroes in the dataset. The function *anova.manyglm* in *mvabund* was used to test the significance of each term within the model and the *p.uni = adjusted* argument was implemented in order to allow univariate "species by species" results to be returned (Wang *et al.* 2012). The *p*-values returned in this argument were adjusted to control for multiple testing, using a Holm's step down resampling algorithm, allowing control over family error rates (Westfall and Young 1993). Parametric bootstrap resampling was applied to test for dietary differences, ensuring inferences took into account correlation between variables (Wang et al. 2012). The independent variables used within the analysis were chosen to represent environmental and biological variation across

differing temporal and spatial scales. Countries were categorised into Denmark, Germany and the UK.

- Country (three categories)
- Sex

All variables were categorical, and no model simplification was performed as the aim of the modelling was significance testing, rather than developing simpler predictive models. Within mainland Europe, dietary variation between age class was not investigated due to a small sample size for juveniles (*n*=2). Intra-regional plant and invertebrate dietary differences were not investigated due to small sample sizes for sites in both Denmark and Germany (Demark site: Kirkevej *n*=8, German sites: Homburg *n*=3 and Velbert *n*=3). For all models, quantile-quantile (Q-Q) diagnostic plots were checked to ensure normality in multivariate data and multivariate homoscedasticity was checked by plotting Dunn-Smyth residuals against fitted linear predicted values (Wang *et al.* 2012; Bates *et al.* 2015).

Plant and invertebrate dietary differences in Hawfinch populations were visualised using non-metric multidimensional scaling analysis (nMDS) via the function *metaMDS* in the *vegan* package (Oksanen *et al.* 2019). The nMDS was performed with Jaccard distance in three-dimensions (*k*=3), due to the presence/absence nature of the data. Spider plots were produced using nMDS results via *ordispider* and plotted through *ggplot2* (Wickham 2016) to visualise the community differences between countries. To allow ease of interpretation, two axes were used for visualisation but interpretation of the plot was carried out with caution.

4.4 Results

A total of 91 faecal samples were collected between January and July 2019 of which ITS2 DNA was successfully amplified from 55 samples and COI DNA from 24 samples. There were 22 samples which contained both ITS2 and COI DNA. Successfully amplified samples from UK Hawfinch populations (286 ITS2 and 120 COI respectively) were included within the analysis of mainland European and UK populations.

4.4.1 Mainland European Hawfinch diet composition

I retrieved 1,970,111 ITS2 and 4,385,796 COI sequences respectively from 120 Hawfinch faecal samples. A total of 90,847 and 119,241 sequences were detected within negative controls included within the ITS2 and COI runs respectively. A total of 61,721 and 555,017 unique ITS2 and COI sequences respectively were removed due to contamination, tag-jumping and poor quality sequences or reads likely to be a result of degradation. After excluding 39 ITS2 taxa (see Appendix 3.1), 55 plant dietary taxa remained in the diet of Hawfinch. Of the taxa identified, 87% were identified to species and 100% to genus. Dietary items most frequently detected were beech, sunflower seed (*Helianthus sp.*) and English oak

(*Quercus robur*) (detected in 49.1%, 47.3% and 47.3% of samples respectively; *n*=55). Within Denmark, beech, sunflower and English oak were the most frequently detected taxa within Hawfinch diet (63.6%, 63.6% and 27.3% respectively, *n*=33), while within Germany, English oak, sessile oak (*Quercus petraea)* and northern red oak (*Quercus rubra*) had the highest prevalence (77.3%, 77.3% and 68.2% respectively, *n*=22). The data were categorised according to sex of Hawfinch (Table 4.1a).

After excluding 21 COI spurious taxa and contamination (see Appendix 3.2), 56 invertebrate prey taxa were identified within the 24 Hawfinch faecal samples, all of which were identified to species level. The most frequently detected prey taxa were winter moth, white-lipped snail (*Cepaea hortensis*) and satellite moth (*Eupsilia transversa*), found in 50.0%, 33.3% and 33.3% of samples respectively (*n*=24). Data were categorised according to the sex of Hawfinch (Table 4.1b).

Among the Danish samples (*n*=11), 56% of prey items were identified as *Lepidoptera*, 18% *Araneae*, 10% *Hymenoptera*, 7% *Coleoptera* and 3% *Diptera*, *Pulmonata* and *Neuroptera* with the most prevalent detected species being white-lipped snail (36.4%), birch sawfly (*Cimbex femoratus*) (36.4%) and weevil species *Polydrusus tereticollis* (27.3%). In German Hawfinch samples (*n*=13) prey taxa detected within faecal samples were identified as *Lepidoptera* (76%), *Coleoptera* (7%), *Hymenoptera* (7%), *Pulmonata* (5%) and *Diptera* (5%) with the most prevalent dietary taxa were the winter moth (84.6%), satellite moth (46.2%) and common quaker (*Orthosia cerasi*) (46.2%).

The negative binomial GLM revealed Hawfinch sampled from Germany had a significantly higher number of overall genera in the diet than Danish populations (Nakagawa R^2=0.34, estimate 0.59 ±0.13, z=4.55, *p*=<0.001).

4.4.2 Hawfinch dietary variation

Multivariate GLM analysis revealed a significant difference in plant dietary composition between Denmark and Germany (LRT=150.0, *p*=<0.001; Figure 4.2a). Univariate analysis revealed seven genera associated with the dietary differences. *Aegopodium* (LRT=10.0, *p*=0.02), *Alnus* (LRT=17.4, *p*=0.001), *Pinus* (LRT=14.3, *p*=0.003) and *Tilia* (LRT=11.9, *p*=0.011) were only detected in German samples. *Carpinus* (LRT=10.0, *p*=0.02), which was detected in 55% of samples from Germany compared with 12% from Denmark. *Helianthus* (LRT=10.0, *p*=0.02), was detected in 64% of Danish samples compared with 23% from Germany, and *Quercus* (LRT=20.1, *p*=0.001) was detected within 77% and 28% of faecal samples from Germany and Denmark respectively. No dietary differences were detected between the sexes of the two countries (LRT=41.23, *p*=0.145).

Multivariate GLM analysis indicated invertebrate prey taxa within the diet also differed significantly between mainland European countries (LRT=98.7, *p*=<0.001; Figure 4.2b). Univariate analysis showed dietary differences between the countries was associated with *Operophtera* (LRT=18.5, *p*=0.001), detected within 85% of German samples compared with 9% from Denmark. Distinct diets were also found between the sexes (LRT=66.2, *p*=0.029), however no specific prey taxa were associated with the dietary distinction detected, indicating more general dietary differences.

4.4.2.1 Comparison of dietary variation between the UK and mainland Europe

Analysis of the plant dataset revealed Hawfinch diet differed between mainland Europe and the UK (LRT=412.1, *p*=<0.001; visualised in Figure 4.3a). Univariate analysis revealed ten genera were associated with driving the dietary differences detected between sites within the UK and mainland Europe (Table 4.2). Distinct invertebrate diets were also found between mainland Europe and the UK (LRT=190.3, *p*=<0.001; visualised in Figure 4.3b). Univariate analysis revealed two genera were associated with the differences; *Cepaea* (LRT=12.3, *p*=0.018), detected in 33% of faecal samples from mainland European birds compared with 6% from the UK, and *Lehmannia* (LRT=15.9, *p*=0.005) which were only detected within faecal samples from UK Hawfinch.

Table 4.1a. The percentage of mainland European Hawfinch faecal samples testing positive for dietary items broken down by sex.

		Percentage of samples testing positive for a dietary item		
Taxon	Common Name	All (*n*= 55)	Males (*n*=33)	Females (*n*= 22)
Fagus sylvatica	European beech	49.1	42.4	59.1
Helianthus sp.	Sunflower	47.3	51.5	40.9
Quercus robur	English oak	47.3	45.5	50.0
Quercus petraea	Sessile oak	36.4	39.4	31.8
Quercus falcata	Spanish oak	34.6	33.3	36.4
Quercus rubra	Northern red oak	34.6	33.3	36.4
Betula pubescens	Downy birch	32.7	36.4	27.3
Quercus sp.	Oak	27.3	24.2	31.8
Betula pendula	Silver birch	25.5	33.3	13.6
Picea abies	Norway spruce	25.5	27.3	22.7
Carpinus betulus	European hornbeam	23.6	21.2	27.3
Quercus pyrenaica	Pyrenean oak	23.6	27.3	18.2
Larix sibirica	Russian larch	20.0	30.3	4.6
Prunus avium	Wild cherry	18.2	15.2	22.7
Carpinus laxiflora	Hornbeam	16.4	12.1	22.7
Alnus glutinosa	Alder	14.6	12.1	18.2
Prunus cerasifera	Cherry plum	14.6	18.2	9.1
Pinus sylvestris	Scots pine	12.7	18.2	4.6
Salix alba	White willow	12.7	18.2	4.6
Salix sp.	Willow	12.7	12.1	13.6
Acer pseudoplatanus	Sycamore	10.9	9.1	13.6
Corylus avellana	Common hazel	10.9	6.1	18.2
Prunus domestica	Common plum	10.9	15.2	4.6
Quercus canariensis	Algerian oak	10.9	9.1	13.6
Tilia platyphyllos	Large-leaved lime	10.9	15.2	4.6
Aegopodium podagraria	Bishop's weed	9.1	9.1	9.1
Larix sp.	Larch	9.1	12.1	4.6
Cardamine bulbifera	Coralroot	7.3	12.1	0.0
Fagus sp.	Beech	7.3	9.1	4.6
Populus sp.	Poplar	7.3	9.1	4.6
Prunus serotina	Black cherry	7.3	12.1	0.0
Ulmus glabra	Wych elm	7.3	6.1	9.1
Prunus padus	Bird cherry	5.5	9.1	0.0
Prunus spinosa	Blackthorn	5.5	6.1	4.6
Sambucus nigra	Elder	5.5	3.0	9.1
Acer campestre	Field maple	3.6	6.1	0.0
Acer platanoides	Norway maple	3.6	3.0	4.6
Carpinus sp.	Hornbeam	3.6	3.0	4.6
Fraxinus angustifolia	Narrow-leaved ash	3.6	6.1	0.0
Hedera helix	Common ivy	3.6	3.0	4.6

Juglans regia	English walnut	3.6	3.0	4.6
Populus nigra	Black poplar	3.6	6.1	0.0
Ranunculus bulbosus	Bulbous buttercup	3.6	0.0	9.1
Urtica dioica	Common nettle	3.6	6.1	0.0
Erigeron annuus	Daisy fleabane	1.8	0.0	4.6
Geum urbanum	Wood avens	1.8	3.0	0.0
Ilex aquifolium	Common holly	1.8	0.0	4.6
Larix decidua	European larch	1.8	3.0	0.0
Oxalis acetosella	Wood-sorrel	1.8	0.0	4.6
Populus tremula	European aspen	1.8	3.0	0.0
Quercus faginea	Portuguese oak	1.8	3.0	0.0
Seseli libanotis	Moon carrot	1.8	3.0	0.0
Stellaria crispa	Starwort	1.8	0.0	4.6
Taraxacum officinale	Dandelion	1.8	3.0	0.0
Veronica triloba	Ivy-leaf speedwell	1.8	3.0	0.0

Table 4.1b. The percentage of mainland European Hawfinch faecal samples testing positive for invertebrate dietary items. The percentage of each prey taxon is broken down by sex.

		Percentage of samples testing positive for a dietary item		
Taxon	Common Name	All (*n*= 24)	Males (*n*= 15)	Females (*n*= 9)
Operophtera brumata	Winter moth	50.0	46.7	55.6
Cepaea hortensis	White-lipped snail	33.3	40.0	22.2
Eupsilia transversa	Satellite moth	33.3	20.0	55.6
Cimbex femoratus	Birch sawfly	29.2	33.3	22.2
Orthosia cerasi	Common quaker	29.2	40.0	11.1
Amphipyra berbera	Svensson's copper underwing	25.0	26.7	22.2
Agriopis marginaria	Dotted border	20.8	26.7	11.1
Orthosia cruda	Small quaker	20.8	20.0	22.2
Amphipyra pyramidea	Copper underwing	16.7	20.0	11.1
Bibio marci	St Mark's Fly	16.7	13.3	22.2
Erannis defoliaria	Mottled umber	16.7	20.0	11.1
Tortricodes alternella	Winter shade	16.7	26.7	0.0
Clubiona brevipes	Sac spider	12.5	6.7	22.2
Conistra vaccinii	Chestnut moth	12.5	6.7	22.2
Epirrita christyi	Pale November moth	12.5	6.7	22.2
Hedya nubiferana	Marbled Orchard Tortrix	12.5	20.0	0.0
Lypha dubia	Fly	12.5	13.3	11.1
Polydrusus tereticollis	Weevil	12.5	6.7	22.2

Anyphaena accentuata	Buzzing spider	8.3	0.0	22.2
Apocheima pilosaria	Pale brindled beauty	8.3	6.7	11.1
Cephalcia arvensis	Sawfly	8.3	6.7	11.1
Coleophora laricella	Western Larch case-bearer	8.3	13.3	0.0
Colotois pennaria	Feathered thorn	8.3	0.0	22.2
Formica pratensis	Black-backed meadow ant	8.3	0.0	22.2
Orthosia incerta	Clouded drab	8.3	13.3	0.0
Poecilocampa populi	December moth	8.3	0.0	22.2
Polydrusus undatus	Weevil	8.3	0.0	22.2
Ptycholoma lecheana	Leche's twist moth	8.3	0.0	22.2
Acrobasis repandana	Warted Knot-horn	4.2	6.7	0.0
Agelastica alni	Alder leaf beetle	4.2	6.7	0.0
Agrochola macilenta	Yellow-line quaker	4.2	0.0	11.1
Aleimma loeflingiana	Yellow oak button	4.2	6.7	0.0
Alsophila aescularia	March moth	4.2	6.7	0.0
Anorthoa munda	Twin-spotted quaker	4.2	6.7	0.0
Archips crataeganus	Brown oak	4.2	6.7	0.0
Coleophora flavipennella	Tipped Oak Case-bearer	4.2	0.0	11.1
Coleophora lutipennella	Common Oak Case-bearer	4.2	0.0	11.1
Coleophora serratella	Common Case-bearer	4.2	0.0	11.1
Cosmia trapezina	Dun-bar	4.2	6.7	0.0
Epirrita dilutata	November moth	4.2	6.7	0.0
Euchoeca nebulata	Dingy shell	4.2	6.7	0.0
Eudemis profundana	Diamond-back marble	4.2	6.7	0.0
Galerucella lineola	Brown Willow beetle	4.2	6.7	0.0
Hemerobius micans	Lacewing	4.2	0.0	11.1
Linaeidea aenea	Leaf beetle	4.2	6.7	0.0
Lymantria dispar	Gypsy moth	4.2	6.7	0.0
Macaria notata	Peacock moth	4.2	6.7	0.0
Nematinus steini	Sawfly	4.2	6.7	0.0
Nematus alniastri	Sawfly	4.2	6.7	0.0
Neriene peltata	Spider	4.2	0.0	11.1
Operophtera fagata	Northern winter moth	4.2	0.0	11.1
Oswaldia muscaria	Fly	4.2	6.7	0.0
Philodromus collinus	Spider	4.2	6.7	0.0

Quercusia quercus	Purple hairstreak	4.2	6.7	0.0
Succinea putris	Snail	4.2	6.7	0.0
Tetragnatha obtusa	Spider	4.2	0.0	11.1

Table 4.2. Results for the univariate *"anova"* test in the *manyglm* model comparing mainland Europe and the UK. Significant ($p < 0.05$) plant genera differences for the test variable "location" in the final model are shown, ordered by taxonomic genera. Likelihood ratio test values (LRT) and p-values are given for the univariate test. Percent frequency of occurrence values (% FOO) for each plant genera across the factor level are indicated.

Predictor variable	Plant genus	LRT	*p*-value	%FOO UK	%FOO Europe
Location (UK/Europe)	*Aegopodium*	18.3	0.001	0	9.1
Location (UK/Europe)	*Alnus*	10	0.045	2.8	14.5
Location (UK/Europe)	*Anacardium*	16.1	0.002	14.7	0
Location (UK/Europe)	*Betula*	24.7	0.001	10.1	40
Location (UK/Europe)	*Cardamine*	14.6	0.005	0	7.3
Location (UK/Europe)	*Larix*	11.9	0.01	6.3	21.8
Location (UK/Europe)	*Picea*	23.6	0.001	3.5	25.5
Location (UK/Europe)	*Populus*	14.6	0.006	0	7.3
Location (UK/Europe)	*Salix*	18.2	0.001	4.9	23.6
Location (UK/Europe)	*Sambucus*	10.9	0.019	0	5.5

Figure 4.2a.

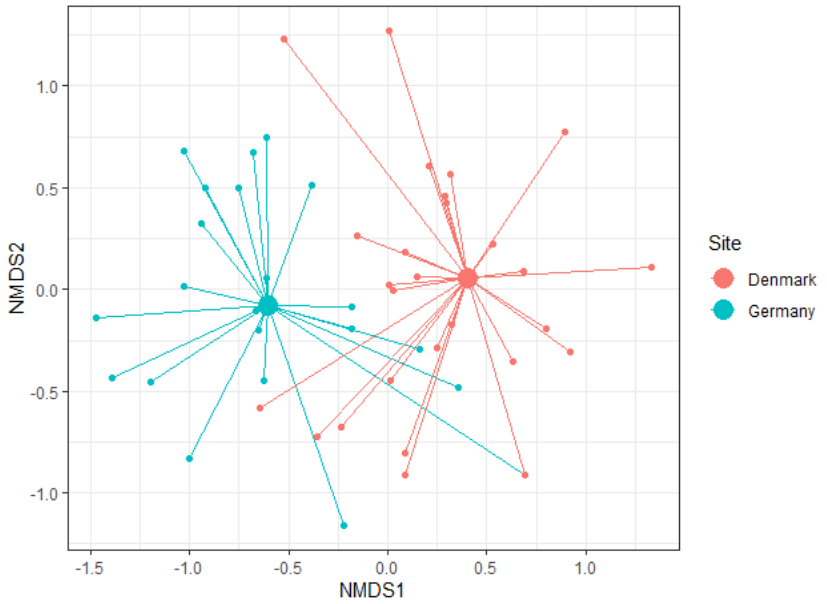


Figure 4.2b.

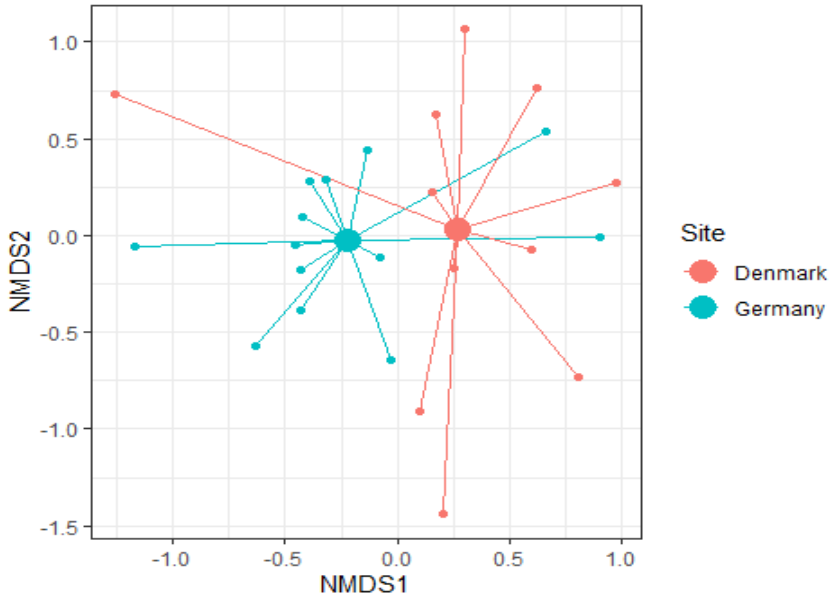


Figure 4.6a. Spider plot for herbivorous taxa consumed by Hawfinch in Denmark and Germany. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its region. Stress = 0.17. **Figure 4.2b.** Spider plot for invertebrate taxa consumed by Hawfinch in Denmark and Germany. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its region. Stress = 0.12.

Figure 4.3a.

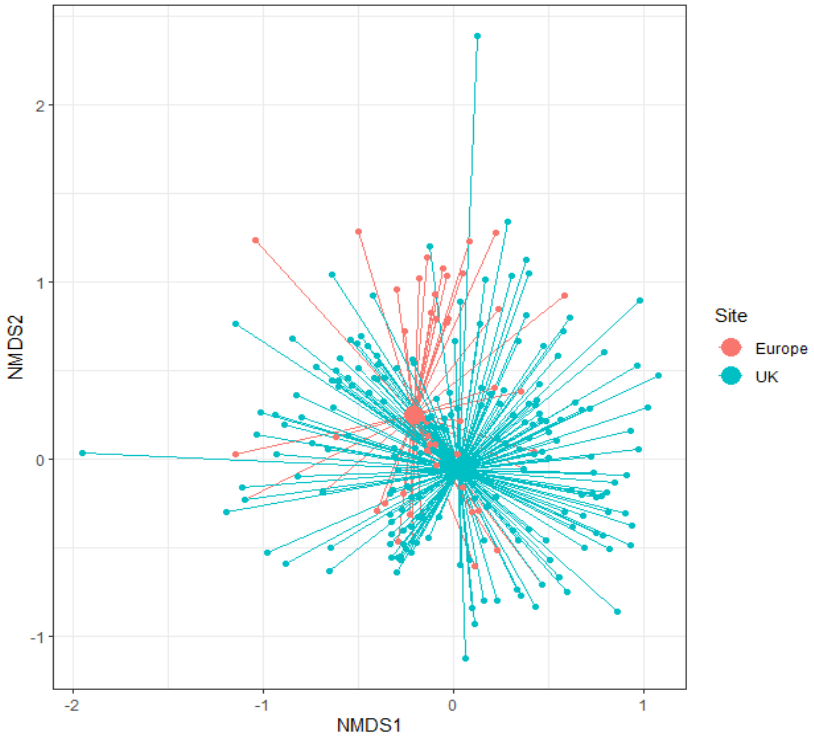


Figure 4.3b.

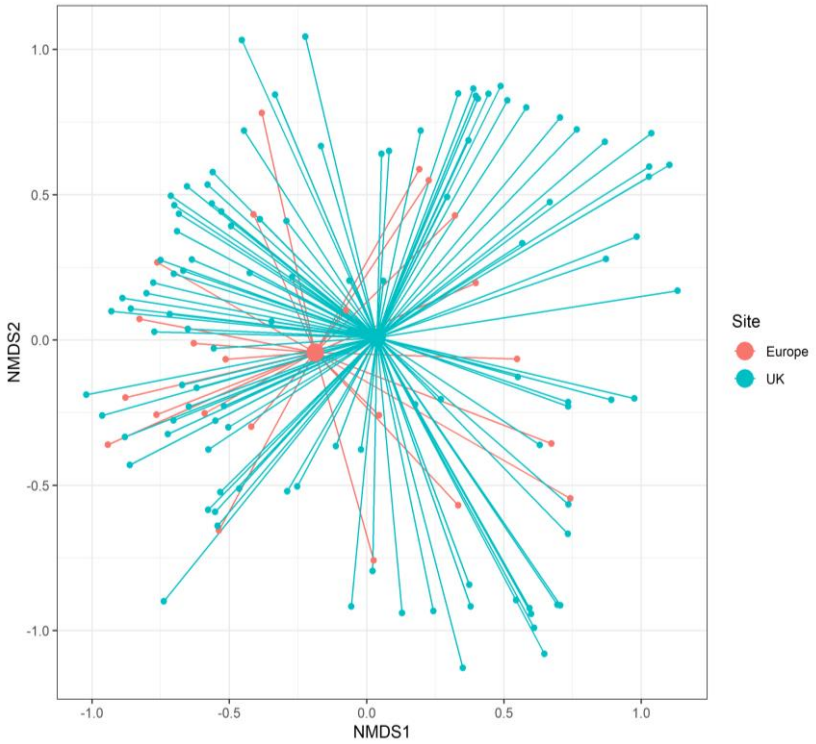


Figure 4.3a. Spider plot for herbivorous taxa consumed by Hawfinch in mainland Europe and the UK. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its region. Stress = 0.17. **Figure 4.3b**. Spider plot for invertebrate taxa consumed by Hawfinch in mainland Europe and the UK. Smaller nodes represent individual Hawfinch with connecting lines joining the individual to the mean centroid (larger nodes) of its region. Stress = 0.16.

4.5 Discussion

This chapter demonstrates the use of high-throughput sequencing in investigating the diet of an avian omnivore, adding to the growing number of studies exploring the implementation of metabarcoding to infer omnivorous dietary information (Robeson *et al.* 2017; da Silva *et al.* 2020; Tercel *et al.* 2022). In this study, which determines the diet of European Hawfinch populations for the first time, 55 plant and 56 invertebrate taxa were identified, resolving 100% of dietary items to species or genus level. Despite a modest level of samples successfully amplified for ITS2 and COI from mainland Europe (*n*=55 and *n*=24 respectively), this study demonstrates the capacity of Hawfinch to exploit a wide range of both plant and invertebrate taxa. The low number of samples testing positive for invertebrate DNA within the diet may be a result of the timing of field sampling. A significant number of faecal samples were collected between January and March (*n*=19) when seasonal invertebrate activity within mainland Europe is lower (Driessen *et al.* 2013). The results support the hypothesis that Hawfinch diet is affected by site at a large geographical scale, as both plant and invertebrate genera consumed differed between Denmark and Germany, as well as between mainland Europe and the UK. There was limited support for the hypothesis that diet composition differed due to demography. No plant dietary composition differences were detected between sexes, however invertebrate prey genera consumed did differ. The data suggests that, while Hawfinch diet is dominated by Fagus and Lepidoptera, several site-specific and demographic factors may influence Hawfinch diet.

4.5.1 Diet composition

This study provides the first molecular based insight into the diet of mainland European Hawfinch, which comprises plant species of (although not limited to) *Fagus sp., Quercus sp.,* and *Betula sp.*, and invertebrate species of (but not limited to) *Lepidoptera*, *Pulmonata* and *Hymenoptera* (Table 4.1a and Table 4.1b). The findings in this study to some degree reinforce previous plant and invertebrate dietary observations of UK Hawfinch (Mountford 1957; Chapters 2 and 3), where high proportions of Fagales and Lepidoptera were detected. However, the high prevalence of oak detected within the diet diverges from previous studies, highlighting the power of metabarcoding to detect previously unknown dietary items (Ando *et al.* 2020). However, as the sample size was small and only taken over one sampling season, these findings should be considered preliminary. Interestingly, the green oak moth (*Tortrix viridana*) was not detected within Hawfinch diet, which has previously been reported as an important food resource (Mountford 1957).

An important aspect to consider within any DNA metabarcoding study is prey detection biases, which can impact the results and subsequent ecological interpretation of metabarcoding

studies (Forsman *et al.* 2022). The invertebrate primer pair used in this study were originally used to characterise the diet of blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*), and subsequently were designed to amplify a broad range of invertebrate taxa, including Lepidoptera (Stockdale 2018). Therefore, the absence of green oak moth in the diet is likely related to availability of the green oak moth within Hawfinch foraging environment rather than a result of false negatives. It is important to note however that no primer pair can provide a completely unbiased and comprehensive account of species' diet due to highly degraded DNA failing to amplify in PCR reactions, primer biases and differences in mitochondrial copy number per cell (reviewed in Clare 2014). A one-locus-several-primer approach should be used more readily within DNA metabarcoding studies in order to maximise taxonomic coverage and minimise false negatives (Corse *et al.* 2019).

Forests cover 33% of mainland Europe, with 96% of forests managed (Thurm *et al.* 2018). Two of the most common tree species within boreal and temperate European forests are European beech and Norway spruce (Thurm *et al.* 2018), with beech being the most frequently detected plant dietary item in this study. The high prevalence of oak detected within Hawfinch faecal samples from German populations may be a result of a high prevalence of oak in deciduous and mixed forest areas due to the economic importance of oak for providing timber (Eaton *et al.* 2016; Woziwoda *et al.* 2019). Oak species rarely form pure forests, with beech, hornbeam and maple present in heterogeneous stands (Eaton *et al.* 2016), all of which were frequently detected within Hawfinch diet. Furthermore, oak has high ecological importance, supporting many invertebrate species (Mitchell *et al.* 2019). Defoliation of oak leaves following bud burst is common by several Lepidoptera taxa, including the winter moth, which was the most frequently detected invertebrate prey taxon within Hawfinch diet. This may reflect which invertebrate taxa are available within the foraging environment of Hawfinch.

Human impact on landscapes can have a direct effect on the quantity and quality of resources (Chace and Walsh 2006; O'Hanlon *et al.* 2020). These changes of resource availability may influence consumer diet and therefore influence the energy and nutrients consumed (Palma *et al.* 2006). This can have subsequent impacts on the survival and reproductive performances of the consumers (White 2008; O'Hanlon *et al.* 2020). While specialist foragers may be negatively affected by landscape changes impacting food availability (Millon and Bretagnolle 2008), generalist foragers can buffer these changes by switching to alternative food resources (Schoener 1971; Pyke *et al.* 1977; O'Hanlon *et al.* 2020). The artificial feed site locations within Denmark were within urban landscapes (back gardens), while the artificial feed site locations within Germany were situated within heterogeneous woodland. Urbanisation has impacted natural habitats through altering vegetation composition, resulting in a shift in species

community dynamics (Narango *et al.* 2018; Jarrett *et al.* 2020). Urban environments offer continuous and abundant food resources throughout the year, favouring euryphagic (broader diets) and granivorous species (Palacio 2020). This could allow Hawfinch to exploit hyperabundant food resources within urban environments, as seen in other generalist species such as the blue tit (*Cyanistes caeruleus*) (Shutt *et al.* 2021).

4.5.2 Dietary variation

Plant and invertebrate dietary composition differed significantly between Hawfinch populations in Denmark and Germany. This indicates that Hawfinch may have more of a generalist diet than previously thought and are likely showing dietary plasticity, however as Hawfinch were only sampled during the spring and summer months our results only represent a snapshot of Hawfinch dietary habits. The results in Chapters Two and Three substantiate the results found within this chapter, which, while analysing Hawfinch populations from the UK, still revealed a high number of plant and invertebrate taxa present within the diet, despite the UK having some of the lowest forest percentage cover in Europe (Raum 2020). The dietary composition differences may further be explained by availability of food resources within the environment, as well as the differing forest communities found between the two sampling countries. Spatial variation in det has been explored extensively in aerial insectivores such as bats, with prey availability found to be an important factor influencing diversity, composition and spatial variation in diet (Czenze *et al.* 2018; Tournayre *et al.* 2021).

This may be a reflection of differences in plant taxa availability within Hawfinch feeding ranges, as a high proportion of the country has undergone deforestation, with only 11% forest land cover remaining (Madsen *et al.* 2005; Stanturf *et al.* 2018). Modern day forestry practices within Denmark are still heavily reliant on non-native species, with all productive conifer species, with the exception of Scot's pine, being non-native (Stanturf *et al.* 2018). Restored forest landscapes have primarily been built on degraded land, and consist of the highly productive mixed stands of Norway spruce and Douglas-fir (Stanturf *et al.* 2018). Norway spruce was detected in 21% of Hawfinch sampled in Denmark, suggesting that Hawfinch can make use of this as a food resource. Widely available food resources may be available within the more heterogeneous woodland environment where the artificial feed sites within Germany were located. Within Europe, Germany is one of the most densely wooded countries, with approximately one third of the landmass forested (Polley *et al.* 2015). Approximately 73% of German forests consist of mixed stands, however the proportions of tree species differ with variation in natural features and site conditions, as well as historic developments (Polley *et al.* 2015). The main species of these heterogenous stands are spruce, pine, beech and oak, with stands of deciduous trees predominantly in lower altitude and coastal areas, which covers the

location of the artificial feed sites used within this study (Polley *et al.* 2015; Schelhaas *et al.* 2018).

Oak was the most prevalent genus detected within the German dataset (77%), indicating oak may be of high availability within the environment. While measuring tree genus abundance within the study sites was beyond the scope of this study, it should be considered for future work to analyse and compare mainland European Hawfinch herbivorous dietary preferences to dietary preferences found for UK populations (Chapter 5). It is important to note, however that without knowing the nutritional importance of these species, their fitness for the consumer is still unknown. Differing species composition and management of forests may also be contributing factors associated with the distinct diets shown. Northern red oak was introduced into Europe from North America in 1691 and is cultivated due to the valuable properties of the wood and the ecosystem services it provides, such as habitat for birds, soil improvement and carbon sequestration (Nicolescu *et al.* 2020). Northern red oak covers over 350,000 hectares in Europe, including 44,550 ha within Germany and 700-1000 hectares within the UK (Wilson *et al.* 2018; Nicolescu *et al.* 2020). Hawfinch have been shown to have a generalist diet (Chapters Two and Three), making use of local food resources when available. Results from this study show that northern red oak was present within 0.4% of Hawfinch faecal samples sampled within the UK, compared with 35% from mainland Europe. The more continuous forest cover of mainland Europe, as well as differing abundances of tree species may allow Hawfinch to access and utilise a differing range of resources than in ASNW areas of the UK. To test this further, tree species composition and abundance should be measured within the Hawfinch artificial feed sites and the surrounding areas, in order to better understanding of Hawfinch feeding ecology and resource utilisation.

Landscape features have been suggested to be important drivers for food availability, as they have been shown to influence dietary composition and spatial variation in insectivorous bat diet (Tournayre *et al.* 2021). Insect species abundance and richness are heavily influenced by landscape features such as plant species richness or heterogeneity of the landscape (Schuldt *et al.* 2019). For example, the lepidopteran *Operophtera brumata* is known to be associated with broadleaved woodlands (Wesołowski and Rowiński 2006) and was shown to have a significant GLM result when analysing dietary composition differences between Hawfinch populations in Denmark and Germany. The presence of invertebrate genera such as *Operophtera* in Hawfinch diet primarily in one sampling site (85% occurrence in faecal samples from German hawfinch populations) partly supports the spatial variation in dietary composition between countries. The pattern of invertebrate dietary composition were likely to reflect site-specific differences in habitat type and sampling locations, with similar patterns being found in spatial variation in the diet of insectivorous bats (Czenze *et al.* 2018). Danish

study sites were located within an urban environment, which during winter, may seem favourable for birds due to scarce natural resources, however during the breeding season, urban environments may lack sufficient high-quality resources such as carotenoids and amino acids available from caterpillars and spiders (Demeyrier *et al.* 2017; Jarrett *et al.* 2020). Furthermore, Lepidoptera have declined substantially across the UK, Finland and Sweden over the last 30 years, with this decline attributed (but not limited to) urbanisation and agricultural expansion (Fox *et al.* 2006; Franzén and Johannesson 2007; Kirkpatrick *et al.* 2017). Both Danish study sites were located in urban environments, with one sampling site approximately 100m from a spruce plantation and agricultural land. Spruce plantations have been shown to have lower species richness of canopy-dwelling beetles when compared with semi natural woodlands of oak and ash (Irwin *et al.* 2014). Furthermore, spruce plantations are generally considered to be species poor, as they are comprised of an intensively managed, non-native tree species (Brockerhoff *et al.* 2008). Within the Danish dataset, there was a lower percentage of lepidopteran taxa detected within the diet (55%) when compared with the German dataset (76%). This may be attributed to decreased Lepidoptera within the immediate foraging area of Hawfinch, and due to the lack of high-quality resources such as caterpillars, Hawfinch are showing dietary plasticity and utilising other invertebrate taxa.

It is important to note however, that these conclusions are based upon a very small number of individual field sites within Denmark (n=2) and Germany (n=4). While the results from this study infer results about Hawfinch diet at a large spatial scale, I appreciate that the results are concluded from a small number of local Hawfinch populations in each country, and therefore the conclusions are somewhat speculative. To increase the spatial coverage shown within this study, future work should incorporate an increased number of field sites across each sampling country, so that Hawfinch populations are better represented across them.

The dietary distinction between UK and European Hawfinch populations may be a result of spatial resource differences, availability and interconnectivity within study sites. From approximately 1918 – 2016, the percentage cover of UK woodlands has grown from 5% of the total land area and now covers approximately 3.16 million hectares (13%) (Forestry Commission 2017; Raum 2020). When compared to European countries forest cover, such as Germany's 33%), the UK is amongst the lowest in Europe (Forestry Commission 2017). The UK has very little natural woodland remaining, with ~340,000 hectares (1.2%) classified as ancient semi-natural woodland (hereafter referred to as ASNW), which is predominantly comprised of broad-leaved species, however this does include pine forests within Scotland (Forestry Commission 2017). ASNW habitats are considered important for Hawfinch feeding, nesting and territory requirements and broader habitat associations (Kirby *et al.* 2015). Within the UK, Hawfinch populations are limited to ASNWs, with low interconnectivity between ASNW

habitat fragments (Kirby *et al.* 2015). As a result, despite utilising ANSWs at a landscape scale, Hawfinch may be limited to the resources available within these habitat fragments (Kirby *et al.* 2015).

Distinct invertebrate, but not plant, taxa were detected between the sexes. Behavioural differences may explain the sexual differences in invertebrate dietary composition (da Silva *et al.* 2020). This is only one of two studies which have used DNA metabarcoding to detect monomorphic passerine species exhibiting sexual dietary differences (see Silva *et al.* 2020). It has been suggested in some bird species that females have reduced foraging ranges in order to be closer to offspring, and as a result, may feed on more abundant or predictable items, even if these items are less nutritious (Sunde *et al.* 2003; da Silva *et al.* 2020). Freeman (2014) found vertical segregation between the sexes of two New Guinean whistlers (*Pachycephala* genus), with little sexual dimorphism, attributed to territory defence and intersexual food resource differentiation. It remains unclear however, how spatial segregation is linked with dietary segregation, and there is little evidence of dietary segregation within monomorphic species. The similarity in plant taxa detected is likely to be related to both sexes, in principle, having access to similar food resources. Although Hawfinch are judged to have minimal sexual dimorphism, biometric measurements such as bill length were not recorded for this study and therefore future work should incorporate this to improve understanding of possible intra-specific variation.

In conclusion, this is one of a small number of studies using metabarcoding approaches to analyse the diet of an omnivorous species and provides a deeper insight into the diet of mainland European Hawfinch populations than previous work. This study has shown Hawfinch diet differs spatially, both between mainland European countries and mainland Europe and the UK. It is likely therefore, that the decline seen in UK Hawfinch populations is unlikely to be based upon dietary choice, as the results shown in this study indicate Hawfinch can expand their diet to include alternative food, possibly enabling reduced competition for resources (Svanbäck and Bolnick 2007). This dietary switching may be particularly useful when food resources are low, such as in more homogenous environments. Identifying the drivers of dietary differentiation within and between populations is important in our understanding of how species adjust to fluctuating environmental conditions.

4.6 Acknowledgements

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Chapter Five – Dietary preferences shown by the Hawfinch (*Coccothraustes coccothraustes*) within mixed woodland habitats

A Hawfinch foraging on the forest floor. Photo credit: Andy Stanbury; Hawfinch Ringing Group.

5.1 Abstract

Diet and dietary preferences are a vital foundation of an animal's life history strategy. Thus, given the importance of diet, establishing knowledge of diet and dietary preferences is vital, especially for declining species. Accurately obtaining this information however is difficult, especially if the study species feeds on a wide range of food items within heterogenous environments, particularly within the tree canopy. Hawfinches (*Coccothraustes coccothraustes*) are one of a suite of rapidly declining woodland passerines, with species-specific drivers behind declines still unknown. This chapter used results from DNA metabarcoding techniques identifying the diet of Hawfinches across five regions of the UK from faecal samples (Chapter Two) and the relative abundance of tree species detected within the diet collected from tree count data from the Wye Valley, north Wales and the New Forest to test for evidence of selective foraging and dietary preferences in Hawfinch populations. Dietary preferences were analysed at both landscape and local scales. The analysis of consumed and available food resources suggested that Hawfinch are selectively feeding, and are consuming certain tree genera regardless of availability. Preferences were shown for cherry (*Prunus sp.*), beech (*Fagus sylvatica*) and hornbeam (*Carpinus betulus*), whilst rowan (*Sorbus aucuparia*), ash (*Fraxinus excelsior*) and hazel (*Corylus avellana*) were avoided. This

information will inform management of woodland in which Hawfinch populations can persist. These data reveal the impact of tree identity and community composition on Hawfinch persistence in broad-leaved woodlands, information that is needed to predict the effects of changing food resources on Hawfinch numbers, both now and in future.

5.2 Introduction

Birds, like all organisms, need to show adaptations to local habitat and resource conditions in order to satisfy their energetic demands (Böhm and Kalko 2009). Individual birds must decide which habitat or forging areas to visit more frequently than others in order to fulfil their daily energy budget (Davison and Jones 1997). Food types are deemed more rewarding if they provide greater energy per handling time than alternative resources, with many species selecting mixed diets in order to meet energetic and nutritional demands (Bolnick *et al.* 2003; Carrillo *et al.* 2007). Partitioning of available resources has been highlighted as a key factor structuring bird communities, and differences in morphological and physiological characteristics result in resource use and foraging strategies differing between species (Tu *et al.* 2020). Additionally, as food availability is often strongly impacted by seasonality, birds can respond to fluctuating temporal and spatial availability of resources through adapting a specialised foraging behaviour (van den Bosch *et al.* 2019). Specialised foraging can reduce resource competition among individuals, with this being a beneficial foraging strategy under strong intra-specific competition pressure (Svanbäck and Bolnick 2007). While specialised foragers may benefit from improved foraging efficiency, they may become vulnerable to fluctuations in abundance of the limited resources exploited (Dall *et al.* 2012). Therefore, the adaptive value of specialisation may vary temporally and spatially, due to fluctuations in resource availability or level of competition (Van De Pol *et al.* 2010; Sheppard *et al.* 2018).

Dietary preferences in birds, or the greater consumption of a certain food resource despite equal opportunity to feed on an alternative food (Bolser *et al.* 2013), can be linked to physiological capabilities and nutritional requirements (Wheelwright 1988; Wilson and Downs 2011). The process of “selection”, unlike preference is where an animal makes a choice among differing resources and consumes them disproportionately to their availability (Johnson 1980). This process is a result of interactions between dietary preferences and a number of factors which modify them (Bolser *et al.* 2013). These include consumer abilities such as handling time, trophic morphology such as gape size, and the subsequent constraints this puts on feeding behaviour (Moran and Catterall 2010). Spatial distribution of resources (Smith and McWilliams 2014), the availability of alternative resources (Blendinger and Villegas 2011), temporal variation of resources, and interaction with other species (Herrera 1982; Carlo 2005) also influence resource use. Increasing understanding of resource use in relation to food availability is a focal point within the study of bird communities (Böhm and Kalko 2009), and

may provide valuable insights into the mechanisms behind the declines seen in woodland passerines.

Previous work on dietary preferences in birds has focused on granivorous and frugivorous bird species (Böhm and Kalko 2009; Molokwu *et al.* 2011; Dunn *et al.* 2018; Rojas *et al.* 2021). Studies on seed selection in birds have suggested that preference is predominantly driven by handling time, as well as distribution and density of seeds (Brown and Mitchell 1989) with seed quality remaining secondary (Diaz 1996). Research on frugivorous species suggest dietary preference is based upon several hypotheses, including optimal foraging theory (Schaefer *et al.* 2003), where frugivores make decisions based upon energy content of fruit eaten; geometry of nutrition (Raubenheimer *et al.* 2009), which states animals balance their macronutrient intake in order to achieve the highest energy gain; and the size-matching hypothesis, where frugivores are more likely to feed on fruits easier to consume (Rojas *et al.* 2021).

To confidently identify the available and consumed food items within the environment of an animal remains one of the biggest challenges within ecology (Lopes *et al.* 2015). This is especially difficult for species which feed on a wide range of food items within heterogenous environments, particularly within the tree canopy, as accurate observations of food resources consumed based on ground level observations is extremely challenging (Matthews *et al.* 2020). Accurate identification becomes even more challenging if the study species utilise a wide range of food resources within diverse environments (Valentini *et al.* 2009; Pompanon *et al.* 2012; Lopes *et al.* 2015).The Hawfinch (*Coccothraustes coccothraustes*) breeds across the Palearctic, where Britain is its western range limit (Kirby *et al.* 2015). Over recent decades, Hawfinch have declined substantially, with a 76% reduction in occupied 10km squares between 1968 and 2011 (Kirby *et al.* 2015). Hawfinch populations are now largely restricted to a small number of westerly locations in England and Wales, with only 4% of 10km squares in Britain occupied (Balmer *et al.* 2013). Hawfinches persist in areas where the landscape is highly wooded, with mature and diverse tree assemblages (Kirby *et al.* 2018).

Hawfinch are thought to be dietary specialists due to their ability to utilise large seeded tree species such as cherry (*Prunus sp.*), hornbeam (*Carpinus betulus*), beech (*Fagus sylvatica*) and elm (*Ulmus sp.*) (Mountford 1957; Newton 1967). During the breeding season (typically from April to June), Hawfinch diet has been observed to include maples (*Acer sp.*), hawthorn (*Crataegus monogyna*), blackthorn (*Prunus spinosa*), wild service tree (*Sorbus torminalis*), dogwood (*Cornus alba*), and larch (*Larix decidua*) (Mountford 1957; von Haartman 1978; Bijlsma 1998; Bryant 2011; Tomiałojć 2012).

This chapter used results from DNA metabarcoding techniques identifying the diet of Hawfinches across five regions of the UK from faecal samples (Chapter Two) and the relative abundance of tree species detected within the diet collected from tree count data from the Wye Valley, north Wales and the New Forest. The aim of this chapter was to determine whether any tree genera are preferred by Hawfinch (do they select food items relative to their availability within woodlands). I tested the hypothesis that previously identified key components of Hawfinch dietary items such as cherry, hornbeam and beech highlighted by Mountford (1957) and identified in the molecular analysis of Hawfinch diet (Chapter 2) will be selected (i.e., these tree genera are more common in Hawfinch diet than would be predicted based on availability). The results from this study may then be used to inform specific management recommendations at a landscape and local scale.

5.3 Methods

5.3.1 Study sites

All tree count surveys were undertaken by RSPB staff prior to the start of this PhD. Three distinct areas were used during this study, corresponding to pre-existing ringing sites in woodland known to contain breeding Hawfinch populations (Figure 5.1). The first study area (sampled 2013-2016) incorporated a segment of the Wye Valley between Monmouth and Chepstow along the border of England and Wales. The second (sampled 2013-2017) was near Dolgellau, Gwynedd in north Wales and the third within the New Forest, Hampshire (sampled 2013). The Wye Valley and north Wales study areas were similar in habitat type, consisting of steep-sloped valleys and heterogeneous, mature woodland. Other notable components of the landscape included farmland, conifer plantations and rural settlements. The New Forest study area was mainly heterogenous mature woodland intersected by roads and forest tracks. Ground cover plants and invertebrate abundance were not recorded when sampling as it was beyond the scope of data collection for this study.

Figure 5.1. Locations of sites where tree surveys were undertaken are shown as green dots. Map was constructed using QGIS (QGIS Development Team 2021).

5.3.2 Hawfinch tracking

Hawfinch caught in 2013 to 2017 were radio-tagged and tracked, as detailed in Kirby *et al.* (2018). To gain a more detailed insight of habitat use GPS archival tags (PathTrack nanoFix© Geo-mini) were additionally attached to a sample of the birds in the Dolgellau study area during post-breeding season 2016, and breeding season in 2017 (Kirby *et al.* 2018).

5.3.3 Tree surveys

Tree count surveys based upon nest site locations (2013-2016) were centred on a nest tree with two to three random locations selected within the same woodland. Tree count surveys based on GPS tag locations were based upon spring/summer Hawfinch locations and compared with randomly selected locations within the same study area. All tree count surveys were undertaken by RSPB staff using a quadrat-based survey methodology. For the 2017 GPS tagged birds, where a tagged bird was known to be nesting (nest found) or suspected to be nesting (suggested by location cluster) all locations within a 50m buffer were excluded, as these were highly likely to be relatable to the nest rather than foraging, for example females often leave the nest and move to a nearby tree to be fed by their mate. For similar reasons locations within a 50m buffer of known feed sites were also excluded, as birds in these areas may have been exploiting the artificially provided seed. Where multiple locations were clustered within 10m of each other a 10m buffer was created around these, with the sampling location defined as the central point. Once all woodlands had been mapped, randomly

selected locations within them were visited to undertake data collection. This was done by assigning numbers to each point and generating random numbers to select which points data were collected from. Control quadrats were randomly generated points within suitable habitat (broadleaved or mixed woodland) across the entirety of the study areas. At all locations visited a 10m * 10m quadrat was marked out with the GPS/random location being the SW corner of the quadrat. Within each quadrat, all tree and shrub species were recorded. Any trees which had a circumference at breast height (CBH) less than 20cm diameter were not recorded to discount saplings which are known not to be utilised as a food resource by Hawfinch. Tree survey count information was analysed in conjunction with genus level plant dietary data results detailed in Chapter 2, produced from DNA metabarcoding techniques identifying the diet of Hawfinches across five regions of the UK from faecal samples.

5.3.4 Statistical analysis

All statistical analyse were carried out in R version 3.6.3 (R Core Team 2020) unless otherwise stated. The *adonis* function was used within the *vegan* package (Oksanen *et al.* 2019) to test for variation in tree genus composition at the landscape scale. Pairwise distances were calculated using Bray-Curtis dissimilarities and the model was run for 999 iterations. In this instance, UK wide analysis is defined as “national scale” while individual study areas are defined as “landscape scale”.

Hawfinch dietary choice may be greatly influenced by food availability. It is possible to elucidate feeding preferences by testing the difference in prevalence of taxa in the diet against a null model based on prevalence of taxa in the foraging environment. This enables us to differentiate between taxa which are consumed in greater, lesser or equal frequencies to their availability. Hawfinch dietary choices were analysed using null models within the *econullnetr* package (Vaughan *et al.* 2018). This was to investigate dietary selectivity and for determining the strength of dietary interactions. The function *generate_null_net* generated a dietary choice selection from the abundance of individual tree genera collected within woodlands where Hawfinches were known to be feeding (tree availability) and the molecular dietary data (tree use). Presence-absence of each tree genus per individual Hawfinch faecal sample was compared to total abundance of the same genus sampled from the tree surveys. Data were analysed at a landscape scale by including all tree quadrats throughout the three study areas where Hawfinch were known to be feeding and comparing this to the equivalent molecular dietary data. Local scale models were run separately using only tree survey and equivalent molecular dietary data from the individual study areas. Models were run for 999 iterations to produce frequency distributions of expected rates of herbivory based on the plant food available. Observed herbivory rates were then compared to those expected by chance. When

these rates were outside the central 95% of simulated values, this indicates deviations from random herbivory. Tree genera not detected during the tree surveys were excluded from the analysis as well as tree genera surveyed which were not detected in the diet. Repeated statistical tests within *econullnetr* analysis can generate Type 1 errors, and up to 5% of significant interactions can be expected to occur purely by chance (Vaughan *et al.* 2018). To minimise Type 1 errors, results generated by *generate_null_net* were scored by standardised effect sizes (SES values) rather than *p*–values. Standardised effect size "measures the number of standard deviations that the observed index is above or below the mean index of the simulated communities" (Gotelli and McCabe 2002). A SES value of 2 is approximately equivalent to a 5% significance level (Gotelli and McCabe 2002). All analysis was done at the genus level in order to standardise the taxonomic level of analysis, as some closely related tree species could not be differentiated in the field. Although this method cannot provide explanations as to the mechanisms underlying resource choice, it can highlight the interactions between food availability and choice (Vaughan *et al.* 2018).

5.4 Results

A total of 27 tree genera were recorded at a national scale. The most frequently recorded genera (number of recorded counts in brackets) were hazel (502), birch (455) and oak (453). At a landscape scale, in north Wales, birch (78), ash (47) and oak (44) were the most commonly recorded tree genera. The Wye Valley was dominated by ash (57), beech (55) and hazel (46), while the genera most frequently recorded within the New Forest were beech (7), holly (4) and oak (3). The *adonis* results revealed a significant difference in tree species composition at a landscape scale (R^2=0.75, *p*=0.01).

From a total of 261 Hawfinch faecal samples, testing for deviations from a random herbivory null model, the resource selection models revealed that Hawfinch had feeding preferences at both a national (Figure 5.2, standardised effect sizes are presented in Table 5.1) and landscape scale (Figure 5.3). Standardised effect sizes at the landscape scale are presented in Appendix 5.1. Analysis of Hawfinch populations at a national scale revealed feeding preferences for five genera: elm, cherry, beech, hornbeam and maples (*Acer*), with the strongest interactions (SES >4) between cherry, beech, hornbeam and elm. Hawfinch were shown to avoid seven genera: lime (*Tilia sp.*), rowan (*Sorbus*), ash (*Fraxinus*), hazel (*Corylus*), chestnut (*Castanea*), birch (*Betula sp.*) and fir (*Abies sp.*).

Analysis of feeding preferences from 108 Hawfinch faecal samples in north Wales showed feeding preferences for four genera: hornbeam, beech, cherry and yew (*Taxus*). Avoidance of six genera: fir, birch, hazel, ash, rowan and elm were also detected. Analysis of 134 faecal

samples within the Wye Valley revealed preferences for five genera: beech, maples (*Acer sp.*), oak, elm and yew. Significantly weaker interactions than expected based upon relative abundance were revealed for four genera: fir, hazel, ash and lime. Conversely, 19 Hawfinch faecal samples analysed from the New Forest showed significant dietary preferences towards oak and did not show significant dietary avoidance for any genus. Hornbeam, beech and cherry all showed large effect sizes (>4) nationally and in at least one individual study area, indicating consistently strong selection, while yew, sycamore, elm and oak showed selection at a landscape scale. Similarly, ash and hazel showed effects sizes <-4 nationally and at a landscape scale, indicating consistent avoidance.

When comparing preferences (SES values) with the abundance of tree genera at a national scale, cherry and hornbeam returned the two highest SES values (17.78 and 14.73 respectively) yet were only recorded 41 and 38 times respectively across the study areas. Conversely, beech had a SES value of 14.13, however was the fifth most frequently recorded genus (399 records). This was also found at a landscape scale, as within the Wye Valley, where beech showed the highest SES value (11.79), however was the second most abundant tree genus surveyed (recorded 55 times). Cherry and hornbeam returned the highest SES values from the north Wales region (14.41 and 12.26 respectively), while being recorded four and six times respectively.

When analysing dietary avoidance at a national level, hazel and birch returned the lowest SES values (-8.81 and -9.68 respectively) and had the highest relative abundance (recorded 502 and 455 times respectively). A similar pattern was also found at the landscape scale, with birch and ash showing the lowest SES values (-6.70 and -5.26 respectively) and highest abundance (recorded 78 and 47 times respectively) within north Wales. Dietary preference shown in the Wye Valley followed a similar relationship, with ash showing the lowest SES value (-8.09) but was the most frequently recorded genus (recorded 57 times).

Table 5.1. Strength of preference comparisons between the tree community detected in the diets of Hawfinch at a national scale. “Weaker”=less of this genus in the diet than expected from its observed frequency. “Stronger”=more of this genus in the diet than expected from its observed frequency. “NS”=reveal genera eaten in proportion to their availability. SES values are standardised effect sizes.

Resource	Common name	Observed	Null	Lower 95% CL	Upper 95% CL	Test	SES
Abies	Fir	4	27.24	18.00	37.00	Weaker	-4.90
Acer	Maple	44	32.23	23.00	42.00	Stronger	2.37
Alnus	Alder	8	9.18	4.00	15.00	ns	-0.40
Betula	Birch	29	83.84	70.00	98.03	Weaker	-7.73
Carpinus	Hornbeam	52	8.73	3.00	15.00	Stronger	14.73
Castanea	Chestnut	2	7.62	3.00	14.00	Weaker	-2.04
Corylus	Hazel	19	90.34	76.00	105.00	Weaker	-9.68
Crataegus	Hawthorn	1	4.69	1.00	9.00	ns	-1.69
Fagus	Beech	173	75.99	63.00	89.00	Stronger	14.13
Fraxinus	Ash	21	82.99	69.00	98.00	Weaker	-8.81
Ilex	Holly	20	15.42	8.00	23.00	ns	1.22
Larix	Larch	15	23.19	15.00	32.00	ns	-1.90
Picea	Spruce	10	14.27	7.00	22.00	ns	-1.15
Pinus	Pine	4	3.32	0.00	7.00	ns	0.38
Prunus	Cherry	62	9.43	4.00	16.00	Stronger	17.78
Quercus	Oak	98	84.11	71.00	98.00	ns	2.00
Salix	Willow	12	7.46	3.00	13.03	ns	1.65
Sorbus	Rowan	5	19.19	12.00	27.00	Weaker	-3.57
Taxus	Yew	15	9.45	4.00	15.03	ns	1.83
Tilia	Lime	10	17.79	10.98	26.00	Weaker	-1.97
Ulmus	Elm	43	20.55	12.00	29.00	Stronger	5.33

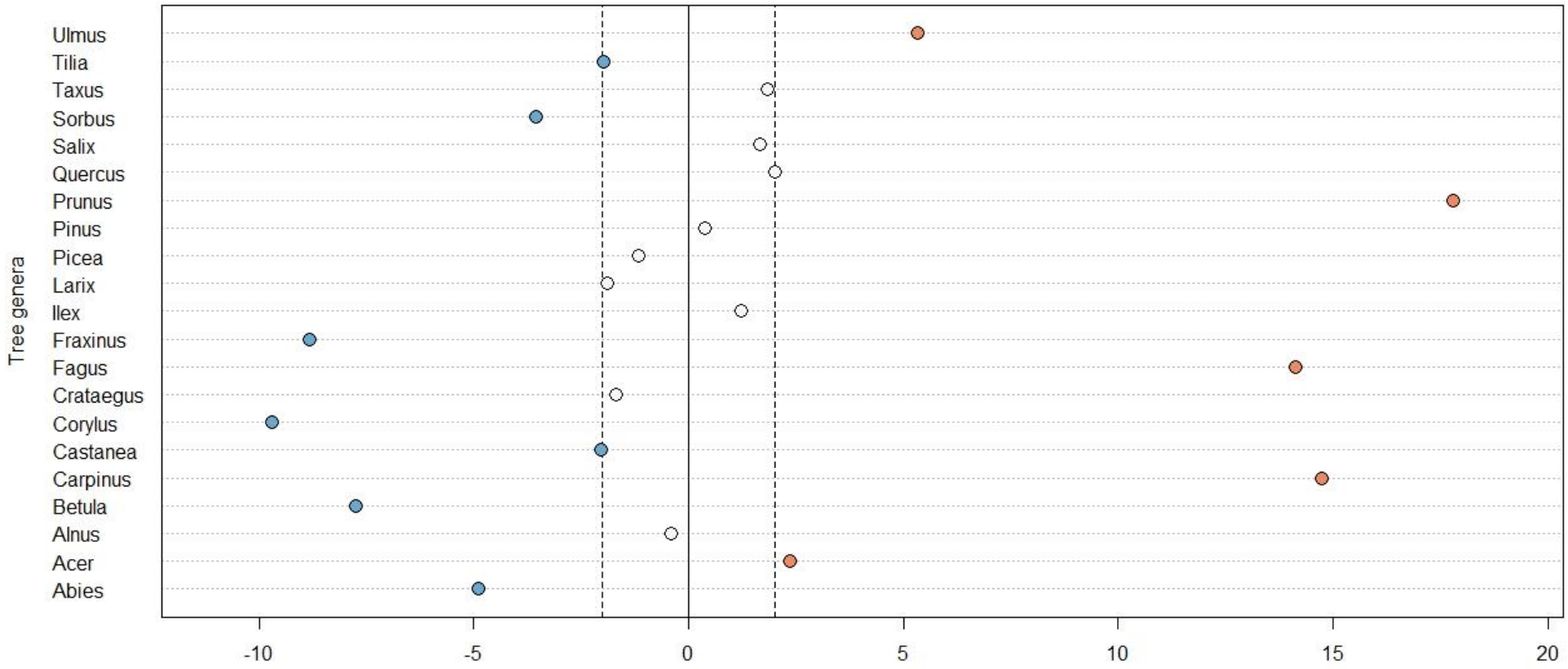

Figure 5.2. Descending alphabetical order preference plot for Hawfinch populations at a national scale comparing the observed interaction frequencies (dots) to the 95% confidence intervals from the null model (vertical dashed lines). The interaction represents occurrences of tree genera within a 10x10m quadrat (resource: available food) and faecal samples (consumed: DNA analysis). The white circles indicate tree genera eaten in proportion to their availability; blue circles: genera eaten in lower proportions than expected; orange circles: genera eaten at a greater proportion than expected. SES values are shown along the x-axis.

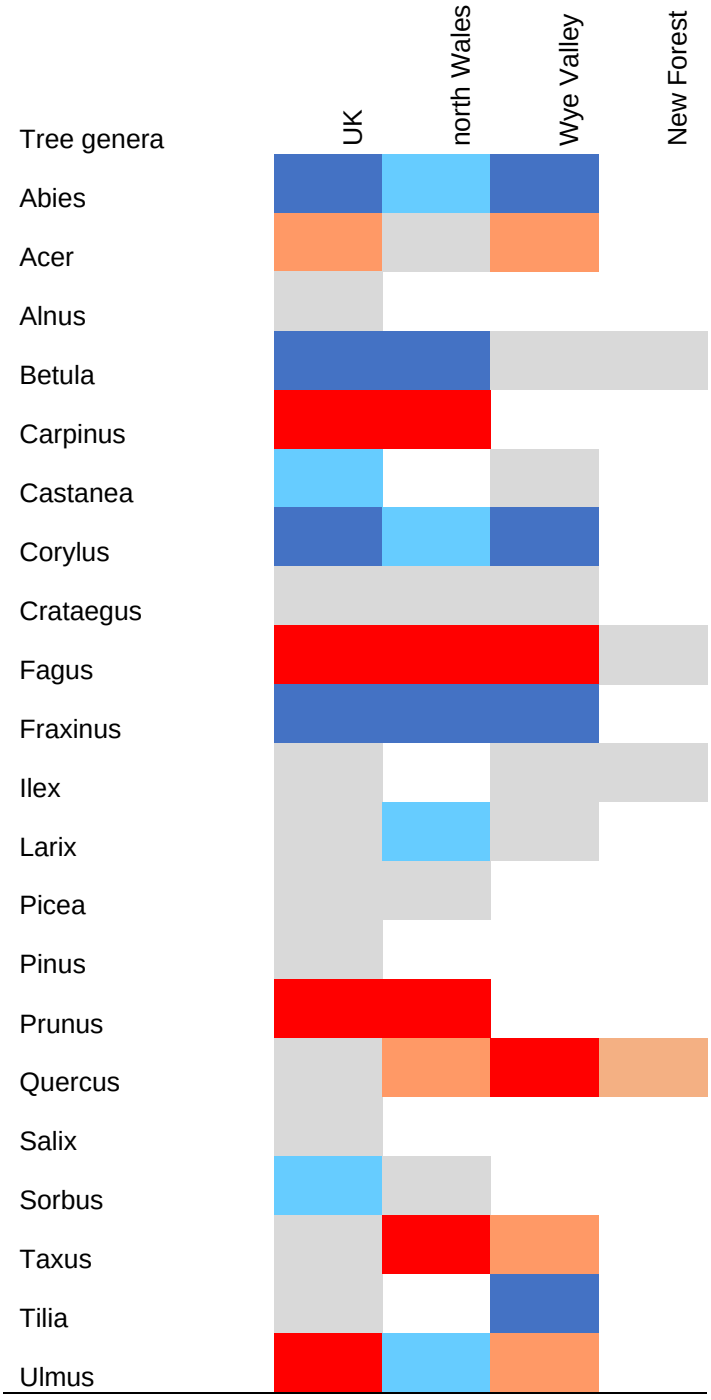

Figure 5.3. Null model standardised effect sizes (SES) for all tree genera at a landscape scale. A white cell indicates genera not included in analyses (either not detected in tree surveys or in molecular dietary analysis). Red cells indicate a SES value of >4, showing strong dietary preference for this genus than expected from the null model, orange cells indicate a SES value of 2 to 4, indicating weak preference for this genus than expected from the null model. Grey cells indicate a SES value of -2 to 2 indicating Hawfinch show no dietary preference for this genus. Light blue cells indicate a SES value of -2 to -4 showing weak avoidance of this genus, while dark blue cells indicate a SES value of <4, showing strong Hawfinch avoidance of this genus than expected from the null model. A SES value of 2 is approximately equivalent to a 5% significance level (Gotelli and McCabe 2002).

5.5 Discussion

Hawfinch consumed certain tree genera (such as beech and cherry) more than expected based upon their relative abundances. Other genera (including ash and hazel) were avoided (Figure 5.1). Our results suggest that Hawfinch are showing selective feeding within woodlands.

Hawfinch were found to be strongly selectively feeding on cherry at both a national and landscape scale, which was not unexpected, considering cherry has previously been highlighted as a key food resource frequently utilised (Mountford 1957). This preference may be due to the high nutritional value of cherry, or that Hawfinch can handle cherry efficiently, giving a high energy reward per handling time (Molokwu *et al.* 2011). Hawfinch may also be preferentially feeding on cherry due to their morphological adaptations of having a large, powerful bill, permitting them to crack cherry stones and subsequently access the kernel within, a food resource unavailable to other bird species. Significant relationships between beak morphology and feeding ecology have been found in Darwin's finches, great tit (*Parus major*), shorebirds and raptors (Gosler, A 1987; Barbosa and Moreno 1999; Bright *et al.* 2016). Local conditions can also result in morphological adaptations within a species, for example in birds, food type and feeding behaviour are strongly linked to bill shape and size (Remsen 2003; García Antón *et al.* 2018). This morphological adaptation and foraging specialisation may allow Hawfinch to coexist with other avian species within the same habitat (in this case broadleaved woodlands) through niche-partitioning, therefore avoiding competitive exclusion and facilitating co-existence (Mansor *et al.* 2021). Hawfinch bill size may also be a result of adaptive evolution, as selection for feeding performances and preferences on certain food types has influenced their bill size and shape (Olsen 2017).

Hawfinch previously frequented traditional orchards in the breeding season and winter months in sufficient abundance to be considered pests, however the area of traditional orchards has declined by 63% and 94% in England and Wales respectively since the 1950's (Kirby *et al.* 2015). While there is no evidence of a direct effect, Hawfinch range decline from south eastern areas of the UK corresponds to the removal of traditional orchards and the subsequent intensive management of those orchards remaining in this area (Mountford 1957; Myczko *et al.* 2013; Kirby *et al.* 2015). Key food resources identified in Chapter 2 such as elm and hornbeam were also shown to be strongly preferred, strengthening the hypotheses in earlier studies that these tree species act as regularly utilised food resources (Mountford 1957; Newton 1967). The impact of Dutch elm disease, which resulted in the estimated loss of 20 million elm trees in the UK (Gibbs *et al.* 1994) may have induced Hawfinch to seek out and hence feed preferentially on the elm trees that remain, especially if the loss of this resource

may reduce Hawfinches ability to retain a suitable condition for breeding (Kirby *et al.* 2015). This may be due to elm providing food resources such as flowers and seeds during early spring, when availability of other resources is low (Kirby *et al.* 2015).

As well as showing dietary preferences, Hawfinch showed dietary avoidance of food resources, including ash. This genus has increased in abundance of broad-leaved woodland since the 1940's, making up 13.1% of total broadleaved area in 2002 (Hopkins and Kirby 2007). Ash has not been highlighted as a frequently utilised food resource (Mountford 1957), and the avoidance shown within this study may be due to other more rewarding food resources (such as cherry) being available. Furthermore, ash seeds are known to contain phenolic compounds which may limit their consumption by Hawfinch (Greig-Smith and Wilson 1985). Throughout much of the country ash trees are dying from ash dieback disease (*Hymenoscyphus fraxineus*) (Mitchell *et al.* 2014). Although these trees form a major component of UK forests, our data suggest the loss of ash trees may not affect Hawfinches. Hazel was also shown to be avoided by Hawfinch, and this result supplements previous observational data showing Hawfinch do not utilise hazel as a food resource (Mountford 1957; Newton 1967). Hazel is predominantly an understorey shrub species, and while Hawfinch require a complex understorey in terms of persistence within woodland, they are not known to feed within the understorey layer, with feeding occurring on the ground and in the canopy (Mountford 1957; Newton 1967). Additionally, the seeds of hazel are large, and as a result only corvids, due to their larger size, and greater spotted woodpecker (*Dendrocopos major*) and nuthatch (*Sitta europaea*) due to their feeding behaviour of "hammering" open the seed, are able to handle them (Laborde and Thompson 2009; P.Bellamy pers. comms).

Analysing dietary preferences at a landscape scale, Hawfinch populations in north Wales showed a strong preference for yew. Due to the temporal range of sampling, it is unlikely that Hawfinch were feeding on yew berries, but can be surmised feeding was on buds or flowers, which are available from February to April (Thomas and Polwart 2003). Yew is a wind pollinated species which produce pollen rich catkins which may be a significant food resource in spring (Thomas and Polwart 2003). Yew may therefore be providing Hawfinch with specific nutritional and energetic benefits over other available food resources. Interestingly, populations in north Wales showed weak dietary avoidance for elm. This may be due to other food resources such as yew and hornbeam meeting Hawfinch nutritional requirements with a higher net energy benefit at the time of sampling. Furthermore, elm has been shown to be less dominant within woodlands containing ash, oak and beech (Thomas *et al.* 2018). The results from this study strengthen this, as elm was not frequently recorded within the north Wales sampling area, with ash, oak and beech recording a higher relative abundance. It is

therefore a possibility that Hawfinch are preferentially feeding on other more abundant tree species such as oak and beech.

Hawfinch populations in the Wye Valley and the New Forest showed dietary preferences for oak, while Wye Valley Hawfinch populations also showed a preference for maples. While maples have been previously highlighted as utilised food resource (Mountford 1957; Newton 1967), oak has not. It was estimated in 2012 that oak makes up 44% of stocked areas within the New Forest national park, covering a total of 5700 hectares, as well as making up 57% of the tree species composition by standing volume (Ditchburn and Brewer 2015). Tree surveys within the New Forest did not record cherry, elm and hornbeam (frequently utilised food resources for Hawfinch), while Wye Valley tree surveys did not record cherry or hornbeam. As a result, it can be tentatively suggested that Hawfinch are showing foraging plasticity to make use of the abundance of oak in the absence of other, preferred food resources. It should be noted however that the sampling effort for the New Forest was smaller than other sites, and these results should be considered in light of this. Future work should increase the sampling effort within the New Forest to achieve more accurate tree abundance estimates.

While the nutritional value and subsequent net benefit of each food resource was not quantified within this study, the dietary preferences shown by Hawfinch may be due to the presence of secondary compounds within certain dietary items (Ríos *et al.* 2012). The presence of toxic secondary compounds may decrease the value of the food resource (Diaz 1996; Molokwu *et al.* 2011). This may be though inhibiting protein uptake through binding with digestive enzymes which may lead to reduced growth rates (Deshpande 2002). Food which contain high levels of toxins is often less preferred and of lower quality, and generalist species will forage on a preferred higher quality food resource, incorporating the lower quality food into the diet only when the preferred food choice has been reduced below a certain threshold (Hochman and Kotler 2006). The impacts of secondary compounds may be dependent on the amount consumed, rather than their concentration (Dearing *et al.* 2005). For example, Bullfinches (*Pyrrhula pyrrhula*) are known to reject seeds containing high levels of phenols (Greig-Smith and Wilson 1985). This study revealed rowan, known to contain secondary compounds (Bobinaitė *et al.* 2020) was strongly avoided by Hawfinch based on its relative abundance. It is possible the concentrations of toxins within rowan limits consumption, therefore when this toxin capacity is exceeded Hawfinch switch to a different food resource. Rowan is considered to be an important tree in the creation of complex understoreys (Hopkins and Kirby 2007). Hawfinch are known to feed primarily in the canopy (Perea and Gil 2014), and therefore it is plausible that this avoidance shown is simply due to their feeding niche within woodlands.

It is important to take into consideration that seasonal diet expansion or switching may occur. This may be due to physiological processes such as gut modulation through the alteration of digestive physiology, allowing more efficient nutrient uptake (Whelan *et al.* 2007). Birds may switch or expand their dietary niche breadth in relation to increasing nutrient requirements for migration or breeding (McWilliams *et al.* 2002; Lahti 2003), or as a result of declining food availability (Dostine and Franklin 2002). The results from this study are only a temporal snapshot, and while focused within the spring and summer months, it can be assumed that during this sampling period Hawfinch would have differing nutrient and energetic requirements than in autumn and winter. In order to fully understand dietary preferences of Hawfinch, tree abundance and dietary data should be collected within autumn and winter to capture a comprehensive picture of Hawfinch dietary preferences throughout the year.

While the methodology used in this study provides a broad overview of Hawfinch dietary preferences, there are limitations to this approach which should be considered. The detection of species abundance and distribution is frequently imperfect within ecological studies (Kellner and Swihart 2014), due mainly to observer error or rarity of species (Dettmers *et al.* 1999; Gu and Swihart 2004). While the tree surveying methodology captured a wide range of tree genera relevant to foraging Hawfinch, dietary items were recorded within Hawfinch diet (Chapter 2) which were not sampled during the tree surveys. While a large number of tree surveys were undertaken, complete surveys of the woodlands were not possible due to the time needed to accomplish this. As a result, random subsets of the woodlands were surveyed. Highly positive SES values seen in this study may be a result of low measured abundance from the tree surveys, potentially due to the patchy distribution of certain tree genera within woodlands. This may result in skewed estimations of overall tree richness and abundance. Nonetheless, without the application of remote sensing data such as Airborne Laser Scanning (ALS), on the ground field quadrats were considered the most appropriate and effective sampling methodology available to quantify the abundance of tree genera, which together form the majority of Hawfinch herbivorous dietary items. Due to the depth of knowledge required to accurately identify closely related and morphometrically similar trees to species level, a broad-spectrum sampling approach was selected identifying trees to genus level. In future, identifying trees to species level will provide a deeper level of understanding when researching dietary preference analysis.

To conclude, Hawfinch populations throughout the UK show dietary preferences for resources previously observed to be highly utilised (Mountford 1957) and frequently occurring within the diet (Chapter 2). This may be due to the net energy benefit gained by Hawfinch from consuming these resources, the presence of secondary compounds which limits consumption

of certain resources, or the seasonal switching of diet in order to match changing nutritional requirements. Whether the dietary preferences found within this study directly translate to dietary importance is determined by the tree tissue type consumed and its nutritional value. While this was not investigated in this study, it is encouraged for future research. Having nutritional information will further knowledge regarding how sensitive Hawfinch are to environmental changes, such as climate change or changes in woodland composition, factors which have been investigated as possible drivers of woodland bird decline (Fuller *et al.* 2005).

The combined use of HTS and tree composition data in this study has enabled the feeding preferences of Hawfinch to be analysed to a greater extent than in previous studies (Mountford 1957). The use of this approach within this study therefore raises the possibility that this method can be applied to future woodland passerine studies. The combination of in-depth dietary data using molecular methodologies and knowledge of feeding preferences can result in more in-depth analyses of woodland bird species diets and trophic interactions, leading to improved understanding of how woodland bird species are interacting within their environment. This has the potential to enhance understanding of the drivers behind the decline of woodland bird species.

5.6 Acknowledgements

Thank you to Ian Vaughan for assistance with the application of econullnetr. Thanks to Will Kirby and all the RSPB fieldwork team for collecting the tree survey data and help with the methodology.

Chapter Six – General discussion

A female Hawfinch. Photo credit: Andy Stanbury: Hawfinch Ringing Group.

6.1 Project aims

The overall aim of this PhD was to use DNA metabarcoding methodologies to document at a fine scale the diet of Hawfinches (*Coccothraustes coccothraustes*) across a broad range of their distribution and to explore whether demographic factors of age and sex were driving intraspecific differences in dietary composition. This PhD also explored the diet of Hawfinches in relation to food availability, to determine whether any tree species are preferred by Hawfinches based on tree species availability within Hawfinch foraging habitat. Results from this research have improved our understanding of Hawfinch feeding ecology and could feed into conservation management strategies aiming to help tackle the decline of Hawfinches within the UK.

Specific aims of this PhD were to: i) compile a comprehensive and fine scale taxonomic overview of both plant and invertebrate taxa within the diet of UK Hawfinches in order to improve understanding of their dietary needs, ii) explore spatiotemporal variation in Hawfinch diet to examine whether variation in resource use is an important adaptive form of dietary or nutritional flexibility when resource availability fluctuates iii) to determine whether Hawfinch demographics are a driver of dietary composition differences iv) determine the diet of the stable mainland European Hawfinch populations and analyse food choice differences between

UK and mainland European populations and finally, v) assess whether Hawfinch show dietary preferences by selecting herbivorous food items more frequently than predicted based on relative availability.

6.2 Completion of aims

6.2.1 Main findings

Two general markers, targeting a 187-387 bp region of the Internal Transcribed Spacer 2 (ITS2) (Moorhouse-Gann *et al.* 2018) and a 406 bp region of the Cytochrome Oxidase One (COI) (Stockdale 2018) were used to amplify and sequence DNA from a range of plant and invertebrate dietary items within Hawfinch faecal samples using high-throughput sequencing (HTS). The output sequences were compared with those held within the ITS2 database and Genbank to identify dietary items. While the ITS2 primer pair have successfully been used in herbivorous dietary studies (Dunn *et al.* 2018; Moorhouse-Gann *et al.* 2018), the COI primer pair used for invertebrate DNA amplification targeted a longer DNA region than previous studies (King *et al.* 2008; Zeale *et al.* 2011; Jedlicka *et al.* 2017; Shutt *et al.* 2020). While it is known that longer DNA sequences give higher taxonomic resolution (Liu *et al.* 2020), primers amplifying shorter DNA fragments are frequently used due to improved amplification of degraded DNA typically found in faecal samples (Alberdi *et al.* 2017). Despite this potential pitfall, a broad range of invertebrate prey items were detected, with all invertebrate prey items detected to either genus or species level. These primers have already been successfully used in the exploration of warbler diet (Davies 2020) and as a result, I recommend this primer pair for use in future studies exploring invertebrate components of avian diet.

The plant and invertebrate diet of Hawfinch was documented to a fine scale across the UK over multiple sites and years and two mainland European countries across multiple sites. This PhD is one of the first to use molecular techniques to explore spatiotemporal variation in the diet of a woodland bird with presumed access to differing food resources, across a broad distribution range. These findings have implications specifically for our understanding of how Hawfinch diet differs spatially and demographically and shows how DNA metabarcoding can be utilised in dietary studies of woodland avian omnivore species in general. I found support for the hypothesis that Hawfinch show adaptive dietary flexibility when resource availability fluctuates, as plant and invertebrate taxa within Hawfinch diet varied between regions of the UK and sampling year, as well as varying significantly between mainland European countries. I also found limited support for the hypothesis that dietary composition is (at least partially) driven by demography. Plant dietary composition differences were detected between adults and juveniles, with juveniles shown to consume supplementary feed more frequently than adults. The data suggest that, while faecal samples of Hawfinch are dominated by Fagales and Rosales, it is likely that several site-specific and demographic variables influence the

dietary composition detected. One potential reason behind the spatial variation seen in Hawfinch dietary composition may be site-specific tree composition differences. For example, sampling sites within the Wye Valley were comprised of a mixture of heterogenous, beech dominated woodland and hornbeam plantation, with sites in the New Forest consisting of oak dominated heterogenous woodland with Hawfinch foraging in each. Thus, the spatial variation seen in Hawfinch dietary composition is likely to be a reflection of food availability, with Hawfinch feeding opportunistically on resources, as seen in many bat species (Vesterinen *et al.* 2016; Czenze *et al.* 2018; Tournayre *et al.* 2021).

There was also support for the hypothesis that Hawfinch dietary composition differed due to demography, with adult and juvenile birds having different dietary compositions. A possible explanation for the differences detected are experience in foraging. Juvenile birds are less experienced foragers than adults and use appropriate learned cues less often (Thornton and Lukas 2012; Franks and Thorogood 2018). This can lead to less efficient foraging, for example if juvenile birds sample a wide number of food sites to acquire information they may return to non-rewarding food sites more frequently (Naef-Daenzer 2000). This lack of experience may be particularly evident during the first spring/summer post fledgling, where plant food resources are unknown (Goss-Custard and Durell 1987). Alternatively, habitats which have a wide range of potential food resources may enable juvenile birds to find food more successfully, and sample a wider number of food resources to gain information about them (Marchetti and Price 1989).

Supplementary food was found to be highly prevalent and ubiquitous within Hawfinch diet. A major issue to address in relation to avian ecology studies is how diet differs between populations occupying urban habitats from conspecifics occupying natural habitats (Coogan *et al.* 2018). Anthropogenic food resources can distort the diets of species which frequent urban areas to certain extents (Coogan *et al.* 2018). Dietary analysis of Australian silver gulls (*Larus novaehollandiae*) found 85% of stomach contents consisted of human discarded food resources, while approximately 38% of suburban Florida scrub jay diet (*Aphelocoma coerulescens*) was comprised of peanuts (Smith and Carlile 1993; Fleischer *et al.* 2003). Dietary differences can have strong implications for fitness of individuals utilising anthropogenic food resources if their diet is imbalanced relative to the nutritional requirements needed for survival within a natural environment (Coogan *et al.* 2018). We found that Hawfinch dietary composition differed between north Wales, where Hawfinch had access to urban garden feeders and the Wye Valley, where anthropogenic food subsidies were reduced. However, it can be suggested that Hawfinch in more urbanised areas still consume a similar amount of macronutrients as populations in natural environments consuming different food resources (dietary generalists and macronutrient specialists), as both populations share

regulatory systems which govern nutrient intake (Coogan *et al.* 2018). Hawfinch breeding success has not been found to be reduced within north Wales (Will Kirby, personal communication), suggesting that Hawfinch may be consuming food items in urban environments which are still beneficial for fitness. To test this further, nutritional studies should be undertaken to understand and predict the nutritional requirements and foraging goals of Hawfinch from contrasting habitats. Having knowledge of Hawfinches nutritional niche within its native range can be used in predicting how Hawfinch may respond to environmental change in the quality and nutritional characteristics of available food within its foraging environment (Raubenheimer *et al.* 2012).

Many invertebrate taxa detected in the diet were rare at the genus level, whereas a smaller number, such as Lepidoptera were detected at very high frequency. This suggests there may be a presence of a core diet (Tournayre *et al.* 2021), which makes up the foundation of Hawfinch dietary composition, and a secondary diet comprised of many rare taxa occurring once at sampling locations. Hawfinch core diet included common genera *Amphipyra*, *Eudemis*, *Orthosia*, *Operophtera* of the Noctuidae, Geometridae and Torticidae families which were shared by all populations. A previous study based on personal observation of Hawfinch feeding suggested that Lepidoptera should be considered as “key” prey (Mountford 1957), however this classification was based upon observation of Hawfinch feeding. The key prey genus *Operophtera* described by Mountford (1957) was found in the core diet of Hawfinch using DNA metabarcoding methodologies, however some differences were seen. We did not detect the oak-roller moth (*Tortrix viridana*) in the core diet, while conversely Diptera (*Bibio)* was detected in this study but not identified by Mountford (1957). This is likely due to biases, either from the inability of personal observation to identify certain prey items within Hawfinch diet (Matthews *et al.* 2020), or biases associated with DNA metabarcoding such as degraded DNA not being amplified (Alberdi *et al.* 2017). Additionally, it is important to note that delineating boundaries between core and secondary diet based on occurrence data remains arbitrary. Moth species present within Hawfinch faecal samples were similar with respect to habitat preferences. All moths detected, including the most frequently detected genera were habitat generalists (e.g., *Operophtera* and *Erannis*), found in widespread habitats such as woodlands. Moths which breed in woodland have increased in distribution by an average of 12% (Fox *et al.* 2021). A potential factor therefore, in the frequent detection of Lepidoptera within the diet is due to the heterogenous foraging environment of Hawfinch, as heterogeneous woodland is important habitat in maintaining diversity of Lepidopterans (Evens *et al.* 2020) through the availability of larval food plants (Sánchez-Bayo and Wyckhuys 2019). Furthermore, the dominance of Lepidoptera may simply reflect local availability of prey type, with Hawfinch showing dietary plasticity to utilise locally abundant food resources.

Molecular dietary data from Chapter 2 was combined with tree genera abundance data obtained from tree count survey data in the Wye Valley, north Wales, and the New Forest. This was to determine whether any tree genera are preferred or avoided by Hawfinch. I tested the hypothesis that Hawfinch would select particular genera of tree (based on occurrence within Hawfinch diet than would be expected based on availability). Results indicated that Hawfinch consumed some tree genera more than expected based on their frequency within their foraging areas and consumed other tree genera less than expected. Hawfinch showed preferences for common tree genera such as cherry (*Prunus sp.*) and beech (*Fagus sylvatica*) which were previously identified as key food resources (Mountford 1957) and which were found to frequently occur in the molecular analysis of diet (Chapter 2). Optimal foraging theory suggests that when food is abundant, individuals are likely to be choosy and will select higher quality food (Pyke *et al.* 1977). Hawfinch are generally considered a habitat specialist, however dietary composition and preference was shown to vary between sites at a relatively local geographical scale with similar foraging environments, as has been seen in the Daubenton's bat (*Myotis daubentonii*) (Kirby *et al.* 2015; Vesterinen *et al.* 2016). The dietary preferences shown may thus be a result of Hawfinch locally adapting to efficiently utilise certain tree genera in order to reduce the cost of associated foraging (Vesterinen *et al.* 2016). These results, combined with *a priori* knowledge highlight the importance of certain key tree genera in likely Hawfinch persistence within their core habitat of ancient semi-natural woodland (Kirby *et al.* 2015).

6.2.2 PhD Chapter summaries

In Chapter 2, the focus was on determining plant dietary taxa found within Hawfinch diet in the UK. Hawfinch faecal samples were collected at 11 feeding stations within five UK regional population hotspots. Hawfinch populations were found to show high levels of dietary plasticity. A total of 84 taxa across 51 genera were identified from faecal samples, of which 92% were identified to species and 100% to genus. Hawfinch diet was found to vary significantly nationally, as well as between sampling years and age-classes. No dietary differences were detected between sexes. At a landscape scale, Hawfinch diet was found to vary significantly between feeding stations within the Wye Valley and north Wales, although only these regions were included in the analysis due to multiple feeding sites within them. Hawfinch were shown to consume supplementary feed frequently, with supplementary feed being present in 44.1% of all faecal samples including 79% of the juvenile faecal samples collected. Supplementary feed prevalence in the diet was found to be significantly different between artificial feed site locations and sampling years.

In Chapter 3, the focus was on the molecular identification of invertebrate prey taxa found within UK Hawfinch diet. A total of 118 prey taxa were identified from faecal samples, 96% to

species level and 100% to genus, achieving a greater taxonomic resolution than previous studies (Shutt *et al.* 2020; da Silva *et al.* 2020; Mitchell *et al.* 2021). As found in other avian studies (Orłowski *et al.* 2014; Rytkönen *et al.* 2019; Shutt *et al.* 2020; Mitchell *et al.* 2021), the order Lepidoptera was the most taxon rich (73 taxa), and the most commonly recorded (present in 61.9% of samples). As observed in Chapter 2, Hawfinch diet varied between populations at a national scale, and between sampling years, while diet did not vary between sexes. Hawfinch were found to be eating fewer plant than invertebrate genera, which may be a result of Hawfinch altering their diets in order to balance nutrient and energy intake (Marshall *et al.* 2016), or simply that invertebrates are more taxon rich than trees in woodlands (Miklín and Čížek 2014). Males were found to have significantly fewer prey taxa within the diet than females. This is likely due to behavioural differences, as females are more limited in foraging due to nesting activities (Freeman 2014). Defoliating larvae were frequently detected within Hawfinch diet, indicating that Hawfinch may be showing switching behaviour (Kjellander and Nordström 2003) by exploiting specific and abundant food resources within their foraging environment.

Chapter 4 explored the diet of mainland European Hawfinch populations collected from populations in Denmark and Germany. A total of 55 and 56 plant and invertebrate taxa respectively were identified from faecal samples, with 87% and 100% of plant and invertebrate dietary items identified to species level respectively. While there was no significant variation in the number of plant or invertebrate taxa detected within Hawfinch diet, plant and invertebrate dietary composition varied significantly between the two countries. This may be a reflection of differences in plant taxa availability within Hawfinch feeding ranges, as the glacial retreat after the last Ice Age left forests in Denmark species-poor when compared to temperate forests such as those in Germany (Stanturf *et al.* 2018). Within the invertebrate dataset, there was a lower percentage of lepidopteran taxa detected within the diet of Hawfinch faecal samples collected from Denmark (55%) when compared with the faecal samples collected from Germany (76%). This may, however, simply be due to an effect of local habitat differences between feeding sites.

Chapter 5 explored whether Hawfinch showed dietary preferences or avoidance for certain tree species. Tree survey data was collected from three areas of the UK, the Wye Valley system between Monmouth and Chepstow, areas of broadleaved woodland near Dolgellau, Gwynedd in north Wales and broadleaved woodland in the New Forest, Hampshire (Kirby *et al.* 2015). All three study sites were similar in their habitat types, which consisted of heterogeneous, mature woodland. At a landscape scale, Hawfinch were shown to have strong dietary preferences for elm (*Ulmus sp.*), cherry, beech, and hornbeam (*Carpinus*). All of these genera have been previously highlighted as frequently utilised food resources for Hawfinch

(Mountford 1957; Newton 1967). At a local scale, Hawfinch dietary preferences were found for cherry, oak (*Quercus sp.*) and yew (*Taxus*) within the north Wales sampling region, and oak, beech, and maples (*Acer sp.*) within the Wye Valley. Hawfinch populations within the New Forest showed weak dietary preferences for oak.

Hawfinch showed dietary avoidance for rowan (*Sorbus*) and hazel (*Corylus*) which had not previously been observed as a food resource (Mountford 1957; Newton 1967). Dietary avoidance for these genera was found at landscape and local scales, indicating consistent avoidance. This may be due to preferred food choices giving a higher energetic or nutritional benefit to Hawfinch, the presence of secondary compounds limiting their consumption or increased handling time (Ríos *et al.* 2012). Furthermore, these results show that mature, broad-leaved heterogenous woodland provide Hawfinch with a suitable diversity of dietary items to exploit, indicating that many tree taxa identified within the diet are important. This indicates that woodland management regimes should be reviewed, to maintain the heterogeneity of broad-leaved woodland, especially within heavily managed areas.

6.2.3 Future implications for Hawfinch conservation

Within ecology, knowledge of "who-eats-what" is of great importance in order to gain better insights into complex trophic interactions (Pompanon *et al.* 2012; Sow *et al.* 2020; van Schrojenstein Lantman *et al.* 2021). The results from this study begin to characterize trophic interactions associated with Hawfinch and heterogenous woodland habitats in the UK and mainland Europe, as well as improving knowledge of the environmental and demographic drivers behind Hawfinch dietary composition. Dietary analysis can provide information on food preferences of individuals, but can also contribute towards building a description of the biodiversity across the foraging area of the study species, especially in the case of omnivores such as the Hawfinch (Boyer *et al.* 2015; Nørgaard *et al.* 2021). The use of DNA metabarcoding methodology within avian dietary studies has already produced novel information, such as the Western bluebird (*Sialia mexicana*) consuming mosquitoes (*Aedes* genus), blue tits (*Cyanistes caeruleus*) consuming winter moth (*Operophtera brumata*) caterpillars earlier than expected in spring (Shutt *et al.* 2020) and European nightjars (*Caprimulgus europaeus*) showing intra- and inter-annual dietary variation (Mitchell *et al.* 2021).

Analysis of Hawfinch faecal samples using molecular methods identified key prey taxa and gave unparalleled insight into Hawfinch dietary breadth. From Chapters 2,3 and 4, it can be concluded that Hawfinch are utilising a broader range of plant and invertebrate taxa than previously recorded (Mountford 1957). Insect diversity is directly impacted by the community composition and diversity of plants, with increased plant diversity known to facilitate co-existence of herbivore and predator species (Rzanny *et al.* 2013; Scherber *et al.* 2014; Hertzog

et al. 2016; O'Brien *et al.* 2017). However, while the general prediction of increased plant diversity results in increased insect diversity (Scherber *et al.* 2010) is still a valid one, specific plant species may have an overall higher net contribution to the community composition of insects than their diversity (Scherber *et al.* 2014; van Schrojenstein Lantman *et al.* 2020). Furthermore, separated from primary producer dynamics, insect variation can be impacted by spatial factors such as habitat size and isolation (Debinski and Holt 2000; Krauss *et al.* 2010). Within fragmented forest environments, tree species diversity, identity and patch size have been shown to strongly define arthropod diversity (Hertzog *et al.* 2019; Hertzog *et al.* 2021; Perring *et al.* 2021). Forests which have a high proportion of oak, sycamore and birch (*Betula sp.*) are well known to support a high number of associated arthropod species, including Lepidoptera (Brändle and Brandl 2001; Shutt *et al.* 2019). Additionally, these stands frequently contain hazel and rowan which also support a high number of suitable prey species (Latimer and Zuckerberg 2017). Hawfinch were found to consume a more expansive range of invertebrates than has previously been recorded, and this may be a direct result of sampling Hawfinch populations within mature heterogeneous woodland. The reduction in invertebrate densities within woodlands has been highlighted as a potential driver of woodland bird decline (Fuller *et al.* 2005), and while woodlands have the highest diversity of invertebrate fauna of any habitat within Britain, many invertebrate species are limited to ancient semi-natural woodland (ASNW) (Neumann *et al.* 2015). The results from this thesis highlight the importance of heterogeneous woodland in order to provide suitable habitat for invertebrate taxa through larval food plant availability, which in turn not only support Hawfinch populations, but may also support other declining woodland bird species such as pied flycatchers (*Ficedula hypoleuca*) and wood warblers (*Phylloscopus sibilatrix*) (Mallord *et al.* 2016).

Changes in breeding phenology, resulting in a temporal mismatch between peak resource abundance and peak resource availability, is a factor which has been linked to the decline of woodland bird species (Mallord *et al.* 2017). The relative importance of phenological mismatch, does however, depend on the degree to which species' survival or productivity is limited by these trophic interactions (Miller-Rushing *et al.* 2010). Birds which show a more generalist diet may not be negatively impacted by shifts in phenology of certain prey species (Mallord *et al.* 2017). For example, Eurasian reed warblers (*Acrocephalus scirpaceus*) extend their breeding season, enabling more nesting attempts (Halupka *et al.* 2008). A broader range of prey also enables more regular food provisioning, as prey will be plentiful throughout the breeding season and subsequently the selection pressure of synchronised breeding with peak invertebrate abundance is lifted (Dunn *et al.* 2011). Across Hawfinches occupied geographical range, a wide variety of prey was taken, with prey choice seemingly adequate for Hawfinch nutritional needs. The sudden increase in detections of St Marks Fly (*Bibio marci*) in April

suggests Hawfinch are capable of consuming a broad range of prey, and alternative prey resources may constitute an ample food resource when the density of caterpillars drops below a certain threshold (Vesterinen *et al.* 2016).

This thesis emphasises the dominance of a select number of caterpillar species within the diet such as the ubiquitous winter moth, with mottled umber and dun-bar also frequently detected. Additionally, I highlight the possibility that the dominant lepidopteran species vary geographically, as I found no evidence of the green oak tortrix, previously described as a commonly consumed food resource by Hawfinch in the south eastern regions of England (Mountford 1957). The common lepidopteran species detected within this study should be regarded as vital species within heterogenous woodland environments. This is due to the spring caterpillar peak frequently dominated by a small number of abundant species such as the winter moth in Europe (Wesołowski and Rowiński 2006). These species make up a significant dietary component for woodland passerines nestlings (Cholewa and Wesołowski 2011; Shutt *et al.* 2019). Therefore, a decline in abundance of these species could lead to serious cascade effects throughout the ecosystem. To assess the relative importance of Lepidoptera within the breeding season, the diet of nestling Hawfinch should be described, and geographic variability of diet assessed to assess the significance of Lepidoptera for Hawfinch and other woodland birds. Furthermore, future research could involve faecal metabarcoding of multiple species from Hawfinch study sites. A large number of co-existing predator species utilise Lepidoptera and other invertebrates during the breeding season including great tit (*Parus major*) (Ramakers *et al.* 2019), blue tit (Shutt *et al.* 2019) and both great spotted (*Dendrocopos major*) and lesser spotted (*Dendrocopos minor*) (Charman *et al.* 2012; Smith and Smith 2013) woodpecker species. Faecal metabarcoding of adults and nestlings from a range of representative woodland bird species at each site (or a single site) would help quantify to what degree these species are competing for resources and the importance of specific dietary items to the woodland bird community.

The prevalence of supplementary food within the diet of Hawfinch was highlighted within this thesis and suggests that it is a commonly consumed food resource. Supplementary food may be utilised as a food resource more regularly when natural food resources are limited, for example in early spring (Shutt *et al.* 2021). Supplementary feed is provided *ad libitum* at various times throughout the year at all artificial feed sites (Will Kirby, pers. comms). While supplementary feeding may offset some losses from the low availability of natural food (Siriwardena *et al.* 2007), there are associated risks with providing a near continuous supply of supplementary food. Hawfinch may become reliant on these food resources, or be subject to increased predation at artificial feed sites (Hanmer *et al.* 2017; Reynolds *et al.* 2017; Lawson *et al.* 2018). Supplementary feeding may also increase the opportunities for disease

transmission, due to a high congregation density of birds over a prolonged period of time, inter-specific mixing which would not naturally occur and poor hygiene levels resulting in contamination of the feed site (Sorensen *et al.* 2014; Murray *et al.* 2016). Furthermore, if supplementary food is of low nutritional value, this could impact overall condition (Murray *et al.* 2016). There is also evidence that sperm quality of finches may be negatively affected by a high intake of sunflower seeds (Støstad *et al.* 2019). Taking these results into consideration, modification of the current supplementary feeding regime should be implemented. A reduced supply of supplementary food resource throughout the year would reduce overall project costs and may benefit Hawfinch fitness by reducing the risk of disease transmission at artificial feeding stations. Furthermore, the presence of finch trichomonosis, caused by the parasite *Trichomonas gallinae* (Lawson *et al.* 2012) should be monitored in Hawfinch populations. This is in order to avoid trichomonas prevalence within Hawfinch populations causing a similar population decline seen in greenfinch (*Carduelis chloris*) and chaffinch (*Fringilla coelebs*) populations (Lawson *et al.* 2012; Lawson *et al.* 2018).

While it is known that Hawfinch can travel large distances to search for food, it can be inferred that the supplementary food provided at the feeding stations will be available to a high proportion of the local Hawfinch population. While supplementary feeding therefore may be benefiting species actively utilising it as a resource, it may also be directly impacting inter-specific competition within ecosystems, benefiting certain species more than others resulting in an anthropogenically homogenized ecosystem (Oro *et al.* 2013). Utilising the dietary data presented in Chapter 2, it is recommended that supplementary food at the artificial feeding stations is provided for shorter periods throughout the year. This strategy may reduce the need for supplementary feeding and provide a health benefit to Hawfinch populations by reducing possible disease transmission. A downside to the reduction of supplementary feeding, however, is that accurate monitoring of Hawfinch populations may become more challenging if Hawfinch stop visiting the feed sites with the same regularity.

Focusing on woodlands within Britain, it is unlikely that the overall cover of deciduous woodland is driving Hawfinch decline, as deciduous woodland cover has increased from 560,000 hectares in 1982 to 881,000 hectares in 2000 (Mason 2007). While there have been alterations to woodland composition and management practices since then, there has been a progression towards more mature woodland in Britain over this time period, with approximately 50,000 hectares of forest stands now over 68 years old (Kirby *et al.* 2015). Research from previous studies suggests this should benefit Hawfinch, however Bijlsma (1998) recorded a population expansion within a young heterogeneous plantation in the Polder woodlands in The Netherlands eight to 18 years after initial planting. Kirby et al. (2015) found that woodlands showing higher levels of forestry management are more likely to have shown a decline or loss

of breeding Hawfinch. This may suggest that Hawfinch are more likely to be present within semi-natural woodlands with lower levels of management.

6.3 Future research directions

To achieve the aims within this thesis, certain aspects of potentially important avian ecology were ignored. Future work should include autumn and winter diet of UK Hawfinch to generate a more complete dietary overview of the species. Furthermore, future work should include sampling a wider range of populations from mainland Europe to build a more complete overview of Hawfinch dietary niche breadth. Without taking these issues into consideration, the conservation and subsequent management of Hawfinch populations cannot be considered with full confidence. However, the approaches used within this thesis provide a clear framework for future work. Integrating approaches such as metabarcoding data with traditional methods such as direct observation of feeding, and morphological identification of dietary items in faecal samples is recommended to minimise knowledge gaps regarding life stage/tissue type of dietary item consumed and to allow a more accurate depiction of diet. This mixed methodology approach has been utilised in studies investigating diet of seabirds and bears (Alonso *et al.* 2014; Waap *et al.* 2017; Bonin *et al.* 2020), however, to the best of my knowledge, no studies exploring passerine diet has utilised this mixed methodology. It is important to note however, that for Hawfinch in particular, this may be difficult to implement, as Hawfinch are very sensitive to disturbance (Mountford 1957). To explore optimal foraging, nutrient contents of dietary items should also be analysed to quantify the contribution of each dietary item to the fitness and dietary choice of Hawfinch. A protocol has recently been developed to quantify macronutrients from invertebrates, but studies implementing this have to date focused only on spiders (Cuff 2020; Cuff *et al.* 2021).

Widespread declines throughout woodland bird populations are well documented and have been associated with a decline in woodland management practices, habitat destruction, reduction of low woody vegetation by deer browsing, climate change and finally, the planting of non-native species at both new and existing woodland sites (Fuller *et al.* 2007; Gill and Fuller 2007; Mason 2007). This thesis has highlighted the requirements for a fine-scale mosaic of woodland habitat which will provide Hawfinch with suitable breeding and feeding habitats, however there are still important caveats to consider. Studies with the aim of elucidating diet should, where possible, take into consideration long term diet studies, especially for studies focusing on single geographical areas. Many management plans are produced based upon data from a single year or season, or in some cases multiple years pooled together (Marzluff and Ewing 2001). The results from very short term datasets can be misleading or too simplistic, as annual variation in resource availability is highly likely to influence the diets of the study species (Durst *et al.* 2008; Gómez *et al.* 2018). Future studies on Hawfinch could

expand on the results within this thesis to identify foraging events by birds and record feeding habitats used in other regions of the UK where Hawfinch population decline has been more pronounced, such as the southeast of England, though catching or observing birds in small and declining populations yields very few results. This approach has the potential to highlight suitable foraging and breeding habitats which can be fortified through habitat and conservation management.

In future, quantification of population densities of invertebrate prey within Hawfinch population strongholds should be undertaken, as distinguishing patterns driven by biological mechanisms such as resource selection and avoidance allows for an increased understanding of ecological processes (Vaughan *et al.* 2018). While this was undertaken for plant taxa, having data regarding dietary preferences and avoidance for invertebrate taxa will increase understanding of how hawfinch utilise invertebrates within their foraging environment. Having invertebrate dietary preference information may also help understand the factors behind Hawfinch prey selection, for example it is well known that high habitat diversity such as that found within heterogenous woodland is important for maintaining diversity and abundance of Lepidoptera (Sánchez-Bayo and Wyckhuys 2021). This is through the availability of larval food plants and nectar sources for adults (Dover and Settele 2009). These results could then be used in conjunction with woodland management and tree genera dietary preference data to highlight key tree species for both Hawfinch and their preferred prey. Finally, the integration of dietary and demographic studies should be attempted to investigate relationships between diet, population dynamics and productivity and the decline seen in Hawfinch.

6.3.1 DNA metabarcoding limitations

DNA metabarcoding can reveal a wide range of taxa within the diet at a finer taxonomic resolution than more traditional methods of dietary monitoring such as observation and microscopic analysis (Alberdi *et al.* 2017). However, there are limitations associated with this methodology. DNA metabarcoding cannot identify the tissue type consumed, or provide any nutritional information (Deagle *et al.* 2019). Combining metabarcoding data with feeding observations to determine which tissue types are being consumed is recommended, as well as observing which life stages invertebrate prey are consumed at, for example larvae or imagos. Following this, nutritional analysis of the tissue types for each species should be undertaken. This combined approach will enable the clear identification of each dietary component's importance to Hawfinch and will improve understanding of the trophic food web interactions within woodlands. This methodology however, may prove to be practically challenging to implement as Hawfinch are primarily arboreal, feeding within the canopy layer (Perea and Gil 2014).

The research presented in this study has revealed Hawfinch have an omnivorous diet, however, some analytical challenges (outlined below) need to be considered. Inherent biases are present throughout the DNA metabarcoding workflow, but the selection of PCR primers has been discussed as the most critical step in minimising biases (Piñol *et al.* 2018; Tercel *et al.* 2021). For the analysis of omnivore diet, using multiple markers is considered optimal in order to elucidate the complete range of taxa within the diet (Tercel *et al.* 2021). While multiple markers were used in this PhD (Chapters 2 and 3), it is important to acknowledge that no primer pair (or combination of primers) can provide an unbiased and fully comprehensive dietary account (da Silva *et al.* 2019; Tercel *et al.* 2021). There may be under-representation or non-detection of certain taxa within the diet, due to DNA of those taxa being highly degraded and subsequently not amplified during PCR reactions (Tercel *et al.* 2021). Furthermore, primer biases, mitochondrial copy number per cells varying between taxa and PCR inhibition of certain taxonomic groups may all occur (Pompanon *et al.* 2012; Taberlet *et al.* 2018; Tercel *et al.* 2021). A further challenge is the co-amplification of DNA shed from the focal consumer and the dietary items being fed upon, which can result in up to 95% of the sample read depths lost to focal consumer DNA (Cuff *et al.* 2021). While the chosen primer pairs amplified a broad taxonomic range of target taxa, there was no co-amplification of Hawfinch DNA, avoiding the issue of focal consumer DNA amplification bias (Piñol *et al.* 2014; Tercel *et al.* 2021).

DNA metabarcoding is unable to accurately provide biomass measurements of dietary taxa (Deagle *et al.* 2019; Lamb *et al.* 2019). At best, a semi-quantitative prediction of biomass consumed can be analysed from calculating the number of samples which contain a given food item, coined frequency of occurrence (FOO), or from calculating the relative frequencies of sequence reads, coined relative read abundance (RRA) (Deagle *et al.* 2019). The RRA methodology is based upon the assumption that the number of sequences generated for a particular dietary taxon is proportional to the relative biomass of the dietary taxon consumed (Deagle *et al.* 2010; Neby *et al.* 2021). Prior dietary information and sequence number produced for each dietary item are then used to generate correction factors for estimating biomass consumed (Deagle *et al.* 2019). This methodology is not without caveats, as a recent meta-analysis by Lamb et al. (2019) showed RRA and ingested food biomass showed a positive correlation in some model systems (Kartzinel *et al.* 2015; Nichols *et al.* 2016), but the same relationship was not found in others (Deagle *et al.* 2013; Elbrecht *et al.* 2017; Piñol *et al.* 2018). This highly variable correlation implies that RRA should not be used as a proxy for diet proportions, with biases arising from DNA extraction and amplification, as well as biases from differential digestion rates of plants and invertebrates (Majaneva *et al.* 2018; Neby *et al.* 2021). Furthermore, sequencing and PCR biases result in further caveats with regards to accurately estimating how much of an organism was consumed (Thomas *et al.* 2016; Piñol *et*

al. 2018; Deagle *et al.* 2019). Taking these caveats into consideration, this methodology is not yet suitable for dietary quantification of highly generalist species such as Hawfinch, which have the potential to consume a high number of different species. The use of frequency of occurrence as a measure of importance can however, conceal the true biological importance to the consumer (Deagle *et al.* 2019). This is due to all taxa (both common and rare) given equal weight, resulting in the importance of rare food taxa being artificially inflated within the dataset (Deagle *et al.* 2019). Rare-item inflation can obscure niche partitioning conclusions, as niche separation may be driven by the partitioning of rare food items, which, given similar weight to commonly consumed items can result in false conclusions of species feeding on separate resources (Deagle *et al.* 2019).

Secondary predation is the detection of species consumed by the prey of a predator (Sheppard *et al.* 2005). The study of omnivorous diet can result in the secondary consumption of plant taxa artificially inflating the presence of plant taxa detected within the diet of the study species (da Silva *et al.* 2020; Tercel *et al.* 2021). If plant tissue consumed by the omnivore and the consumer is from the same plant species, for example leaves and fruits, this often results in detections being indistinguishable, with this issue being heightened if the omnivore is feeding on herbivorous insects (Guenay *et al.* 2021). This results in difficulty determining species level interactions as well as coarser dietary patterns. Secondary predation within metabarcoding studies can positively skew the proportion of plant taxa consumed if herbivorous insects are commonly consumed (da Silva *et al.* 2020; Tercel *et al.* 2021). This may dilute the ecology of the study omnivore and subsequent species interactions. Additionally, if the diet of the study organism is largely unknown, but it is assumed to be omnivorous, secondary predation may lead to a false conclusion of herbivory, or heighten the importance of plants within the diet (Tercel *et al.* 2021). Conversely, secondary predation may be viewed as a false problem. Secondary predation is almost impossible to detect within DNA metabarcoding studies, however within foraging studies, even if not directly hunted, consumed taxa which are indirectly ingested will still contribute towards the nutritional intake, therefore, it can be argued are valid within dietary categorisation of the consumer (Bowser *et al.* 2013; Nielsen *et al.* 2017).

6.4 Concluding statement

Overall, this thesis advances knowledge regarding ecological interactions of Hawfinch within deciduous woodlands. It also shows that using DNA metabarcoding methodologies can be highly effective in gaining crucial biological insights into rare and cryptic species. Results from this thesis indicate Hawfinch consume a broad diversity of herbivorous and invertebrate taxa in their diet. I have shown that dietary composition differs demographically, and that Hawfinch show regional and site-specific differences in their dietary composition. I also found that

Hawfinch are selective in their foraging, showing both preference and avoidance for certain tree genera. This thesis shows that the use of DNA metabarcoding techniques can generate an in-depth insight into the entire dietary breadth of a rare species which may help conservation efforts of the species, as well as the landscape management of where Hawfinch populations persist.

A wide diversity of fruit and kernel-bearing tree species has been shown as an important factor in Hawfinch persistence within woodlands (Kirby *et al.* 2015). While woodlands in which Hawfinch persist often contain a canopy layer dominated by oak and beech, the combination of complex understorey layer of yew, hornbeam and wych elm coupled with mature canopy trees has been shown to be important habitat structure (Mountford 1957; Kirby *et al.* 2015; Kirby *et al.* 2018). In order to persist within these landscapes, Hawfinch need woodland sites which provide them with suitable feeding opportunities, but also where food availability is high enough to sustain breeding colonies (Mountford 1957; Kirby *et al.* 2019). Woodland composition has altered dramatically over the last 50 years (Fuller *et al.* 2007), with many tree species shown in this thesis to be important for Hawfinch such as cherry, hornbeam, elm and yew all showing declines (Rackham 2020). This simplification of woodland habitats may also be driving the decline of other woodland species such as the lesser spotted woodpecker through invertebrate food shortages during the breeding season (Charman *et al.* 2012).

The knowledge gained from this thesis should be applied to conservation management strategies for Hawfinch and the methodology considered for other declining woodland bird populations throughout the UK. Conservation measures should focus on the preservation of key plant and invertebrate dietary taxa detect from this study. The observation that a large proportion of tree and plant taxa consumed by Hawfinch were native species could indicate a preference for native species over exotic or invasive taxa. To decide whether focused species planting, and rejuvenation efforts should be undertaken will require further research in order to determine nutritional value and commercial viability. However, future recommendations for woodland management remains difficult. Actions which benefit Hawfinch, for example the incorporation of wild cherry and beech during planting of new broad-leaved woodland, may be detrimental for other woodland species. Due to the lack of certainty in predictions of how future forest ecosystems will develop, woodland management options should explore the possibility of maintaining high structural diversity at a range of spatial scales. These options should include the consideration of which landscape and habitat structures will promote persistence of populations, as well as continuing to develop opportunities for woodland expansion (Fuller *et al.* 2007). There should also be consideration as to how suitable diversity of habitat structures should be maintained within woodlands (Fuller *et al.* 2007). Complex habitat

structures should be maintained, as these have been proven to be beneficial for Hawfinch and other woodland species (Fuller *et al.* 2012; Kirby *et al.* 2015).

Appendix One – Supplementary information relating to Chapter 2

Appendix 1.1. Modifications to the QIAGEN QIAmp® DNA Stool Mini Kit protocol

DNA extraction from warbler faecal material was carried out following the standard protocol, including all recommended steps with modifications by Zeale *et al*. 2011, Nicholls *et al*. 2019, Shutt *et al.* 2020 and Davies 2020. The following modifications were used.

i) Uric acid was removed from each stool sample by scraping the sides of the faecal pellet. Either the whole pellet, or up to 220mg of the pellet (for larger samples) was used in the extraction.

ii) 500µL of InhibitEx Buffer was added to each stool sample, then mixed manually using a pestle for 30 seconds before adding a further 500µL of InhibitEx Buffer. The samples were then homogenized by vortexing for 3 minutes.

iii) 20µL of proteinase K, 400µL of supernatant and 400µL Buffer AL was added to a new 2 ml microcentrifuge tube in step 5 and samples were incubated at 70°C for 15 minutes before adding 400µL molecular grade (96-100%) ethanol.

iv) 100µL of Buffer AE was added to each spin column membrane, or 50µL for samples with small amounts of faecal material. Samples were incubated at room temperature for 1 minute and then centrifuged at full speed for 1 min to elute DNA.

Appendix 1.2. Forward and reverse MID-tag oligos used for metabarcoding

Forward identifier

F1 ACGAGTGCGTTGTGAATTGCARRATYCMG

F2 ACGCTCGACATGTGAATTGCARRATYCMG

F3 AGACGCACTCTGTGAATTGCARRATYCMG

F4 AGCACTGTAGTGTGAATTGCARRATYCMG

F6 ATATCGCGAGTGTGAATTGCARRATYCMG

F7 CGTGTCTCTATGTGAATTGCARRATYCMG

F8 CTCGCGTGTCTGTGAATTGCARRATYCMG

F10 TCTCTATGCGTGTGAATTGCARRATYCMG

F11 TGATACGTCTTGTGAATTGCARRATYCMG

F14 CGAGAGATACTGTGAATTGCARRATYCMG

F17 CGTCTAGTACTGTGAATTGCARRATYCMG

F18 TCTACGTAGCTGTGAATTGCARRATYCMG

F19 TGTACTACTCTGTGAATTGCARRATYCMG

F21 CGTAGACTAGTGTGAATTGCARRATYCMG

F22 TACGAGTATGTGTGAATTGCARRATYCMG

F24 TAGAGACGAGTGTGAATTGCARRATYCMG

F25 TCGTCGCTCGTGTGAATTGCARRATYCMG

F27 ACGCGAGTATTGTGAATTGCARRATYCMG

F28 ACTACTATGTTGTGAATTGCARRATYCMG

F31 AGCGTCGTCTTGTGAATTGCARRATYCMG

F32 AGTACGCTATTGTGAATTGCARRATYCMG

F34 CACGCTACGTTGTGAATTGCARRATYCMG

F35 CAGTAGACGTTGTGAATTGCARRATYCMG

Reverse identifier

R1 ACTAGCAGTACCCGHYTGAYYTGRGGTCDC

R3 ACAGTATATACCCGHYTGAYYTGRGGTCDC

R4 TGTGAGTAGTCCCGHYTGAYYTGRGGTCDC

R5 TGACGTATGTCCCGHYTGAYYTGRGGTCDC

R6 TCTATACTATCCCGHYTGAYYTGRGGTCDC

R7 TCTAGCGACTCCCGHYTGAYYTGRGGTCDC

R9 TCGATCACGTCCCGHYTGAYYTGRGGTCDC

R10 TAGTGTAGATCCCGHYTGAYYTGRGGTCDC

R11 TACGCTGTCTCCCGHYTGAYYTGRGGTCDC

R12 TACAGATCGTCCCGHYTGAYYTGRGGTCDC

R13 TACACGTGATCCCGHYTGAYYTGRGGTCDC

R15 CGACGTGACTCCCGHYTGAYYTGRGGTCDC

Appendix 1.3. Shell and perl scripts for metabarcoding data used in the bioinformatics pipeline

The following scripts were written by Drake *et al.* 2021 (modified from Helen Hipperson at NBAF, University of Sheffield). The entire pipeline was repeated for each indexing library (library 1 shown below).

Script 1 – Trimming and aligning paired reads to generate complete amplicon sequence

we will do FastQC quality check, merge the paired end reads and trim the sequences in one go using FastP to get the complete amplicon sequence

/mnt/data/GROUP-sabwocs/d1006888/ITS2_2016/fastp -i Lib1ITS2R1.fastq -I Lib1ITS2R2.fastq -l 125 -m --discard_unmerged -o merged_2016.fastq

next convert the fastq file to fasta format

module load fastx_toolkit/0.0.14

fastq_to_fasta -i merged_2016.fastq -Q 33 -o merged_2016.fasta

Script 2 – Allocate MID-tag combinations to their respective samples and remove primer sequences

we will identify the sequences that match the oligos used, allowing for 1 mismatch. oligos = text file where the first column reads #‘primer’, the second and third columns are the forward and #reverse primer and MID-tag combinations for a particular #sample, and the fourth column is the sample ID annotated with #an additional ‘a’ or ‘b’. ‘a’ is used when the forward primer #is in column 2 and the reverse is in column 3. ‘b’ is used #when this order is reversed. This means that the total number #of rows should be twice the number of samples.

#Run Mothur
module load mothur/1.39.5
mothur "#trim.seqs(fasta=merged_2016.fasta,oligos=UKoligos.txt, checkorient=t,pdiffs=1)"

#split. groups file into A and B
grep 'a$' merged_2016.groups > merged_2016A.groups
grep 'b$' merged_2016.groups > merged_2016B.groups

#remove 'a' and 'b' labels
sed -i 's/a//g' merged_2016A.groups
sed -i 's/b//g' merged_2016B.groups

Script 3 – Demultiplexing - getting one fasta file per MID-tag combination
Part 1. Perl script

#!/usr/bin/perl

unless ($#ARGV == 0)

{

  print "Usage: 3_Demultiplex.pl Fastalist_2016.txt";

die;
}

open (INLIST, "<$ARGV[0]") || die;

replace 'XXX' with your username, and if you want to put the output into another directory you can add that to the 'outdir' path here

```
$indir = "/mnt/scratch/d1006888/deplex";
$outdir = "/mnt/scratch/d1006888/deplex";

# Loops through the list fo your samples ('SampleList') and performs the commands for each
one
while (<INLIST>) {
$lib = $_;
chomp($lib);

# A shortcut to read or write a file for each of your samples, each file having the same
extension
$readidsa = $lib . "_a_ids.txt";
$readidsb = $lib . "_b_ids.txt";
$readidsab = $lib . "_ab_ids.txt";

$fa1 = $lib . ".fa";
$fa2 = $lib . ".fasta";

# split fasta read IDs into files grouped by sample ID. Replace 'XX' with the name of you
'.groups' file (output from mothur)
system("grep -w $lib $indir/merged_2016A.groups | awk '{print \$1}' > $outdir/$readidsa");
system("grep -w $lib $indir/merged_2016B.groups | awk '{print \$1}' > $outdir/$readidsb");

# combine the list of sequence names for 'a' and 'b' matches
system("cat $outdir/$readidsa $outdir/$readidsb >> $outdir/$readidsab");

# split the trimmed fasta file into reads specific to each sample. Replace 'XX' with the name
of your trimmed fasta file (output from mothur)
my $command1 = 'perl -ne'."'".'if(/^>(\S+)/){$c=$i{$1}}$c?print:chomp;$i{$_}=1 if'."
@ARGV'"." $outdir/$readidsab $indir/merged_2016.trim.fasta > $outdir/$fa1";

system ($command1);

system("awk '{print \$1}' $indir/$fa1 > $indir/$fa2");


}

exit;
```

Part 2. Shell script

```
perl 3_Demultiplex.pl Fastalist_2016.txt
```

Script 4 – Editing headers so each file has its sample ID at the start of each sequence

Part 1. Perl script.

```
#!/usr/bin/perl

unless ($#ARGV == 0)

{

print "Usage: 4_Edit_Headers.pl Fastalist_2016.txt";

die;
```

```
}
open (INLIST, "<$ARGV[0]") || die;


$indir = "/mnt/scratch/d1006888/deplex/FastaFiles";


$outdir = "/mnt/scratch/d1006888/deplex/FastaFiles";


while (<INLIST>) {
$lib = $_;
chomp($lib);
$fa1 = $lib . ".fasta";
$fa2 = $lib . "_edit.fasta";
system( qq(sed "s/^>/>$lib;/g" "$indir/$fa1" > "$indir/$fa2"));
}
exit;
```

Part 2. Shell script

```
perl 4_Edit_Headers.pl Fastalist_2016.txt
```

Script 5 – USEARCH

```
# removes identical replicates from the fasta input, output for next step =
SampleName_rc_uniques.fasta
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -fastx_uniques Allmerged.fasta -
fastaout Unique.fasta -sizeout -strand both -relabel Uniq -threads 4

# sort by size
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -sortbysize Unique.fasta -fastaout
Sorted.fasta

# Cluster OTUs
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -cluster_otus Sorted.fasta -otus
OTU.fasta -relabel Out

# denoise and cluster using unoise3 to make zOTUs
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -unoise3 Sorted.fasta -zotus
zOTU.fasta

# make list of zOTU's and the number of sequences per zOTU (size)
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -otutab Allmerged.fasta -zotus
zOTU.fasta -otutabout zOTUtable.txt -strand both -threads 4

# make list of OTU's and the number of sequences per OTU (size)
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -otutab Allmerged.fasta -otus
OTU.fasta -otutabout OTUtable.txt -strand both -threads 4
```

Script 6. BLAST

```
# blast the clusters from usearch

module load blast/2.7.1

export ITS2_database=/mnt/data/GROUP-sabwocs/d1006888/ITS2_Database/ITS2_database

blastn -query zOTU.fasta -db /mnt/data/GROUP-sabwocs/d1006888/ITS2_Database/ITS2_database -num_threads 4 -evalue 0.00001 -perc_identity 97 -outfmt 6 -out zOTU_blastoutput.txt

blastn -query OTU.fasta -db /mnt/data/GROUP-sabwocs/d1006888/ITS2_Database/ITS2_database -num_threads 4 -evalue 0.00001 -perc_identity 97 -outfmt 6 -out OTU_blastoutput.txt
```

Script 7. Filter the BLAST results

```
# only keep results with over 95% identity and remove and sequences with less than 100bp in length

awk '$3 >= 95' OTU_blastOutput.txt | awk '$4 >= 100' > OTU_2016_blast_filtered.txt.

awk '$3 >= 95' zOTU_blastOutput.txt | awk '$4 >= 100' > zOTU_2016_blast_filtered.txt.
```

Script 8. Add taxon information to diet zOTU matrix (R-script)

```
#Add in taxon information to your zOTU and OTU tables: Open R and run the following code on your blast output to get only the top hit for each motu based on bitScore (combination of e-value and percentage identity):

blast_ITS2_2016 <- read.table("zOTU_2016_blast_filtered.txt")

summary(blast_ITS2_2016)

library(dplyr)

blast_filter <- blast_ITS2_2016 %>%

  group_by (V1) %>%

  filter (V12 == max(V12))

write.table (blast_filter, "ITS2_2016_zOTU_TopHit_blastOutput.txt")

#Next use the program MEGAN to assign ids to each zOTU from the BLAST top hit output.
#Use VLOOKUP in Excel to add taxon ids to each zOTU in the diet matrix.
#Calculate maximum contamination/tag jumping from NAs and negative controls and apply this to all samples in that row. Convert negative values to 0.
#Remove all reads with a read count of less than 10.
#Remove zOTUs that have highest reads in positive controls from the remaining diet matrix.
#Remove non-dietary data
#Convert matrix to csv file for aggregating in R.
```

Script 9. Aggregate zOTUs in diet matrix based on taxon ID (R-script)

```
ITS2_2016_to_Agg <- read.csv("ITS2_2016_Just_samples_Aggregated.csv", header = T)

Agg <- aggregate(.~Taxon, data=ITS2_2016_to_Agg, sum)

write.csv(Agg, "ITS2_2016_Just_samples_Aggregated.csv")
```

Appendix 1.4. Taxa removed from Hawfinch ITS2 metabarcoding dataset

Table A1.4.1. Taxa removed from the Hawfinch ITS2 dataset.

Taxon	Common name	Reason for removal	Accession Code
Acer sempervirens	Cretan maple	Non target taxa	AM238344
Arachis hypogaea	Peanut	Bird ringer food	AF156675
Arrhenatherum palaestinum	Grass	Non target taxa	AJ632238
Brassica carinata	Ethiopian mustard	Non target taxa	DQ003700
Bromus diandrus	Great brome	Non target taxa	AY367936
Carpinus polyneura	Hornbeam	Non target taxa	AF081517
Carpinus tientaiensis	Chinese hornbeam	Non target taxa	JF796532
Citrus maxima	Pomelo	Bird ringer food	JN681155
Citrus reticulata	Mandarin orange	Bird ringer food	JN661212
Citrus x paradisi	Grapefruit	Bird ringer food	FJ641956
Coriandrum sativum	Coriander	Bird ringer food	KM051454
Cucumis pubescens	Cucumber	Bird ringer food	AM981116
Cucurbita pepo	Pumpkin	Bird ringer food	AF013349
Fagus engleriana	Chinese beech	Non target taxa	AF457021
Fagus grandifolia	American beech	Non target taxa	AY232922
Fagus hayatae	Taiwan beech	Non target taxa	AY232935
Geum canadense	White Avens	Non target taxa	DQ006033
Heracleum vicinum	Cow parsley	Non target taxa	FJ812126
Hesperocyparis stephensonii	Cuyamaca cypress	Non target taxa	U60751
Hexachlamys emerichii	Ubajay	Non target taxa	JQ033295
Iris dichotoma	Iris	Non target taxa	DQ277638
Lachnagrostis ammobia	Grass	Non target taxa	AY705907
Lolium rigidum	Rigid ryegrass	Non target taxa	AJ240142
Lolium temulentum	Darnel	Passerine sup. Feed filler	AJ240145
Picea rubens	Red spruce	Non target taxa	AF136613
Plantago leiopetala	Madeira plantain	Non target taxa	AJ548985
Prunus domestica	Common plum	Bird ringer food	EU669097

Prunus mandshurica	Manchurian apricot	Non target taxa	EF211082
Pterocarya tonkinensis	Tonkin Wingnut	Non target taxa	AF179586
Quercus dentata	Daimyo oak	Non target taxa	AY042935
Quercus vulcanica	Kasnak oak	Non target taxa	FM244270
Rosa abyssinica	Rose	Non target taxa	AB048592
Rubus scissoides	Bramble	Non target taxa	KM037547
Rubus tabanimontanus	Bramble	Non target taxa	KM037595
Salix schwerinii	Narrow-leaf willow	Non target taxa	FR693629
Salix turczaninowii	Willow	Non target taxa	FR693631
Tilia hyrcana	Lime	Non target taxa	JX051606
Triticum turgidum	Durum wheat	Passerine sup. Feed filler	KF482091
Viola acuminata	Speedwell	Non target taxa	AY928273

Appendix 1.5. % Frequency of occurrence tables for dietary items detected in the five sampling regions in Chapter 2

Table A1.2.1. The % Frequency of occurrence (%FOO) of plant dietary items in faecal samples from Hawfinch sampled within the five population sampling areas.

Taxon	Common Name	%FOO Dolgellau	%FOO Wye Valley	%FOO New Forest	%FOO north Cardiff	%FOO Norfolk
Abies concolor	White fir	2.6	0.7	0	0	0
Abies delavayi	Delavay's silver-fir	0.9	0	0	0	0
Acer campestre	Field maple	1.7	10.1	0	28.6	0
Acer japonicum	Amur maple	0.9	0	0	0	0
Acer platanoides	Norway maple	1.7	2.9	0	0	0
Acer pseudoplatanus	Sycamore maple	14.8	2.9	21.1	14.3	57.1
Acer velutinum	Persian maple	0	0	0	0	28.6
Alnus glutinosa	Black alder	1.7	2.2	15.8	0	0
Amelanchier lamarckii	Juneberry	0.9	3.6	10.5	0	0
Anacardium occidentale	Cashew	2.6	19.6	57.9	0	14.3
Arctium minus	Lesser burdock	0	0.7	0	0	0
Aucuba japonica	Japanese laurel	1.7	0	0	0	0
Betula pendula	Silver birch	13	4.3	26.3	0	0
Betula pubescens	Downy birch	13.9	1.4	26.3	0	0
Bidens sp.	Beggarticks	0	0.7	0	0	0
Carpinus betulus	European hornbeam	30.4	13	10.5	42.9	100

Castanea sativa	Sweet chestnut	0.9	0	5.3	0	0
Cerastium fontanum	Mouse-ear chickweed	0.9	0	0	0	0
Chenopodium album	Pigweed	0	1.4	0	0	0
Corylus avellana	Common hazel	7.8	8	0	0	14.3
Crataegus monogyna	Common hawthorn	0	0.7	0	0	0
Cupressus macrocarpa	Monterey cypress	0	0.7	0	0	0
Cupressus sempervirens	Mediterranean cypress	0.9	0	0	0	0
Eucalyptus sp.	Eucalyptus	0	0	5.3	0	0
Fagus sylvatica	European Beech	47	83.3	52.6	85.7	28.6
Fraxinus excelsior	European Ash	10.4	5.8	10.5	28.6	0
Geranium robertianum	Roberts geranium	0.9	0	0	0	0
Geum urbanum	Wood avens	0	0.7	0	0	0
Hedera helix	Common ivy	3.5	10.1	0	14.3	14.3
Helianthus sp.	Sunflower	68.7	23.9	36.8	14.3	85.7
Heracleum sphondylium	Hogweed	0	0.7	0	0	0
Ilex aquifolium	Common holly	5.2	3.6	52.6	0	28.6
Larix decidua	European larch	4.3	6.5	0	14.3	0
Larix kaempferi	Japanese larch	0.9	5.1	0	14.3	0
Nothofagus obliqua	Patagonian oak	3.5	1.4	10.5	0	0
Picea abies	Norway spruce	2.6	5.1	0	0	0
Pinus luchuensis	Luchu pine	0	0	5.3	0	0
Pinus sylvestris	Scots pine	0.9	0	15.8	0	42.9
Plantago lanceolata	Ribwort plantain	2.6	0.7	0	0	0
Primula veris	Cowslip	0.9	0	0	0	0
Prunus avium	Wild cherry	18.3	21.7	5.3	0	0
Prunus cerasifera	Cherry plum	1.7	0.7	5.3	0	0
Prunus domestica	Common plum	5.2	0.7	5.3	0	0
Prunus laurocerasus	Cherry laurel	0.9	0	0	0	0
Prunus padus	Bird cherry	0.9	0	0	0	14.3
Prunus persica	Peach	0	1.4	0	0	0
Prunus serotina	Black cherry	2.6	0	0	0	0
Prunus spinosa	Blackthorn	0.9	0.7	5.3	0	0

Quercus canariensis	Algerian oak	13	10.9	36.8	0	14.3
Quercus cerris	Turkey oak	2.6	0	0	0	14.3
Quercus faginea	Portuguese oak	1.7	0	0	0	0
Quercus petraea	Sessile oak	27.8	26.1	78.9	0	42.9
Quercus robur	English oak	24.3	21.7	78.9	0	42.9
Quercus rubra	Northern red oak	0	0.7	0	0	0
Ranunculus repens	Creeping buttercup	0.9	2.9	0	0	0
Rhododendron caucasicum	Rhododendron	0.9	0	0	0	0
Rhododendron ponticum	Common rhododendron	2.6	0	0	0	0
Ribes nigrum	Blackcurrant	0.9	0	0	0	0
Rosa arvensis	Field rose	0	0.7	0	0	0
Rosa caesia	Hairy dog rose	0	0.7	0	0	0
Rosa canina	Dog-rose	0.9	0.7	0	0	0
Rosa moschata	Musk rose	1.7	0	0	0	0
Rubus idaeus	Red raspberry	0.9	0	0	0	0
Rubus silvaticus	Bramble	0	0.7	0	0	0
Rubus sp.	Bramble	4.3	7.2	15.8	0	0
Salix caprea	Goat willow	1.7	0	0	0	0
Salix sp.	Willow	10.4	1.4	0	0	0
Solanum sp.	Nightshade	1.7	0	0	0	0
Sonchus oleraceus	Common sowthistle	0.9	1.4	0	0	0
Sorbus aucuparia	Rowan	3.5	0.7	0	0	0
Stellaria media	Chickweed	0	0.7	0	0	0
Taraxacum officinale	Common dandelion	0	0.7	0	0	0
Taraxacum sp.	Dandelion	0.9	2.2	5.3	0	0
Taxus baccata	English yew	4.3	6.5	5.3	0	0
Taxus x media	Anglojap yew	0	2.9	0	0	0
Tilia cordata	Small-leaved lime	0.9	4.3	0	0	0
Tilia platyphyllos	Large-leaved lime	0	2.2	0	0	14.3
Tilia sp.	Lindens	0	0	0	0	14.3
Ulmus glabra	Wych elm	3.5	30.4	0	42.9	0
Urtica dioica	Common nettle	3.5	2.9	0	14.3	0
Veronica chamaedrys	Bird's-eye speedwell	0.9	0	0	0	0
Vicia sepium	Bush vetch	0	0.7	0	0	0
Viola lactea	Pale dog-violet	0.9	0.7	10.5	0	0

Viola reichenbachiana	Early dog-violet	0.9	0	5.3	0	0

Appendix 1.6. *Manyglm* post-hoc pairwise comparisons between sampling regions and years

Table A7.3.1. Post-hoc pairwise comparisons of plant dietary differences found between Hawfinch at different sampling regions.

Site comparison	Observed statistic	Free Stepdown Adjusted *p*-value
Dolgellau vs Wye Valley	240.14	0.001
Dolgellau vs New Forest	149.86	0.001
New Forest vs Wye Valley	132.56	0.001
north Cardiff vs New Forest	80.10	0.001
Norfolk vs Wye Valley	68.39	0.001
New Forest vs Norfolk	57.75	0.001
north Cardiff vs Norfolk	52.12	0.001
Dolgellau vs north Cardiff	43.98	0.003
Dolgellau vs Norfolk	43.20	0.003
north Cardiff vs Wye Valley	33.58	0.033

Table A1.4.2. Post-hoc pairwise comparisons of plant dietary differences found between Hawfinch sampled between years.

Year comparison	Observed statistic	Free Stepdown Adjusted *p*-value
2018 vs 2019	224.96	0.001
2017 vs 2018	207.71	0.001
2016 vs 2018	184.32	0.001
2017 vs 2019	136.29	0.001
2016 vs 2019	107.71	0.001
2016 vs 2017	78.12	0.001

Appendix Two – Supplementary information relating to Chapter 3

Appendix 2.1. Forward and reverse MID-tag oligos used for metabarcoding

Forward identifier

F2: ACGCTCGACAGGWACWGGWTGAACWGTWTAYCCYCC

F3: AGACGCACTCGGWACWGGWTGAACWGTWTAYCCYCC

F4: AGCACTGTAGGGWACWGGWTGAACWGTWTAYCCYCC

F5: ATCAGACACGGGWACWGGWTGAACWGTWTAYCCYCC

F6: ATATCGCGAGGGWACWGGWTGAACWGTWTAYCCYCC

F7: CGTGTCTCTAGGWACWGGWTGAACWGTWTAYCCYCC

F8: CTCGCGTGTCGGWACWGGWTGAACWGTWTAYCCYCC

F10: TCTCTATGCGGGWACWGGWTGAACWGTWTAYCCYCC

F11: TGATACGTCTGGWACWGGWTGAACWGTWTAYCCYCC

F13: CATAGTAGTGGGWACWGGWTGAACWGTWTAYCCYCC

F15: ATACGACGTAGGWACWGGWTGAACWGTWTAYCCYCC

F16: TCACGTACTAGGWACWGGWTGAACWGTWTAYCCYCC

F17: CGTCTAGTACGGWACWGGWTGAACWGTWTAYCCYCC

F18: TCTACGTAGCGGWACWGGWTGAACWGTWTAYCCYCC

F19: TGTACTACTCGGWACWGGWTGAACWGTWTAYCCYCC

F20: ACGACTACAGGGWACWGGWTGAACWGTWTAYCCYCC

F21: CGTAGACTAGGGWACWGGWTGAACWGTWTAYCCYCC

F22: TACGAGTATGGGWACWGGWTGAACWGTWTAYCCYCC

F23: TACTCTCGTGGGWACWGGWTGAACWGTWTAYCCYCC

F24: TAGAGACGAGGGWACWGGWTGAACWGTWTAYCCYCC

F25: TCGTCGCTCGGGWACWGGWTGAACWGTWTAYCCYCC

F26: ACATACGCGTGGWACWGGWTGAACWGTWTAYCCYCC

F27: ACGCGAGTATGGWACWGGWTGAACWGTWTAYCCYCC

F30: AGACTATACTGGWACWGGWTGAACWGTWTAYCCYCC

F31: AGCGTCGTCTGGWACWGGWTGAACWGTWTAYCCYCC

Reverse identifier

R1: ACTAGCAGTACCCGGTAAAATTAAAATATAAACTTC

R4: TGTGAGTAGTCCCGGTAAAATTAAAATATAAACTTC

R5: TGACGTATGTCCCGGTAAAATTAAAATATAAACTTC

R7: TCTAGCGACTCCCGGTAAAATTAAAATATAAACTTC

R8: TCGCACTAGTCCCGGTAAAATTAAAATATAAACTTC

R9: TCGATCACGTCCCGGTAAAATTAAAATATAAAC TTC

R10: TAGTGTAGATCCCGGTAAAATTAAAATATAAACTTC

R11: TACGCTGTCTCCCGGTAAAATTAAAATATAAACTTC

R12: TACAGATCGTCCCGGTAAAATTAAAATATAAACTTC

R13: TACACGTGATCCCGGTAAAATTAAAATATAAACTTC

R14: TACACACACTCCCGGTAAAATTAAAATATAAACTTC

R15: CGACGTGACTCCCGGTAAAATTAAAATATAAACTTC

Appendix 2.2. Shell and perl scripts for metabarcoding data used in the bioinformatics pipeline

The following scripts were written by Drake *et al.* 2021 (modified from Helen Hipperson at NBAF, University of Sheffield). The entire pipeline was repeated for each indexing library (library 5 shown below).

Script 1 – Trimming and aligning paired reads to generate complete amplicon sequence

we will do FastQC quality check, merge the parired end reads and trim the sequences in one go using FastP to get the complete amplicon sequence

/mnt/scratch/d1006888/COI_UK/FastP/fastp -i COIUK_2019R1.fastq -I COIUK_2019R2.fastq -l 300 -m --discard_unmerged -o merged_COI_2019.fastq

next convert the fastq file to fasta format

module load fastx_toolkit/0.0.14

fastq_to_fasta -i merged_COI_2019.fastq -Q 33 -o merged_COI_2019.fasta

Script 2 – Allocate MID-tag combinations to their respective samples and remove primer sequences

we will identify the sequences that match the oligos used, allowing for 1 mismatch. oligos = text file where the first column reads #‘primer’, the second and third columns are the forward and #reverse primer and MID-tag combinations for a particular #sample, and the fourth column is the sample ID annotated with #an additional ‘a’ or ‘b’. ‘a’ is used when the forward primer

#is in column 2 and the reverse is in column 3. ‘b’ is used #when this order is reversed. This means that the total number #of rows should be twice the number of samples.

```
#Run Mothur
module load mothur/1.39.5
mothur
"#trim.seqs(fasta=merged_COI_2019.fasta,oligos=UKoligos.txt,checkorient=t,pdiffs=1)"

#split .groups file into A and B
grep 'a$' merged_COI_2019.groups > merged_COI_2019A.groups
grep 'b$' merged_COI_2019.groups > merged_COI_2019B.groups

#remove 'a' and 'b' labels
sed -i 's/a//g' merged_COI_2019A.groups
sed -i 's/b//g' merged_COI_2019B.groups
```

Script 3 – Demultiplexing - getting one fasta file per MID-tag combination
Part 1. Perl script

```
#!/usr/bin/perl


unless ($#ARGV == 0)

{

   print "Usage: 3_Demultiplex.pl UK_2019_FastaList.txt";

die;
}


open (INLIST, "<$ARGV[0]") || die;

# replace 'XXX' with your username, and if you want to put the output into another directory you can add that to the 'outdir' path here
$indir = "/mnt/scratch/d1006888/COI_2019/Deplex";
$outdir = "/mnt/scratch/d1006888/COI_2019/Deplex";

# Loops through the list fo your samples ('SampleList') and performs the commands for each one
while (<INLIST>) {
$lib = $_;
chomp($lib);

# A shortcut to read or write a file for each of your samples, each file having the same extension
$readidsa = $lib . "_a_ids.txt";
$readidsb = $lib . "_b_ids.txt";
$readidsab = $lib . "_ab_ids.txt";

$fa1 = $lib . ".fa";
$fa2 = $lib . ".fasta";
```

```
# split fasta read IDs into files grouped by sample ID. Replace 'XX' with the name of you
'.groups' file (output from mothur)
system("grep -w $lib $indir/merged_COIA.groups | awk '{print \$1}' > $outdir/$readidsa");
system("grep -w $lib $indir/merged_COIB.groups | awk '{print \$1}' > $outdir/$readidsb");

# combine the list of sequence names for 'a' and 'b' matches
system("cat $outdir/$readidsa $outdir/$readidsb >> $outdir/$readidsab");

# split the trimmed fasta file into reads specific to each sample. Replace 'XX' with the name
of your trimmed fasta file (output from mothur)
my $command1 = 'perl -ne'."'".'if(/^>(\S+)/){$c=$i{$1}}$c?print:chomp;$i{$_}=1 if'."
@ARGV'." $outdir/$readidsab $indir/merged_COI.trim.fasta > $outdir/$fa1";

system ($command1);

system("awk '{print \$1}' $indir/$fa1 > $indir/$fa2");


}

exit;
```

Part 2. Shell script

```
perl 3_Demultiplex.pl UK_2019_FastaList.txt
```

Script 4 – Editing headers so each file has its sample ID at the start of each sequence

Part 1. Perl script.

```
#!/usr/bin/perl


unless ($#ARGV == 0)

{

   print "Usage: 4_Edit_Headers.pl UK_2019_FastaList.txt";

die;
}


open (INLIST, "<$ARGV[0]") || die;


$indir = "/mnt/scratch/d1006888/deplex/FastaFiles";

$outdir = "/mnt/scratch/d1006888/deplex/FastaFiles";

while (<INLIST>) {
$lib = $_;
chomp($lib);
```

```
$fa1 = $lib . ".fasta";
$fa2 = $lib . "_edit.fasta";

system( qq(sed "s/^>/>$lib;/g" "$indir/$fa1" > "$indir/$fa2"));


}

exit;
```

Part 2. Shell script

```
perl 4_Edit_Headers.pl Fastalist_2016.txt
```

Script 5 – USEARCH

```
# removes identical replicates from the fasta input, output for next step =
SampleName_rc_uniques.fasta
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -fastx_uniques Allmerged.fasta -
fastaout Unique.fasta -sizeout -strand both -relabel Uniq -threads 4

# sort by size
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -sortbysize Unique.fasta -fastaout
Sorted.fasta


# Cluster OTUs
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -cluster_otus Sorted.fasta -otus
OTU.fasta -relabel Out

# denoise and cluster using unoise3 to make zOTUs
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -unoise3 Sorted.fasta -zotus
zOTU.fasta

# make list of zOTU's and the number of sequences per zOTU (size)
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -otutab Allmerged.fasta -zotus
zOTU.fasta -otutabout zOTUtable.txt -strand both -threads 4

# make list of OTU's and the number of sequences per OTU (size)
mnt/scratch/d1006888/deplex/FastaFiles/usearch_11 -otutab Allmerged.fasta -otus
OTU.fasta -otutabout OTUtable.txt -strand both -threads 4
```

Script 6. BLAST

```
# blast the clusters from usearch

module load blast/2.7.1

export BLASTDB=/mnt/data/GROUP-sabwocs/c1638428/scripts/BLAST-DB

blastn -query zOTU.fasta -db nt -num_threads 4 -evalue 0.00001 -perc_identity 97 -outfmt 6
-out zOTU.txt

blastn -query OTU.fasta -db nt -num_threads 4 -evalue 0.00001 -perc_identity 97 -outfmt 6 -
out OTU_blastOutput.txt
```

Script 7. Filter the BLAST results

```
# only keep results with over 95% identity and remove and sequences with less than 100bp in length

awk '$3 >= 95' OTU_blastOutput.txt | awk '$4 >= 100' > OTU_COI_2019_blast_filtered.txt.

awk '$3 >= 95' zOTU_blastOutput.txt | awk '$4 >= 100' > zOTU_COI_2019_blast_filtered.txt.
```

Script 8. Add taxon information to diet zOTU matrix (R-script)

```
#Add in taxon information to your zOTU and OTU tables: Open R and run the following code on your blast output to get only the top hit for each motu based on bitScore (combination of e-value and percentage identity):

library(dplyr)

blast <- read.table("zOTU_COI_2019_blast_filtered.txt")

summary(blast)

blast_filter <- blast %>%

 group_by(V1) %>%

 filter(V12 == max(V12))

write.table(blast_filter, "COI_2019_zOTU_TopHit_blastOutput.txt")

#Next use the program MEGAN to assign ids to each zOTU from the BLAST top hit output.
#Use VLOOKUP in Excel to add taxon ids to each zOTU in the diet matrix.
#Calculate maximum contamination/tag jumping from NAs and negative controls and apply this to all samples in that row. Convert negative values to 0.
#Remove all reads with a read count of less than 10.
#Remove zOTUs that have highest reads in positive controls from the remaining diet matrix.
#Remove non-dietary data
#Convert matrix to csv file for aggregating in R.
```

Script 9. Aggregate zOTUs in diet matrix based on taxon ID (R-script)

```
COI_to_Agg <- read.csv("zOTUtable_COI_2019_No_contamination.csv", header = T)

Agg <- aggregate(.~Taxon, data=COI_to_Agg, sum)

write.csv (Agg, "COI_2019_Aggregated.csv")
```

Appendix 2.3. Taxa removed from Hawfinch COI metabarcoding dataset

Table A2.1.1. Taxa removed from the Hawfinch COI dataset.

Taxon	Common name	Reason for removal	Accession Code
Aspergillus campestris	Fungi	Not dietary taxa	EU982130.1
Aspergillus versicolor	Fungi	Not dietary taxa	EU982147.1
Leotiomyceta sp.	Fungi	Not dietary taxa	FJ590524.1
Penicillium digitatum	Penicillin fungus	Not dietary taxa	HQ622809.1
Penicillium polonicum	Penicillin fungus	Not dietary taxa	EF180426.1
Penicillium rubens	Penicillin fungus	Not dietary taxa	EF180211.1
Penicillium sp.	Penicillin fungus	Not dietary taxa	FJ004524.1
Proctophyllodes sp.	Feather mite	Parasite	KU203128.1
Pythiales sp.	Oomycete	Not dietary taxa	JN660054.1
Pythium aff. diclinum	Oomycete	Not dietary taxa	EU350526.1
Pythium apiculatum	Oomycete	Not dietary taxa	HQ708490.1
Pythium aquatile	Oomycete	Not dietary taxa	HQ708492.1
Pythium attrantheridium	Oomycete	Not dietary taxa	GU071826.1
Pythium folliculosum	Oomycete	Not dietary taxa	HQ708477.1
Pythium mamillatum	Oomycete	Not dietary taxa	GU071819.1
Pythium rostratifingens	Oomycete	Not dietary taxa	HQ708803.1
Pythium sp.	Oomycete	Not dietary taxa	HQ708533.1
Pythium viniferum	Oomycete	Not dietary taxa	HE797904.1
Saprolegnia sp.	Water mould	Not dietary taxa	HQ709052.1
Saprolegnia unispora	Water mould	Not dietary taxa	HQ709056.1
Tetracladium furcatum	Fungi	Not dietary taxa	EU883404.1

Appendix 2.4. *Manyglm* post-hoc pairwise comparisons between sampling regions and years

Table A2.2.1. Post-hoc pairwise comparisons of invertebrate dietary differences found between Hawfinch at different sampling regions.

Site comparison	Observed statistic	Free Stepdown Adjusted *p*-value
Dolgellau vs Wye Valley	267.92	0.001
Dolgellau vs New Forest	249.54	0.001
New Forest vs Wye Valley	171.26	0.001
Forest Ganol vs New Forest	83.28	0.002
Dolgellau vs Forest Ganol	74.10	0.003
Forest Ganol vs Wye Valley	65.00	0.033
New Forest vs Norfolk	63.41	0.05

Forest Ganol vs Norfolk	55.28	0.05
Dolgellau vs Norfolk	49.50	0.05
Norfolk vs Wye Valley	47.47	0.05

Table A8.4.2. Post-hoc pairwise comparisons of invertebrate dietary differences found between Hawfinch sampled between years.

Year comparison	Observed statistic	Free Stepdown Adjusted *p*-value
2018 vs 2019	419.50	0.001
2017 vs 2019	272.69	0.001
2017 vs 2018	261.63	0.001
2016 vs 2019	51.03	0.043
2016 vs 2017	47.35	0.043
2016 vs 2018	12.57	0.431

Appendix Three – Supplementary information relating to Chapter 4

Appendix 3.1. Taxa removed from Hawfinch ITS2 metabarcoding dataset

Table A3.1.1. Taxa removed from the Hawfinch ITS2 dataset.

Taxon	Common name	Reason for removal	Accession Code
Aspergillus campestris	Fungi	Not dietary taxa	EU982130.1
Aspergillus versicolor	Fungi	Not dietary taxa	EU982147.1
Leotiomyceta sp.	Fungi	Not dietary taxa	FJ590524.1
Penicillium digitatum	Penicillin fungus	Not dietary taxa	HQ622809.1
Penicillium polonicum	Penicillin fungus	Not dietary taxa	EF180426.1
Penicillium rubens	Penicillin fungus	Not dietary taxa	EF180211.1
Penicillium sp.	Penicillin fungus	Not dietary taxa	FJ004524.1
Proctophyllodes sp.	Feather mite	Parasite	KU203128.1
Pythiales sp.	Oomycete	Not dietary taxa	JN660054.1
Pythium aff. diclinum	Oomycete	Not dietary taxa	EU350526.1
Pythium apiculatum	Oomycete	Not dietary taxa	HQ708490.1
Pythium aquatile	Oomycete	Not dietary taxa	HQ708492.1
Pythium attrantheridium	Oomycete	Not dietary taxa	GU071826.1
Pythium folliculosum	Oomycete	Not dietary taxa	HQ708477.1
Pythium mamillatum	Oomycete	Not dietary taxa	GU071819.1
Pythium rostratifingens	Oomycete	Not dietary taxa	HQ708803.1
Pythium sp.	Oomycete	Not dietary taxa	HQ708533.1
Pythium viniferum	Oomycete	Not dietary taxa	HE797904.1
Saprolegnia sp.	Water mould	Not dietary taxa	HQ709052.1
Saprolegnia unispora	Water mould	Not dietary taxa	HQ709056.1
Tetracladium furcatum	Fungi	Not dietary taxa	EU883404.1

Appendix 3.2. Taxa removed from Hawfinch COI metabarcoding dataset

Table A3.2.1. Taxa removed from the Hawfinch COI dataset.

Taxon	Common name	Reason for removal	Accession Code
Aspergillus campestris	Fungi	Not dietary taxa	EU982130.1
Aspergillus versicolor	Fungi	Not dietary taxa	EU982147.1
Leotiomyceta sp.	Fungi	Not dietary taxa	FJ590524.1
Penicillium digitatum	Penicillin fungus	Not dietary taxa	HQ622809.1

Penicillium polonicum	Penicillin fungus	Not dietary taxa	EF180426.1
Penicillium rubens	Penicillin fungus	Not dietary taxa	EF180211.1
Penicillium sp.	Penicillin fungus	Not dietary taxa	FJ004524.1
Proctophyllodes sp.	Feather mite	Parasite	KU203128.1
Pythiales sp.	Oomycete	Not dietary taxa	JN660054.1
Pythium aff. diclinum	Oomycete	Not dietary taxa	EU350526.1
Pythium apiculatum	Oomycete	Not dietary taxa	HQ708490.1
Pythium aquatile	Oomycete	Not dietary taxa	HQ708492.1
Pythium attrantheridium	Oomycete	Not dietary taxa	GU071826.1
Pythium folliculosum	Oomycete	Not dietary taxa	HQ708477.1
Pythium mamillatum	Oomycete	Not dietary taxa	GU071819.1
Pythium rostratifingens	Oomycete	Not dietary taxa	HQ708803.1
Pythium sp.	Oomycete	Not dietary taxa	HQ708533.1
Pythium viniferum	Oomycete	Not dietary taxa	HE797904.1
Saprolegnia sp.	Water mould	Not dietary taxa	HQ709052.1
Saprolegnia unispora	Water mould	Not dietary taxa	HQ709056.1
Tetracladium furcatum	Fungi	Not dietary taxa	EU883404.1

Appendix Four – Supplementary information relating to Chapter 5

Appendix 4.1. *econullnetr* output showing the strength of trophic interactions between consumers (Hawfinch) and resources (tree genus)

Table A4.1.1. Strength of preference comparisons between the tree community detected in the diets of Hawfinch at a landscape scale. "Weaker" = less of this genus in the diet than expected from its observed frequency. "Stronger" = more of this genus in the diet than expected from its observed frequency. "NS" = reveal genera eaten in proportion to their availability. SES values are standardised effect sizes.

Site	Resource	Observed	Null	Lower 95% CL	Upper 95% CL	Test	SES
north Wales	*Abies*	3	15.16	9.00	22.00	Weaker	-3.72
north Wales	*Acer*	19	25.30	17.00	34.00	ns	-1.51
north Wales	*Betula*	17	46.53	38.00	55.03	Weaker	-6.70
north Wales	*Carpinus*	33	5.58	2.00	10.00	Stronger	12.26
north Wales	*Corylus*	8	17.15	10.98	24.00	Weaker	-2.57
north Wales	*Crataegus*	0	1.93	0.00	5.00	ns	-1.40
north Wales	*Fagus*	51	12.64	7.00	19.00	Stronger	12.05
north Wales	*Fraxinus*	11	32.93	25.00	41.00	Weaker	-5.26
north Wales	*Larix*	6	17.19	11.00	24.00	Weaker	-3.14
north Wales	*Picea*	3	7.91	3.00	14.00	ns	-1.84
north Wales	*Prunus*	30	3.55	0.00	7.00	Stronger	14.42
north Wales	*Quercus*	40	31.45	24.00	40.00	ns	2.13
north Wales	*Sorbus*	4	5.22	1.00	10.00	ns	-0.55
north Wales	*Taxus*	5	0.95	0.00	3.00	Stronger	4.28
north Wales	*Ulmus*	4	10.53	5.00	16.00	Weaker	-2.20
Wye Valley	*Abies*	1	24.59	17.00	32.03	Weaker	-5.69
Wye Valley	*Acer*	21	12.43	7.00	20.00	Stronger	2.55
Wye Valley	*Betula*	7	5.74	2.00	10.03	ns	0.55
Wye Valley	*Castanea*	0	2.22	0.00	6.00	ns	-1.48
Wye Valley	*Corylus*	11	44.64	34.98	55.00	Weaker	-6.45
Wye Valley	*Crataegus*	1	1.15	0.00	4.00	ns	-0.13
Wye Valley	*Fagus*	112	50.67	41.00	61.00	Stronger	11.79
Wye Valley	*Fraxinus*	8	52.18	41.00	63.00	Weaker	-8.09
Wye Valley	*Ilex*	5	5.82	2.00	11.00	ns	-0.36
Wye Valley	*Larix*	9	6.81	2.00	12.00	ns	0.87
Wye Valley	*Quercus*	42	5.89	2.00	11.00	Stronger	15.02
Wye Valley	*Taxus*	9	3.50	0.00	8.00	Stronger	2.89
Wye Valley	*Tilia*	9	35.86	26.00	46.00	Weaker	-5.43
Wye Valley	*Ulmus*	39	22.50	14.00	31.00	Stronger	3.98
New Forest	*Betula*	5	8.51	5.00	12.03	ns	-1.80
New Forest	*Fagus*	10	13.53	10.00	17.00	ns	-1.99
New Forest	*Ilex*	10	10.39	7.00	14.00	ns	-0.20
New Forest	*Quercus*	16	8.57	5.00	12.00	Stronger	3.86

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