Interactions between Acute Oak Decline and woodland birds - examining behavioural, microbial and trophic level changes in response to a tree disease

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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The culmination of this thesis has been far from a solo effort. Collecting huge amounts of data, and processing thousands of samples was not possible for one PhD student, so thank you to the undergraduate research assistants who helped me throughout my work. I hope the promise of holding a baby bird made the gruelling weeks worth it. Thank you to the team at Epping Forest, particularly Andy Froud and Jeremy Dagley who took the time to show me around their wonderful site and allowed me to spend four summers in the most magical woodland. To the team at NEOF in Sheffield, in particular Gavin Horsburgh and Kathryn Maher, who helped me with my molecular analysis. Additional thanks to Bartlett's Tree Experts for their contribution.

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Aside from obtaining a PhD, a goal of mine through this work was to become a C permit bird ringer, which, thanks to Colin Wilson and everyone at Frimley ringing group, I was able to obtain in April 2022. A massive thanks to the ringing group, in particular Colin and Mary, for the most enjoyable breaks away from the lab or the computer, and for becoming good friends over the last

few years. Also for entertaining my controversial love of woodpigeons, but I will be getting my hands on that kingfisher after I submit.

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Impact Statement

Grants

I was successful in securing a grant to carry out microbiome analysis at the NERC Environmental Omics Facility (NEOF) at the University of Sheffield.

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Personal Impact

It seems almost cliche to say that this research has been the hardest thing I have ever done, and in fact I believe it will likely be the hardest thing I will ever do. I came into this project with very little lab experience and a secret hatred for genetics, but it was all part of a bigger plan to pursue a PhD working with UK woodland birds after several years of working on conservation in the tropics. I come out of this work with a great appreciation for molecular analysis, and a plethora of skills I would have never dreamed of. During my studies I have gained confidence in my abilities, including pursuing avenues that interested me such as finding opportunities to learn metabarcoding and self-teaching of R. I have had the chance to explore areas outside of my field, notably attending COP25 in Madrid in the first couple of months of my PhD. I have been fortunate to attend and present at many conferences, making connections with great people from the wonderful worlds of woodlands and birds. Aside from the scientific skills gained in this project, I will be the first to admit that the last few years have been incredibly challenging both professionally and personally, with many challenges to face at home. I am not sure if it is a compliment when people repeatedly mention how resilient you are, but I certainly have grown stronger and surer of myself throughout this PhD work. In May 2024 I started a research fellowship within the Tree Health team at DEFRA, becoming the specialist lead in Acute Oak Decline, a position I would never have dreamed of obtaining. The knowledge, confidence and skills gained in the last 4 years have been integral to this and I am enjoying seeing behind the curtain how the interplay between research and policy works.

Contents

Glossary

Abbreviation	Full Form
AOD	Acute Oak Decline
ASV	Amplicon Sequence Variant
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BTO	British Trust for Ornithology
COD	Chronic Oak Decline
DBH	Diameter at breast height
DNA	Deoxyribonucleic acid
ddH20	Double distilled H20 (water)
KOH	Potassium Hydroxide
NCBI	National Center for Biotechnology Information
NEOF	NERC Environmental Omics Facility
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PVC	Polyvinyl chloride
PIT	Passive Integrated Transponders
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNPs	Single nucleotide polymorphisms
VOCs	Volatile organic compounds

Definitions

Term	Definition
Endophyte	Endosymbiont that lives within a plant in a non-pathogenic state, notably bacteria or fungi
Endosymbiont	An organism that lives within another organism
Enteric	Associated with the stomach or digestive tract
Folivorous	Relating to an organism that specialises in eating leaves
Holobiont	Ecological unit of an individual and all the organisms living within and on it
Hologenome	The collective genomes of the organisms forming the holobiont
Microbiome	Collection of genomes of the organisms within a microbiome (the microbiota)
Microbiota	Community of microorganisms within a particular habitat or environment
Mist net	Fine nets used to capture free flying animals, mainly wild birds and bats
Pathosystem	An ecological system containing host species and parasites or pathogens
Proteomics	Study of proteins
Putative pathogen	A pathogen which causes a particular disease
Transcriptomics	The study of transcriptomes, the mRNA molecules expressed from genes
v3-v4 region	Hypervariable region of the 16S bacterial gene, commonly used to distinguish between bacterial species

Abstract

Tree diseases have the potential to cause severe disruption to landscapes and ecosystems by reducing the number of healthy trees within an ecosystem, which act as foundations for a plethora of other species. The mechanism behind the spread of plant pathogens which cause such diseases is variable across pathosystems but is less understood for tree diseases. Acute Oak Decline (AOD) is a disease of oak trees which is fast acting and can result in the death of trees within a few years from the presentation of symptoms. AOD is believed to be caused by the action of a few pathogenic bacteria, however the mechanism behind the spread of these bacteria is not fully understood. The close association between some woodland bird species and oak trees allows for an ideal system where the wider ecological impacts of AOD can be investigated. In this thesis the current knowledge of plant pathogens and their vectors is explored, finding significant gaps in the research around tree diseases and vertebrate vectors. The role of woodland birds as vectors of the bacteria of AOD is then explored, using both culture based and molecular techniques. The results from this work allow me to explore variations in the microbial communities of birds in areas with differing levels of AOD. In order to examine wider ecosystem impacts of AOD, insect oak herbivory was quantified, along with bird breeding success across areas of woodland with differing AOD severity levels, finding higher insect herbivory levels but lower bird breeding intention in areas with higher AOD. Overall, this thesis utilises a variety of techniques to explore the wider ecosystem impacts of AOD and gives us an insight into how tree diseases may have knock-on consequences for other organisms within a habitat.

Chapter 1 – Introduction

1.1 - The importance of trees and forests

There is an estimated global tree species richness of 73,300, with approximately 9,000 undiscovered species (Cazzolla Gatti et al., 2022). Mature, large trees contribute to over half of worldwide forest biomass (Lutz et al., 2018) with old-growth forests being found to have highest species richness within temperate forests (Zeller et al., 2023). The importance of trees cannot be disputed, not just ecologically but also socially and culturally (Seth, 2003). Trees are keystone species (Lindenmayer et al., 2014; Manning et al., 2006; Shackleton et al., 2018; Stagoll et al., 2012), in that they have a large effect on other species within their ecosystem, and their removal would have negative effects for many other species. Within this, trees are primary foundation species (Ellison et al., 2005), providing habitats for other species, with their presence often being the “foundation” of an ecosystem. The idea that heritability and genetic variation within a keystone, or foundation species, can impact the wider ecosystem lead to the idea of community genetics and extended ecosystem effects (Whitham et al., 2003). This is important as it includes not only the individual tree, but all the other organisms that use it, such as microbes and insects. Therefore, death and dieback of trees and forests can impact a wide range of species that depend on these habitats for survival (Fleming et al., 2021).

There is a plethora of threats facing forests and trees which result in early death and decline, both biotic and abiotic. A number of human impacts are behind global forest loss, including direct habitat degradation and removal of trees through felling and deforestation (Curtis et al., 2018). Land use changes and logging have been estimated to reduce tree numbers by 15 billion per year, with an overall loss of 46% in the number of trees (Crowther et al., 2015) since the practices began. It is not just the direct removal of tree biomass which has detrimental impacts. Habitats with diverse ranges of foundation species support higher levels of biodiversity (Thomsen et al., 2022). Decreasing species composition of foundation trees within a forest has knock-on consequences for the surrounding biodiversity, which is an ever-increasing issue, due to a substantial amount of forest being cleared to create monocultures (Felton et al., 2010; Wang et al., 2019; Wright et al., 2021). Other abiotic factors can include larger environmental events such as wildlife and storms (Fischer et al., 2013). Biotic factors impacting trees and forests include pests and pathogens (Balla et al., 2021), and these can work in combination with abiotic environmental pressures to exacerbate the extent of forest and tree decline.

1.2 - Tree diseases and declines

Tree diseases and declines represent a threat to a wide variety of tree species, and threats from new pathogens are increasing with climatic shifts, increased world trade, and human influences (Potter & Urquhart, 2017; Roy et al., 2017). Declines and diseases of trees can be caused by a culmination of pressures upon an individual, when exposure to certain stressors over time weakens the individual, making it more likely to be degraded by pathogens or pests. There can be a threshold after which the individual plant cannot recover, at which point the tree decline leads to tree death. This idea is known as the 'disease decline spiral', originally presented by Manion, (1991), and recently updated by Denman et al., (2022). However, diseases and declines are not necessarily always a combination of factors and can be caused by a single agent acting upon a healthy and robust tree as can happen with ash dieback. This fungal disease of ash trees has been found to result in the death of over 90% of affected individuals in some habitats (Kowalski, 2006), highlighting how tree diseases can have devastating impacts if they are not managed and controlled.

The major groups of plant pathogens which cause disease are fungi, viruses, bacteria and oomycetes. Pathogens impacting trees and forests have become much more prevalent in the past fifty years and are being introduced at rapidly increasing rates (Freer-Smith & Webber, 2017). Tree pathogens and pests are ever evolving, particularly due to increases in human activity such as trade and changing climates, resulting in range and habitat shifts for these pathogens (Ogden et al., 2019). Countries with higher levels of human activity and international trade have the highest rates of occurrence of these pathogens, likely due to higher rates of surveillance (Lutz et al., 2018). As a result, it is difficult to quantify all species that are or will become forest pests and pathogens due to these constantly shifting goalposts (Boyd et al., 2013). With increasing human activity and climate change increasing the prevalence of plant pathogen occurrence, there is a call for more research into plant pathogen spread, especially with new and emerging diseases (Guégan et al., 2023).

As trees are considered foundation species, tree diseases can not only impact the individual host species, but can also drastically alter ecosystems as a whole, through an overall decline in ecosystem functioning and a restructuring of tree species composition within an ecosystem (Boyd et al., 2013). This has consequences for many other species, with degraded forests generally having lower levels of biodiversity (Gibson et al., 2011), highlighting the importance of understanding the wider impacts of tree diseases.

1.3 - The importance of oak trees

Oak tree species (*Quercus* spp.) are native to the Northern hemisphere and there are 469 species worldwide (Royal Botanic Gardens, Kew, 2024). In the UK there are two native species of oak - *Quercus robur* and *Quercus petraea*. Both of these species have long lifespans, with the average age being around 600 years, but they have been documented as living up to 800-900 years (Nolan et al., 2020). A recent inventory of birds, bryophytes, invertebrates, fungi, lichens and mammals associated with oak in the UK concluded that 2300 species within these taxonomic groups use oak trees (Mitchell et al., 2019a). Of these species 326 were obligate associates, meaning that they are not found on any other tree (Mitchell et al., 2019b). Loss of oak would be especially devastating to these specialist species. Oaks support more invertebrate species than any other tree species (Southwood, 1961), and large mature oak trees provide a variety of microhabitats for organisms including bats (Sauerländers Verlag, 2017), and cavity constructing birds such as woodpeckers (Domokos & Cristea, 2014). Dense oak canopies also host a wide variety of arthropods, which then serve as a food source for birds within the wider habitat (Bereczki et al., 2014).

The inventory of species associations with oak by (Mitchell et al., 2019b) recorded 38 bird species that use oak trees, 14 of which are of conservation concern as per the International Union for the Conservation of Nature's (IUCN)UCN Red List of threatened species. Two species of birds in the UK known for their associations with oak trees are blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) (Betts, 1955). This is due in particular to their preference for oak dominated woodlands for breeding (Lambrechts et al., 2004; Wilkin et al., 2009). These species are known to time their egg laying to coincide with oak tree budburst, with earlier laying dates in areas with earlier budburst (Nilsson & Källander, 2006). The caterpillar prey of these birds feed on newly emerged leaves, therefore this synchronisation of laying eggs to coincide with budburst ensures maximum food availability for the nestlings (Van Dongen et al., 1997). The tri-trophic system of oak tree / caterpillar / great tits and blue tits has been studied for decades and has become a notable system to study in UK woodland ecology. Long term studies have documented that climate shifts and increasing temperatures are pushing bud bursts and caterpillar emergence earlier, whilst egg laying dates are failing to match (Burgess et al., 2018). If a changing climate that impacts oak phenology can have cascading effects for other trophic levels such as folivorous insects and birds, then we can assume that other changes in oak condition could also impact other trophic levels.

Oaks are sensitive to environmental stressors such as drought and increased soil moisture (Besson et al., 2014; Perkins et al., 2018), which can weaken trees so that they are more susceptible to degradation by pests and pathogens, as discussed earlier with the disease decline spiral. Pressures on oak trees vary across countries. In Europe, oak species are threatened by a range of pests and pathogens including oak processionary moth *Thaumetopoea processionea* (Godefroid et al., 2020) and powdery mildews (Bert et al., 2016). In the US there are additional pressures on oak trees including *Phytophthora* species (Frisullo et al., 2018; Rizzo & Garbelotto, 2003). *Phytophthora* species are known to cause diseases and decline in other tree species in the UK, such as elm and ash, but have not yet been documented affecting oak trees in the region (Green et al., 2021). These pests and pathogens can lead to diseases and syndromes such as chronic oak decline (COD) (Camilo-Alves et al., 2017) and acute oak decline (AOD) (Brown et al., 2017).

1.4. - Acute Oak Decline

Acute oak decline (AOD) is a decline disease of oak trees which was distinguished in the UK from other diseases and declines around 15 years ago (Denman & Webber, 2009). It is defined as a "decline disease" rather than a disease as it is caused by the combination of multiple stressors rather than a single infectious agent. As such, following the onset of symptoms it can take four – six years for the disease to fully develop and for the tree to die. A tree suffering from AOD will typically present in a weakened state, which can be indicated by a declining canopy, followed by the expression of the following external and internal symptoms (Denman et al., 2014):

- **Stem bleeds** - weeping patches which are vertically aligned on tree trunks and result in staining of the external bark. These can either be actively weeping (active bleeds) or have just the remnants of the stain (inactive bleeds).

- **Bark plate cracks** - cracks in-between plates of bark are characteristic of AOD symptomatic trees, and these cracks have dark fluid seeping from them.

- **Inner bark necrosis and larval galleries** - most symptomatic trees have larval galleries of the oak buprestid beetle *Agrilus biguttatus* between the phloem and sapwood layers of the tree trunk. These galleries are associated with necrosis of the inner bark layer.

- **'D-shaped' beetle emergence holes** - these emergence holes are not always associated with symptomatic trees, but they are left behind following emergence of adult *A. biguttatus* from the larval galleries.

- **Poor crown condition** - although not always characteristic of AOD symptomatic trees, there can be thinning of the canopy with more advanced stages of AOD, and is generally a useful metric to infer the health of oak trees (Brown et al., 2016).

Bacteria have been isolated from the lesions of trees that show symptoms of AOD, and these have been identified as being pathogenic to induce the symptoms of AOD. These bacteria are *Brenneria goodwinii, Gibbsiella quercinecans*, and *Rahnella victoriana*, all of which are within the Enterobacteriaceae family (Brady et al., 2017). *B. goodwinii* is believed to be the most important pathogen and the causative agent behind the symptomatic lesions, whereas *G. quercinecans* and *R. victoriana* increase the severity of existing lesions (Denman et al., 2018; Doonan et al., 2019).

Members of the Enterobacteriaceae are most often found as part of gut microbial communities, and in particular are associated with the guts of warm-blooded animals (Wiley et al., 2017). It is therefore interesting that several species within this bacterial family have been identified as the causative agents of a tree disease, as one could assume that the conditions in UK woodlands and trees would not be optimal for their survival. Little is known about the source of these bacteria, although experimentally they have been shown to survive on different oak tissues, including leaves, acorns and within the soil surrounding oak (Maddock et al., 2023). Work on the bacteria's survival, however, has found that *B. goodwinii* is unable to survive in soil and rainwater, whereas *G. quercinecans* is much better at surviving in both soil and rainwater for several months (Pettifor et al., 2020). As *B. goodwinii* appears to be the main causative agent of AOD, it is interesting that this bacterial species is unable to persist in open environmental conditions. This pattern of persistence poses the question of whether or not this bacteria is transmitted between trees within a warm-blooded animal vector, which would suit the conditions for this member of the Enterobacteriaceae to persist between hosts.

As birds such as great tits and blue tits have such close associations with oak trees and could act as a suitable vector for the bacteria which act as putative pathogens of this disease, there is scope

to investigate if these species are indeed vectors of these bacteria, and to subsequently analyse the impacts AOD has on the birds' ecology.

1.5 - Research gaps, aims and objectives

Research into tree diseases has been increasing in recent decades and there is a pressing need to understand the pathology of tree diseases, alongside their epidemiology and impact on the wider community. This thesis will focus on one tree disease, acute oak decline (AOD), and aims to examine how this disease can impact higher trophic levels and ecosystem functions in oak woodlands in the UK.

Chapter 2: A scoping literature review examining the vectors of plant pathogens

Plant pathogens come in a variety of forms, many of which utilise a range of dispersal methods to spread between individual host plants. This dispersal can be passive, for example through wind and water, or active, for example the use of vectors such as animals. There has been considerable research into pathogens vectored by arthropods, in particular insects, but much less work into pathogens vectored by vertebrates. Certain forms of pathogens, such as bacteria, could be potentially vectored by warm-blooded animals, but there appears to be little in the literature about these vectoring avenues. In this chapter I review the current knowledge of birds and other warm-blooded animals as vectors of plant pathogens, and in particular tree diseases. I found very little evidence of birds acting as vectors for plant pathogens and therefore expanded my search to include all animal vectors of plant pathogens. As a result, I categorised the current knowledge of plant pathogen vectors according to the plant type, pathogen form and the vector type, and found a bias towards literature on pathogens of crops. The majority of classified vectors were within the class Insecta, and within this the most researched vector were species within the order of Hemiptera. The review highlighted the lack of research into bacterial plant pathogens, warm-blooded animal vectors, and pathogens of trees.

Chapter 3: The role of woodland birds as vectors of bacteria associated with Acute Oak Decline

Following the results from Chapter 2, there was a clear research gap in relation to birds as potential vectors of bacterial plant pathogens. Birds such as blue tits and great tits have close associations with oak trees for breeding and foraging. A handful of the results in

Chapter 2 referred to the ability for some plant pathogens to be vectored by birds externally, however none of these pathogens were bacterial. Acute oak decline (AOD) is a tree disease associated with the bacterial pathogens *Brenneria goodwinii, Gibbsiella quercinecans, Rahnella victoriana*. These bacteria are within the family Enterobacteriaceae, which contains species associated with the gut microbiome, therefore it is plausible that bacterial species within this family could be vectored internally by birds. This chapter examined the extent of AOD within a UK woodland, Epping Forest, and the potential for birds to be carriers of the putative pathogens of acute oak decline. Samples were taken from adult and nestling blue tits and great tits - buccal swabs, back and foot swabs, and faecal samples. These were cultured under selective conditions to attempt to isolate the bacteria associated with AOD. Samples were identified using sequencing but less than 1% of the bacteria were those associated with AOD, and these were not the primary AOD pathogens. Culture based methods are not consistently reliable for bacterial isolation, especially when culturing environmental samples as not all bacteria grow well from mixed culture samples, therefore, to investigate fully if the samples taken from the bird contained the AOD associated bacteria, non-culture based identification needed to take place.

Chapter 4: The impact of Acute Oak Decline on avian gut microbiomes

Building on the work in Chapter 3, this chapter examines the unculturable microbiome of the faecal samples from blue tit and great tit adults and nestlings. Following a successful grant application to the NERC Environmental Omics Facility (NEOF), I used metabarcoding to examine which bacteria were present in samples taken from areas of woodland with differing areas of AOD. This not only served to determine if the AOD associated bacteria were present, but also allowed insights into how the presence of the tree disease could result in a shift in the birds' gut microbial community. All bacteria detected in the samples were classified taxonomically to genus level where possible using the DADA2 bioinformatics pipeline and comparing retrieved results to the Silva database. I examined the gut microbial communities in birds associated with forest patches of different degrees of AOD incidence for their richness and alpha-diversity and the beta diversity between them. No further AOD bacteria were identified using the culture-free methods. There were also no significant differences in the richness or alpha diversity between woodland patches with different degrees of AOD or the beta diversity within and

among such patches. Taxonomically there was a higher proportion of Enterobacteriaceae in samples from areas with advanced levels of AOD, which is especially interesting as this is the Family the AOD to which bacteria belongs. Funding was only available to analyse the faecal samples collected in this study, and further work should investigate the buccal and body swabs to determine if the AOD bacteria are able to be vectored orally or on a bird's body.

Chapter 5: The impact of Acute Oak Decline on oak insect herbivory damage

Moving away from examining microbial communities , here I start to examine the impact of AOD on the wider ecosystem. This thesis largely focuses on birds and how they respond to tree diseases within their habitat. The birds examined in this thesis are insectivores, therefore they rely on insects such as caterpillars for food, which is especially important during the breeding season. Oaks, caterpillars and birds such as those in the *Paridae* family form a well-studied tri-trophic chain in woodland ecology. As such, the impacts of diseases affecting oaks should be examined across trophic levels. In this chapter I examined insect herbivory levels of trees with differing AOD statuses and found that herbivory damage was higher on trees which were symptomatic for AOD than on trees which were asymptomatic. This result could indicate either that symptomatic oak trees have lower defences to mitigate the impacts of herbivores, or that predation levels are potentially lower by insect folivores on symptomatic trees. Identification of the herbivores was not possible in this study, but work identifying insect herbivore community assemblages would help to recognise which species favour AOD symptomatic trees.

Chapter 6: The impact of Acute Oak Decline on breeding success of birds

In the final chapter of my thesis, I investigate the impact of AOD on the breeding success of great tits and blue tits in nest boxes across a woodland with varying levels of AOD. Nest boxes were monitored from 2020-2023 to determine if birds chose to breed in those areas, if the breeding was successful, how healthy the nestlings were, and what the fledging success was. It was found that birds were more likely to build nests in areas with lower levels of AOD and higher habitat health, incorporating canopy density, whereas no other breeding metrics were affected by the presence of AOD. Nestbox occupancy can be used to infer a birds' intent to breed, thereby we can conclude that in this system birds showed a preference to breeding in sites with lower levels of AOD.

Chapter 7: Discussion

This thesis has combined several ideas and methods surrounding the impact of acute oak decline on the wider ecosystem, with a particular focus on avian ecology. In this discussion chapter I bring together all the findings and examine how the results from this thesis enhance our understanding of how changes to important tree species such as oak can have knock-on consequences for their ecosystem. I highlight how this project could be developed further using additional techniques and where the gaps in research still exist.

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Chapter 1

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Chapter 2 – A scoping literature review examining the vectors of plant pathogens.

2.1 - Abstract

Plant pathogens are found in a range of forms such as bacteria, viruses, fungi and oomycetes, and can cause plant disease in a wide variety of plant types. These microorganisms can spread naturally between hosts through wind, rain, water, and other natural processes. Plant pathogens can be devastating to many different types of plants, and the diseases they cause can have drastic ecological and economic impacts. The spread of these pathogens by animal vectors has been explored in plant pathosystems, but little work to date has explored the role of animal vectors in the transmission of tree pathogens. Here I conduct a semi-systematic scoping literature review, examining what evidence is currently available on the spread of plant pathogens by animal vectors. Initially aimed at examining the evidence that birds can act as vectors of tree pathogens, the review was extended to include all vectors of all plant pathogens. Gaps in the literature were identified, with the overwhelming majority of research into plant pathogens focussing on crops, and very few studies identifying animal vectors of tree pathogens. This review highlights where further research should be directed to fully understand the transmission of plant pathogens in less studied systems.

Keywords; plant pathogen, plant pathosystem, vectors, tree pathogens, bird vectors.

2.2 - Introduction

Plant diseases can be caused by a range of microbes including bacteria, viruses, and fungi. Plant damage is largely caused by pests such as invertebrates via feeding, but these pests can also facilitate the spread of plant disease via encouraging infection in plants and acting as vectors of pathogens, indirectly attributing to plant disease. This review will focus on pathogens rather than invertebrate pests, but it is important to acknowledge their contribution to plant damage. Microorganisms can cause plant disease through a variety of mechanisms, including cellular and tissue degradation. Damage can be specifically adapted to the plant > pathogen > host pathosystem, or pathogens can be more generalist (Lacaze & Joly, 2020). Endophytes are microorganisms which reside naturally within a plant in a non-pathogenic state and can often serve as plant growth promoting bacteria (Ryan et al., 2008). Endophytes include latent pathogens, which are found within plant tissues but are opportunistically pathogenic, meaning

they can become pathogenic due to favourable shifts in the bacteria's host conditions, such as developmental changes in the plant (Turner et al., 2013). This therefore implies that the detection of a pathogen or endophyte within a plant is not enough to infer disease on its own.

The impact of plant diseases can be both ecological and economic, along with being potentially hazardous and disruptive to humans. Economic impacts are often related to plant pathogens of agricultural and horticultural crops (Juroszek & Tiedemann, 2013; Mansfield et al., 2012), and there has been much research into pathogen transmission in this field. Plant pathogens can be particularly devastating to crops and have resulted in historical events such as the Great Famine in Ireland in the 1800s, a result of potato blight caused by the oomycete, *Phytophthora infestans*, leading to over one million deaths due to starvation, and displacement of millions more of the Irish population (Mokyr, 2023). Looking forward to more recent disease outbreaks, plant pathogens have been estimated to result in losses of between 9-16 % of crops globally (Oerke & Dehne, 1997).

The main ways in which plant pathogens are spread are highlighted in Figure 1, however the mechanisms of pathogen spread can vary across habitats (Tack et al., 2014; Condeso & Meentemeyer, 2007). A woodland or forest habitat would have different types of vectors. For example, rain, water and root extension would be similar in a woodland to an agricultural setting, but it is possible that wind dispersal would play a less significant role depending on the density and composition of the woodland. Human vectors could have a very small contribution, particularly in dense patches of woodland or those with little to no public access. As the relative importance of these pathogen dispersal tactics would vary depending on the individual ecosystem, it is difficult to assume that one mode of dispersal is more significant than another within a specific habitat type. Dispersal also depends on the form of the pathogen and its life stage. Fungi and oomycetes are primarily wind and water-borne pathogens (Borgmann-Winter et al., 2023; Fawke et al., 2015) and most plant viruses are transmitted by insects (Roosien et al., 2013). Bacterial pathogens may require direct transmission from one plant to another by a living vector, arguably making transmission slower and less widespread (West, 2014).

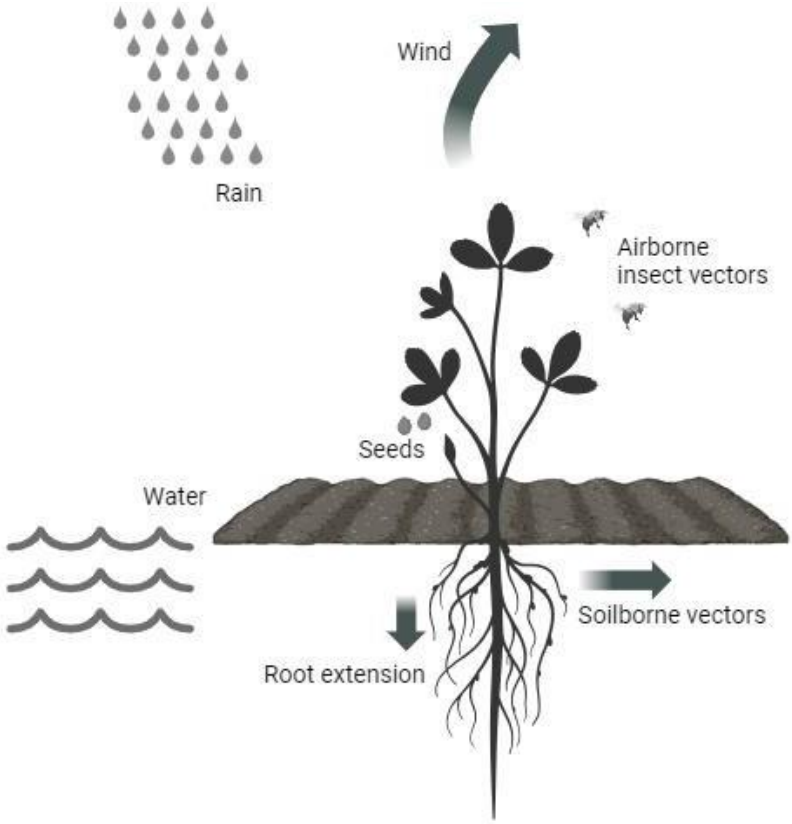

Figure 1. Main mechanisms by which plant pathogens are spread between individual plants, adapted from Lucas, (2009). Created with BioRender.com.

Plant pathogens can pose problems in a range of habitats and plants, including woodlands and trees. Tree diseases such as ash dieback (Mitchell et al., 2014) and acute oak decline (Denman et al., 2014) have been linked to pathogenic microorganisms such as fungi and bacteria, which can either cause disease of a healthy tree, or act opportunistically upon damaged or more vulnerable trees (Brown, et al., 2017). Although tree diseases and their complexity have been documented for decades (Manion, 1981), much of the original research focussed on diseases at an individual level. Expanding this to include the field of forest- and landscape pathology is a relatively new concept (Holdenrieder et al., 2004). There is little evidence in the literature that points towards the transmission of tree pathogens between individual plants, however, this could be an oversight as most research focuses on wind-borne tree pathogens. As more bacterial tree pathogens are being discovered, research on the transmission of these pathogens is needed.

Many birds have close associations with certain tree species, for example great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) frequently use oak trees (*Quercus* spp.) for feeding and breeding (Hinsley et al., 2008). Other birds such as woodpeckers (*Picidae* spp.) can cause physical damage to trees, boring into trunks to predate the insects inside and to create nesting cavities (Spring, 1965). Nuthatches (*Sitta* spp.) and treecreepers (*Certhiidae* spp.) will spend a significant amount of their time foraging on the trunks of trees, and will also nest and roost in tree cavities, along with many other bird species (González-Varo et al., 2008; Norberg, 1986). Birds which have close associations with plants, such as blue tits and great tits associating with oak trees, could have the potential to act as vectors of plant pathogens through mechanical transmission.

2.3 - Aims

This literature review serves to address the current knowledge around the following questions;

1) What is the role of birds as vectors of tree diseases?
2) What are the most commonly studied plant pathogen vector interactions (pathosystems)?
3) What importance do these pathosystems hold for human society (economic, ecological value etc.)?
4) Are there gaps in research into certain plant pathogen vector interactions, and if so, what does the current research primarily focus on?

2.4 - Methods

A semi-systematic literature review was carried out to address what current knowledge there is on the role of birds as vectors of plant pathogens, specifically pathogens causing tree diseases. The following databases were utilised to carry out a comprehensive literature search; Web of Science, Scopus, Google Scholar, PubMed, and Science Direct. ProQuest was also used as a source of grey literature. The outcomes of the initial search indicated there were only 10 papers examining the role birds play in the transmission of plant pathogens, which was not sufficient to answer the research question. This search was then widened to include all plant > plant pathogen > vector pathosystems, not just limited to birds as vectors.

Table 1 summarises the search terms identified and used in a naive search of the databases. Terms in italic indicate the search terms used for an initial search on birds as vectors. The terms in Table 1 were incorporated into Boolean search strings which were inputted into Web of Science and Scopus. The search strings and number of results generated can be seen in Table 2.

Table 1. Search terms used in the first naive search. Terms in italics refer to search terms used in the initial search focussing on birds as vectors

Synonyms for "plant pathogen"	**Synonyms for "vector"**
Plant pathogen	*Vector*
Tree disease	*Bird*
Plant disease	Animal
Tree pathogen	Transmitter
Pathogen	Carrier
Disease	Invertebrate
	Insect
	Mammal

Table 2. Boolean search strings used for naive search and total resulting number of papers identified before removing duplicates from across databases

Search String	***Number of results***
(("plant pathogen*" **OR** "tree disease*" **OR** "plant disease*" **OR** "tree pathogen*" **OR** "pathogen*" **OR** "disease*") **AND** ("vector*" **OR** "transmitter*" **OR** "carrier*") **AND** ("bird*" **OR** "animal*" **OR** "invertebrate*" **OR** "insect*" **OR** "mammal*"))	35,230
(("plant pathogen*" **OR** "tree disease*" **OR** "plant disease*" **OR** "tree pathogen*" **OR** "pathogen*" **OR** "disease*") **AND** ("bird*" **OR** "animal*" **OR** "invertebrate*" **OR** "insect*" **OR** "mammal*"))	445,340
(("plant pathogen*" **OR** "tree disease*" **OR** "plant disease*" **OR** "tree pathogen*") **AND** ("vector*" **OR** "transmitter*" **OR** "carrier*") **AND** ("bird*" **OR** "animal*" **OR** "invertebrate*" **OR** "insect*" **OR** "mammal*"))	924

Following the naive search focussing on birds as vectors, three "gold standard" papers were identified. As the naive search produced limited results, these were the only recovered papers which experimentally demonstrated the role of birds as vectors of plant pathogens. Gold standard papers are used as a means to test the reliability of the search strings and keywords, therefore if these papers are retrieved when amending the search terms, the reliability of the search string is increased.

Gold standard papers;

1 - (Kusunoki et al., 1997) - Role of birds in dissemination of the thread blight disease caused by *Cylindrobasidium argenteum*

2 - (Malewski et al., 2019) - Role of avian vectors in the spread of *Phytophthora* species in Poland

3 - (Scharf & DePalma, 1981) - Birds and mammals as vectors of the chestnut blight fungus (*Endothia parasitica*)

The first search string in Table 2 generated 35,230 results, however after analysing the results from these searches, it appeared a large proportion of these papers were focussed primarily on biomedical sciences. For example, the use of "pathogen/disease" "transmitter/carrier" and

"animal/insect" yielded a lot of results about arboviruses and other insect borne human diseases. Removing the "vector" synonyms only increased these results to 445,340. By reducing the synonyms for "disease" these results were reduced to 924.

The package litsearchr (Grames et al., 2019) in R (v. 4.3.1) was used to ensure a thorough and robust search of the literature was carried out. Litsearchr uses text-mining to extract terms and keywords from the results of an initial naive search that have been missed otherwise. This helps to improve the accuracy of subsequent searches. The results from the third Boolean search string were analysed using litsearchr, and the additional search terms generated can be seen in Table 3 in bold. Where there were multiple variations of terms, for example "pathogen" "pathogens" "pathogenic" etc., these terms were truncated by an asterisk which allowed all variations to be included in the search, for example "pathogen*" would include all three of these variations.

Table 3. Complete set of search terms determined both prior to using litsearchr and those identified from litsearchr (bold). Terms truncated by * indicate there are several varieties of this term, for example pathogen* will include pathogen, pathogens, pathogenic etc.

Synonyms for "plant pathogen"	***Additional synomyms for "plant pathogen" identified from litsearchr***		***Synonyms for "vector"***
Plant pathogen	**Bacterial plant pathogen***	**Plant pathogenic**	Vector
Tree disease	**Insect-transmitted plant**	**fungi**	Bird
Plant disease	**pathogen**	**Plant pathogen***	Animal
Tree pathogen	**Insect-vectored plant**	**Plant pathology**	Transmitter
Pathogen	**pathogen**	**Plant virus***	Carrier
Disease	**Plant-pathogenic bacterium**	**Soilborne plant**	Invertebrate
	Plant disease*	**pathogenic**	Insect
	Plant pathogen*		Mammal
	Plant pathogenic bacteri*		

Litsearchr was used to compile a Boolean search string that would result in the highest number of reliable search results. This can be seen below in Search String 1.

Search String 1

((“plant* pathogen*” **OR** “tree* diseas*” **OR** “plant* diseas*” **OR** “tree* pathogen*” **OR** “bacterial plant pathogen*” **OR** “plant-pathogenic bacteri*” OR “plant pathogenic bacteri*” **OR** “plant pathogenic fung*” **OR** “plant-pathogenic fung*” **OR** “plant pathology” **OR** “plant virus”) **AND** (“vector*” **OR** “transmitter*” **OR** “carrier*”) **AND** (“bird*” **OR** “animal*” **OR** “invertebrate*” **OR** “insect*” **OR** “mammal*”))

Not all the search platforms detected the gold standard papers using Search String 1, however the databases used have different advantages and disadvantages. PubMed mainly includes citations for biomedical science, and Google Scholar has been found to be less reliable in retrieving all literature than more established databases (Haddaway et al., 2015), but was still able to detect the gold standard papers. The abstracts of the gold standard papers were screened and compared to Search String 1 to determine why they had not been detected in PubMed. As a result, Search String 2 was constructed to increase the likelihood of the gold standard papers being retrieved.

Search String 2

((“tree” **OR “**plant” **OR** “plant* pathogen*” **OR** “tree* diseas*” **OR** “plant* diseas*” **OR** “tree* pathogen*” **OR** “bacterial plant pathogen*” **OR** “plant-pathogenic bacteri*” OR “plant pathogenic bacteri*” **OR** “plant pathogenic fung*” **OR** “plant-pathogenic fung*” **OR** “plant pathology” **OR** “plant virus”) **AND** (“vector*” **OR** “transmitter*” **OR** “carrier*”) **AND** (“bird*” **OR** “animal*” **OR** “invertebrate*” **OR** “insect*” **OR** “mammal*”))

Search Strings 1 and 2 were used together across all databases, with search results being combined. In R, Litsearchr was used to remove any duplicates across the results and all gold standard papers were detected.

The online platform Rayyan (Ouzzani et al., 2016) was used to assign each paper as “included” or “excluded” in the literature review according to the inclusion criteria in Table 4. This was conducted by screening paper titles first, followed by abstracts and full text.

Table 4. Inclusion criteria used to determine if papers should be included in the systematic literature review.

Criteria	**Restrictions**
Date Range	None
Geographic Range	None
Language	English (due to language limitations of reviewer)
Population restrictions	Peer-reviewed, primary research study Review papers will also be examined for any additional systems
Outcome restrictions	All plant pathogen/vector systems Pathogens including - Viruses - Bacteria - Fungi - Oomycete - Protists Vectors - All living vectors (non-human)
Other restrictions	Living systems Naturalistic settings

The reference lists of relevant papers were then manually scanned to ensure no relevant papers had been missed out during the database search, and their reference lists then searched manually, a technique known as snowballing (Ali & Tanveer, 2022). This was carried out with reference lists iteratively until no new papers were uncovered.

Search results were assessed and categorised according to the following criteria to form a database of plant > plant pathogen > vector interactions, to give a full picture of the current knowledge of plant pathogens and their vectors.

1) Plant affected
2) Type of plant (agricultural crop, tree, etc.)
3) Plant disease and corresponding plant pathogen (if different)
4) Form of pathogen (bacteria, virus, etc.)
5) Vector (categorised to Order or lowest classification possible)
6) Specific vector (species if specified)

Plants were grouped according to the following categories.

- **Agricultural crops** - plants which are grown for agricultural purposes
- **Trees** - plants that have the common characteristics as trees and are referred to as such in the research paper
- **Flowers** - Flowering plants not used as agricultural crops (may have horticultural importance)
- **Other** - Plants that did not fit into the above categories and did not commonly occur in the search results, including grasses

Where plants fit in multiple categories, the categorisation depended largely on what the plant is primarily used for by humans, and how it was described in the context of the paper. Apple trees for example, could be categorised as trees or crops, but if the research carried out on them was from an agricultural and economic standpoint, they would be categorised as crops.

Each individual interaction was then categorised according to plant > plant pathogen > vector systems, to ensure that if one specific system had a large body of individual papers behind it, this would not bias the overall results.

No detailed analysis was carried out on specific plant pathogens, as many diseases and syndromes of plants had different names depending on where they were researched and the time in which they were researched, therefore it was difficult to accurately distinguish between specific diseases. For example, Sweet Chestnut Blight caused by the ascomycete fungus *Cryphonectria parasitica* had previously been classified as being in the genus *Endothia* (Rigling & Prospero, 2018).

In order to visually represent where research specifically focuses, the results were visualised for example by plant type, vector type etc. This was to show where research may be lacking. Research over time was also visualised using time series plots to show the momentum behind certain areas of research. Results were visualised graphically using ggplot2 in R (v. 4.3.1).

2.5 - Results

2.5.1 - Birds as vectors of plant pathogens

The initial naive search of birds acting as vectors of plant pathogens yielded only ten results, details of which can be found in Table 5. Of these papers only one focussed on bacterial plant pathogens - *Pseudomonas syringae, Xanthomonas campestris pv. vesicatoria, Alternaria macrospora* - and there were no specific papers linking bacterial plant pathogens, trees and birds as a pathosystem.

Table 5. Summary of the current literature on the role of birds as vectors of plant pathogens.

Reference	Plant	Plant Disease	Plant Pathogen	Form of Pathogen
(Kusunoki et al., 1997)	Broad Leaved Trees		*Cylindrobasidium argenteum*	Fungus
(Scharf & DePalma, 1981)	Chestnut	Chestnut Blight Fungus	*Endothia parasitica*	Fungus
(Menning et al., 2020)	Eelgrass (*Zostera marina*)		*Labyrinthula zosterae*	Protist
(Lara & Ornelas, 2003)	*Moussonia deppeana*		*Fusarium moniliforme*	Fungus
(Peters et al., 2012)	Rice	Rice Yellow Mottle Virus	Rice Yellow Mottle Virus	Virus
(Peters et al., 2012)	String Beans	Southern Bean Mosaic Virus	Southern Bean Mosaic Virus	Virus
(Jackson & Jackson, 2004)	Hardwood trees		Red Heart Fungus (*Phellinus pini*)	Fungus

Reference	Plant	Plant Disease	Plant Pathogen	Form of Pathogen
(Bashan, 1986)	Various		*Pseudomonas syringae, Xanthomonas campestris pv. vesicatoria, Alternaria macrospora*	Bacteria
(Dadam et al., 2020)	Various		*Phytophthora ramorum*	Oomycete
(Keast & Walsh, 1979)		Forest Dieback Disease	*Phytophthora cinnamomi*	Oomycete
(Malewski et al., 2019)			*Phytophthora cactorum, P. plurivora, P. alni, P. multiformis*	Oomycete

2.5.2 - All vectors of plant pathogens

By expanding the search to include all potential vectors of plant pathogens, a total of 11,419 papers were identified. By removing duplicates in litsearchr, this number was reduced to 10,865 entries. Figure 2 shows the number of papers removed at each stage of the filtering process, which resulted in 723 papers being identified.

Figures 3 and 4 demonstrate how the research is spread across pathogen type (virus, bacteria etc.), vector type (taxonomic class), and plant type (agricultural crops, trees etc.). Across the total number of papers screened in this review, there was an overwhelming focus on the class *Insecta* as vectors of plant pathogens (78%). Crops were also the main plant type of interest in the recovered papers (66%). These percentages increase when you remove “unspecified” papers from the analysis, which had no clear categorisation of plant/pathogen/vector. When removing “unspecified” results, 84% of retrieved papers focused on crops, and 91% of papers focused on the class Insecta as vectors. 1.5% of the results focussed on birds as vectors, and 12% of papers focused on tree as plants of interest (1.7% and 9.4% respectively when including “unspecified” results).

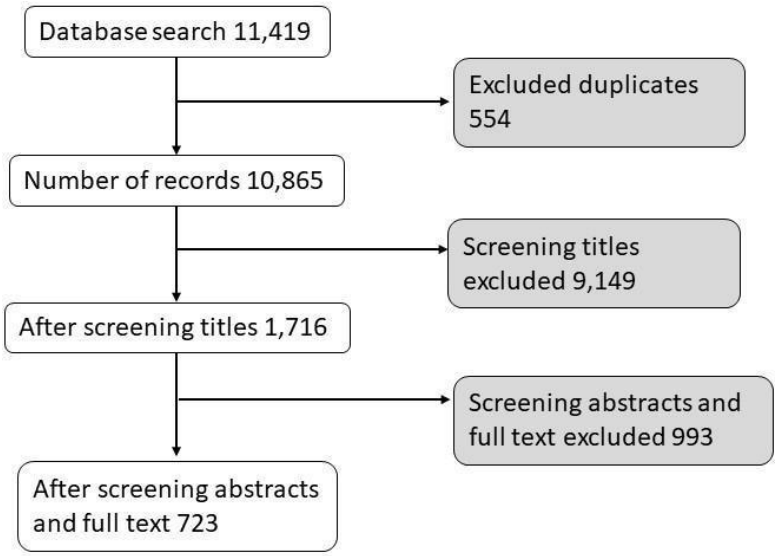

Figure 2. Flowchart demonstrating the number of articles identified and subsequently excluded, eliminating duplicates and applying the exclusion criteria in Rayyan.

Of the 723 papers included in this literature review, 215 unique plant > plant pathogen > vector interactions were detected (See 2.8 Supplementary material, supplementary table 2.1), which are displayed in Figure 4.

Figures 3 and 4 show that the majority of research focuses on plant pathogens affecting agricultural crops. In the absence of agricultural crops when focussing on the remaining plant types, Figures 5 and 6 highlight that Insecta remains the most studied class of pathogen vector, however there is a decrease in the proportion of research on viruses. The diversity of orders within the class Insecta can be seen in Figure 7, which indicates that the majority of vectors are within the order Hemiptera.

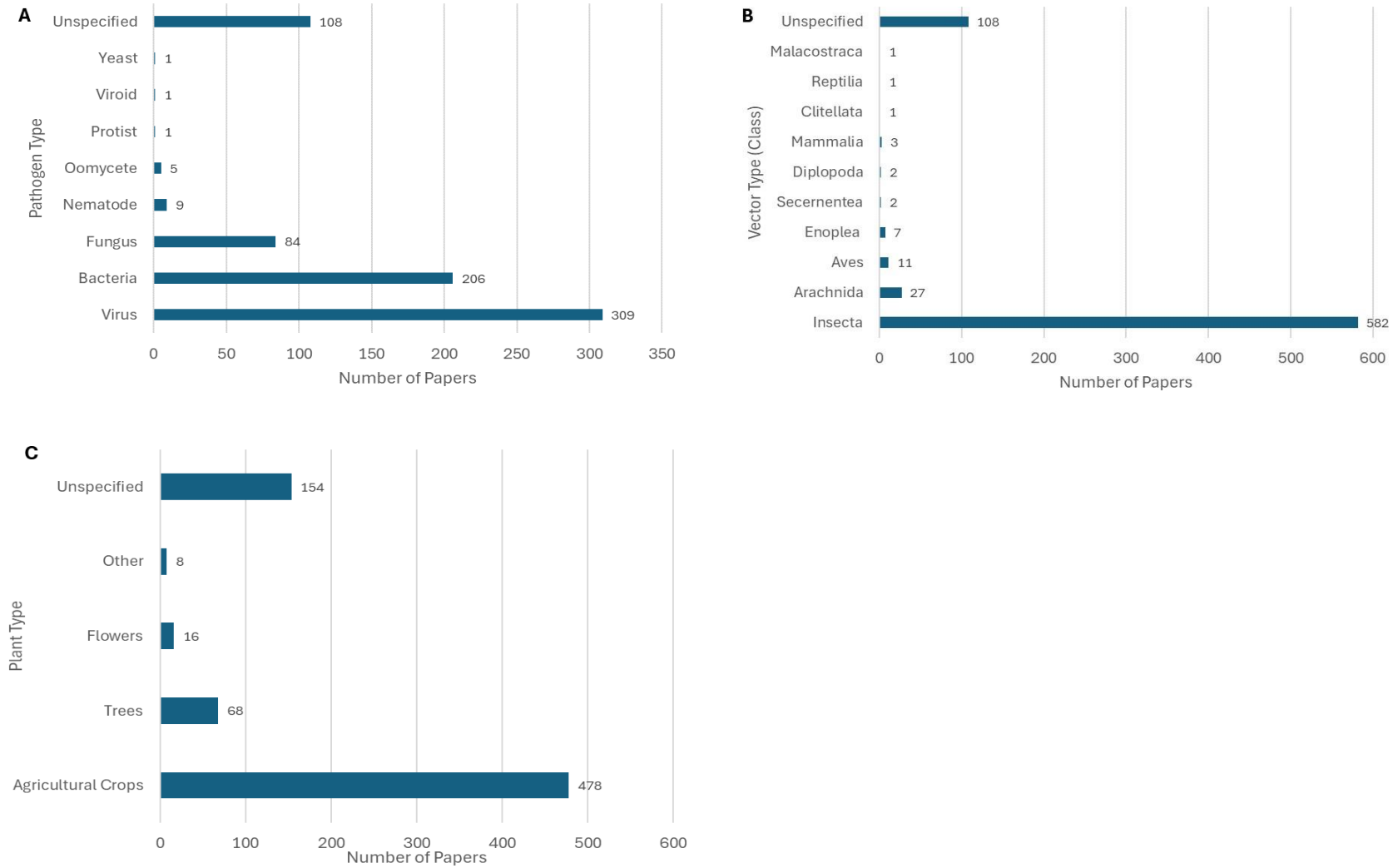

Figure 3. Total number of papers in systematic literature review categorised by A) pathogen type, B) vector type, C) plant type.

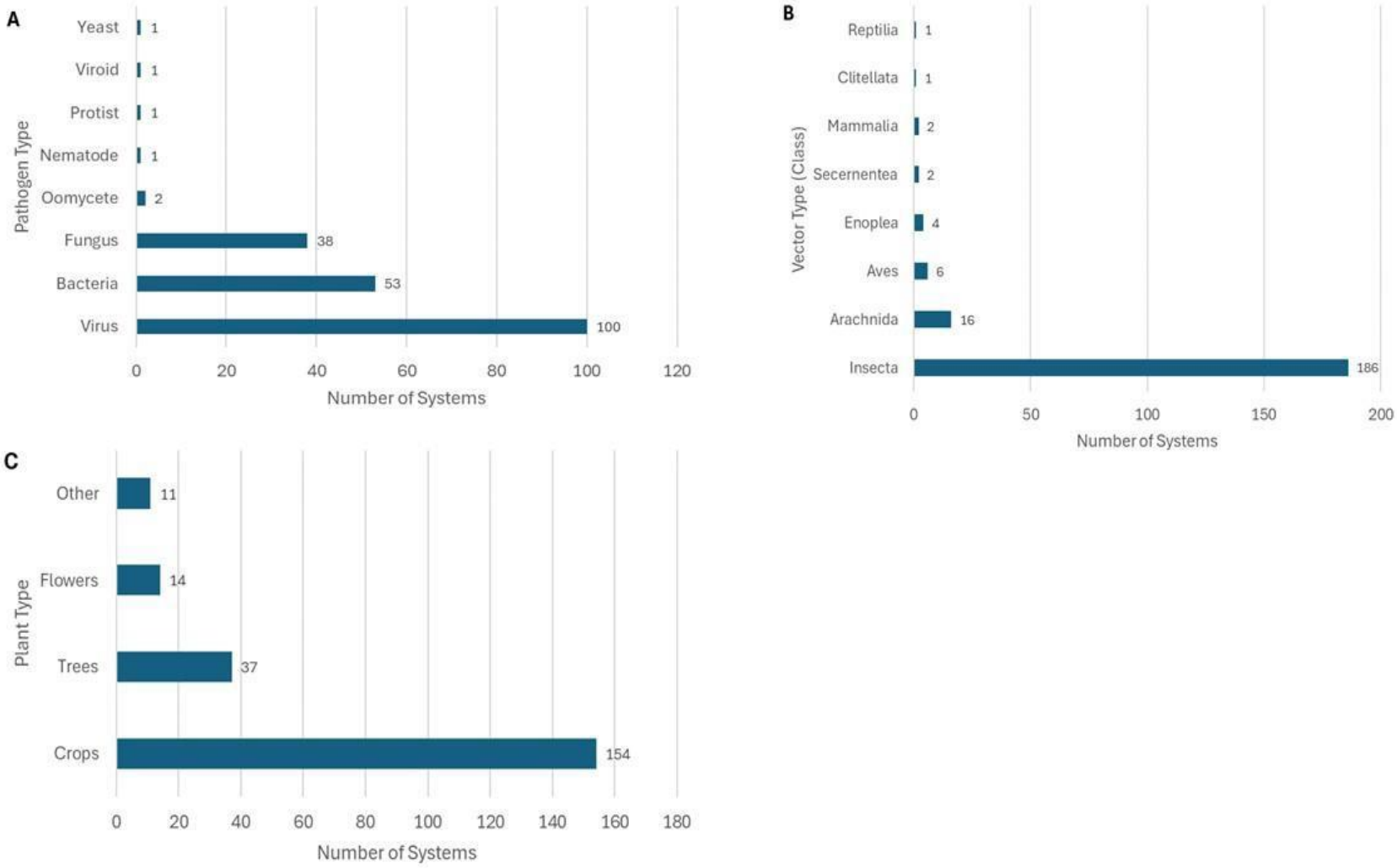

Figure 4. Number of unique plant > plant pathogen > vector interactions identified in systematic literature review categorised by A) different pathogen types, B) different vector types, C) different plant type

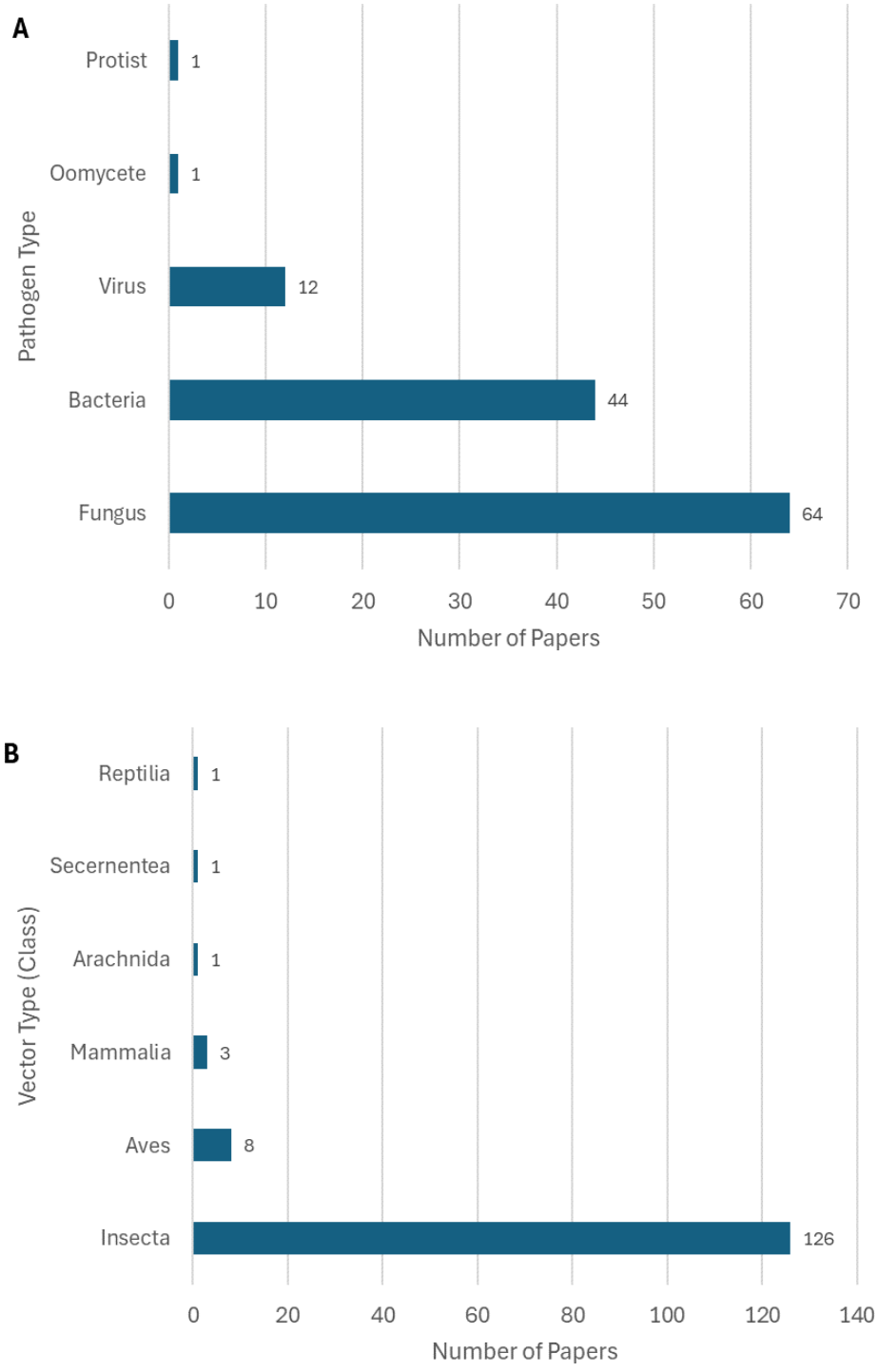

Figure 5. Total number of papers in systematic literature review when papers referring to agricultural crops are removed, categorised by A) different pathogen types, B) different vector types

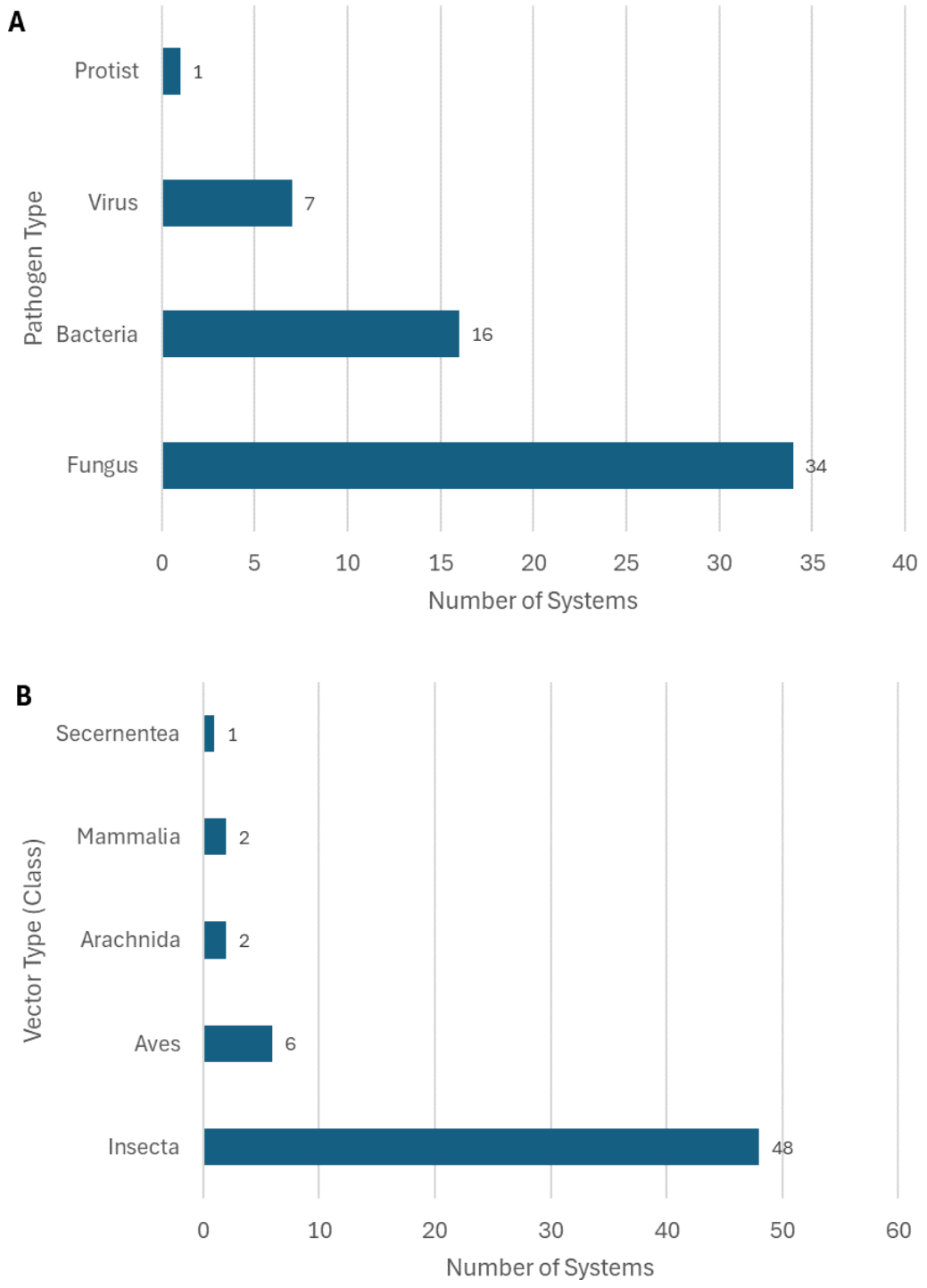

Figure 6. Number of unique plant > plant pathogen > vector systems identified in systematic literature review, when interactions involving agricultural crops are removed, categorised by A) different pathogen types, B) different vector type

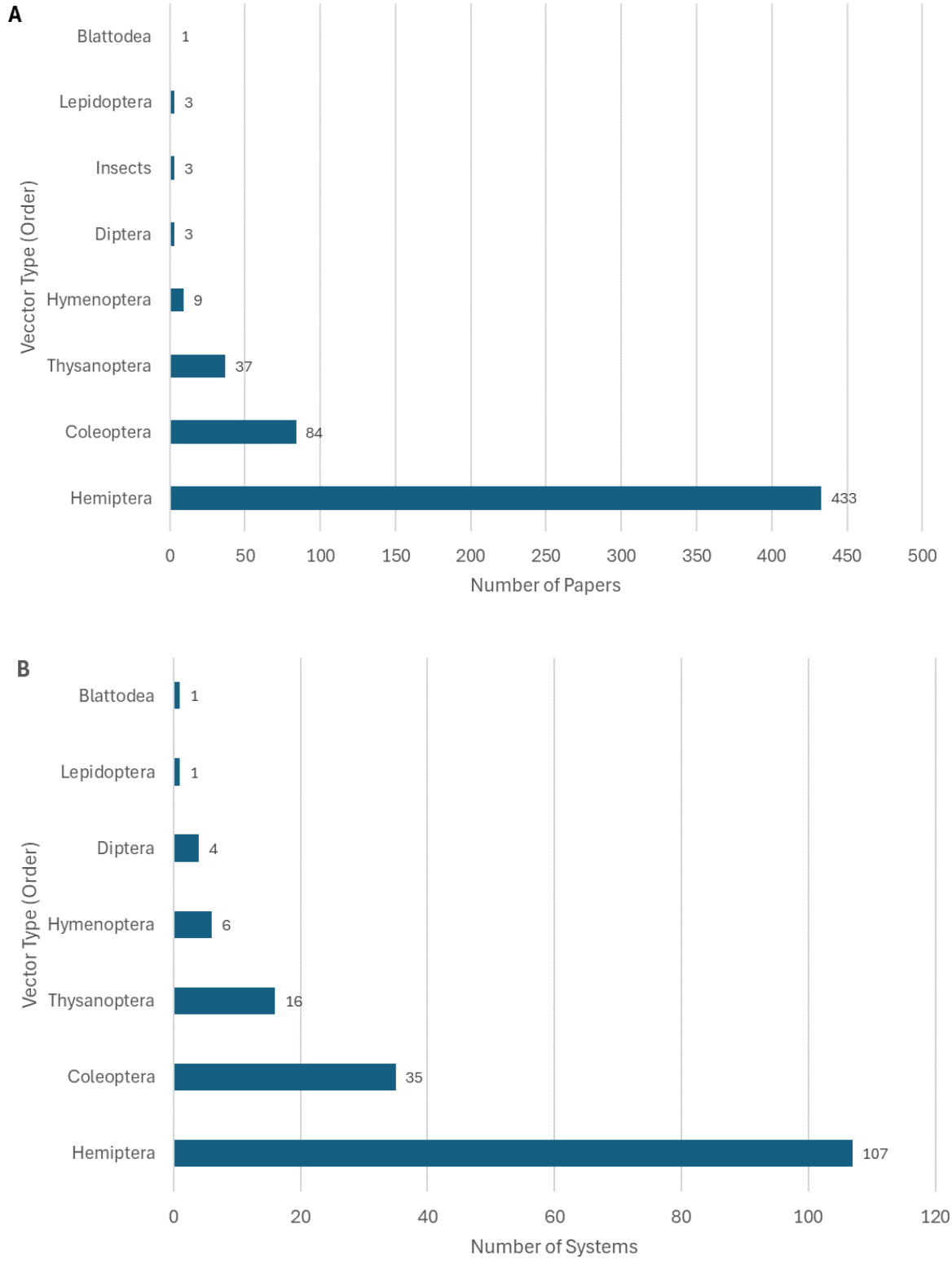

Figure 7. Distribution of orders within the class 'Insecta' A) in the total number of papers, B) by the number of interactions

2.5.3 - Research over time

The rate at which research in each of the plant types has changed over time can be seen in Figure 8, with research into crops as the focal plant group having the largest amount of research which is growing the fastest. Research into plant pathogens of trees has started to increase in recent decades (Fig. 8B), whereas research into pathogens of flowers and other plants has stayed somewhat consistent over time.

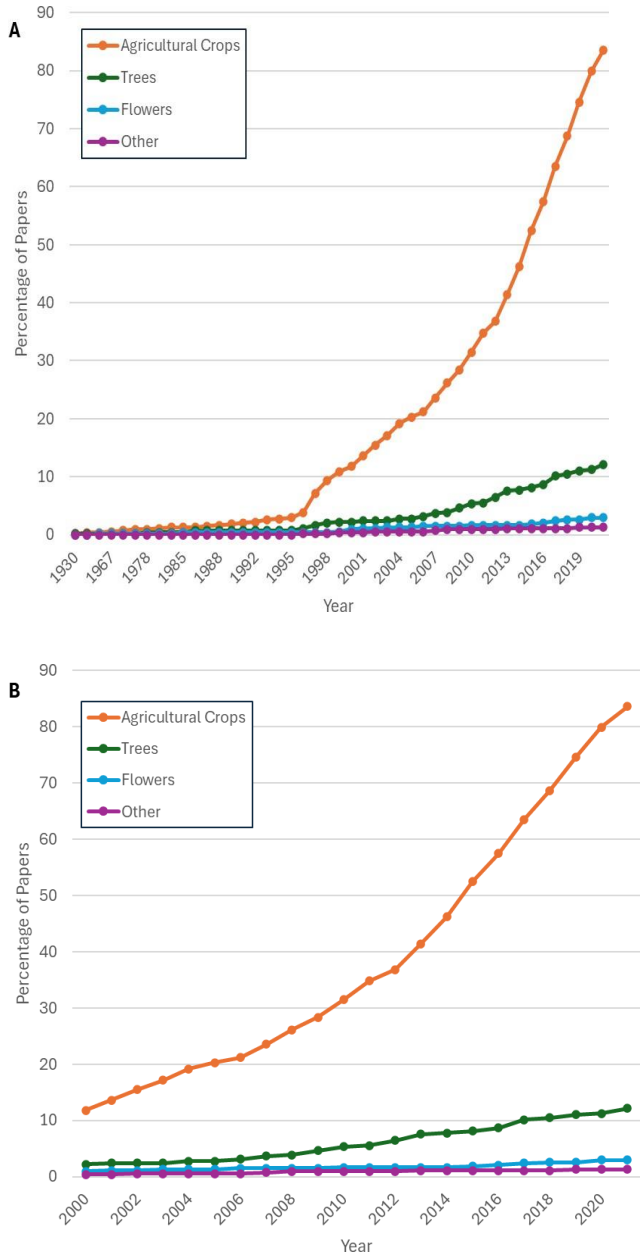

Figure 8. Percentage of papers retrieved in systematic review focussing on different crop types A) since 1930, B) since 2000.

2.6 - Discussion

This literature review served to answer four primary questions;

1) What is the role of birds as vectors of tree diseases?
2) What are the most commonly studied plant pathogen vector interactions(pathosystems)?
3) What importance do these pathosystems hold for human society (economic, ecological value etc.)?
4) Are there gaps in research into certain plant pathogen vector interactions, and if so what does the current research primarily focus on?

This review has served well to answer these questions and has given considerable insight into the current knowledge of the role of birds as vectors of plant pathogens. The review has identified that there is limited research into the role of birds as vectors of plant pathogens, and what information is available appears to be somewhat anecdotal. The most published plant pathogen vector systems favour agricultural crops as the plant type, insects as the primary vector, and viral pathogens, which may not be especially surprising due to the economic value of agricultural crops. There appears to be gaps in the literature when focussing on non-arthropod vectors of plant pathogens, and research into plants other than crops has only been seen to increase in more recent years, but still not at the rate of crop research.

2.6.1 - The role of birds as vectors of plant pathogens

As indicated in Table 5, the current literature on the role of birds as vectors of plant pathogens is very limited, with only 10 citations being recovered. Of these papers, only two (Malewski et al., 2019) sampled wild birds directly in the field for plant pathogens. Using feather and foot swabs, these researchers were able to detect *Phytophthora* DNA on a range of woodland passerines using qPCR. *Phytophthora* represents a genus of important oomycete plant pathogens which have been attributed to a wide range of plant diseases and syndromes (Kroon et al., 2012), including dieback of black alder in Poland, which was the focus of Malewski et al.'s work. Their work demonstrated that plant pathogen DNA could be detected in woodland passerines, indicating a potential avenue for the transmission of this plant pathogen. However, the recovery of DNA from a sample does not necessarily indicate viability of the associated pathogen. The

direct mechanism of transmission is also not entirely clear - it is possible that samples taken from the bird's body or feet could have contained DNA that had passed through the gut of the bird.

A later study by Dadam et al., (2020), employed culture-based assays in an attempt to isolate *Phytophthora* from swab samples taken from birds feathers and feet, however this method was abandoned in favour of molecular identification using DNA sequencing. Whilst yield was low, their analysis did recover *Phytophthora* from the samples, and while not implying that the pathogen is viable, this does offer an association between birds and these plant pathogens. Dadam et al., investigated different birds, including ground-dwelling migratory passerines such as blackbirds and other thrush species which had the potential to acquire *Phytophthora* from the soil. The low incidence of *Phytophthora* detection from Dadam's work could be due to opportunistic sampling across a range of already established ringing sites, looking at migratory species, rather than targeted sampling of sites known to harbour the pathogen. Conversely Malewski's sampling site was already established to have dieback of black alder due to *Phytophthora*, thereby indicating a potentially high load of the pathogen already in the environment. Dadam's paper indicates that *Phytophthora* outbreaks are found "around" the ringing sites, but the scale and proximity to these sites is not clear.

Of the other results which focussed on birds as vectors, the older papers seemed to implement more of an experimental methodology. Keast & Walsh, (1979) examined the ability of *Phytophthora* to be retrieved in the faeces of birds fed on mealworms (*Tenebrio* spp.) which had been injected with *Phytophthora* chlamydospores. The sample size for this paper was just two birds which were housed in an aviary. Although a useful experiment to demonstrate that the pathogen is indeed able to survive through a bird's gastrointestinal tract, this may have been due to the concentration of chlamydospores injected into the mealworms. What is not clear from the Keast & Walsh's paper is how accurately the dose of chlamydospores they injected into the mealworms reflects concentrations found in nature. Is it likely that a bird would ingest these chlamydospores in similar concentrations in the wild, and would this concentration be enough to cause an infection in the plants? Molecular methods such as Sanger sequencing were not available at the time of Keast & Walsh's study, therefore they relied on culture-based methods to retrieve and detect the spores morphologically. This again presents a bias in the methods as the spores were given up to 24 days to grow from the retrieved species, and the authors had no way

of determining the quantity or concentration of spores that survived through the birds' gastrointestinal tract before being grown.

Moving away from *Phytophthora* and oomycetes as the plant pathogen of interest, the second most common form of pathogen studied in relation to birds was fungus. By combining culture based and molecular techniques, Scharf & DePalma, (1981) examined birds and mammals for the presence of the chestnut blight fungus (*Cryphonectria* (*Endothia*) *parasitica*). The relative detection of the fungus from the number of samples was very low, with 153 dead animal specimens being examined following destructive sampling, and only four animals being identified as carrying the fungus - two mammals and two birds (a cedar waxwing and a treecreeper).

Subsequent papers examining what role birds play in the spread of fungal plant pathogens seemed to focus on isolated field observations rather than experimental sampling. Lara & Ornelas, (2003) observed *Moussonia deppeana* plants which were infected with *Fusarium moniliform,* a fungal plant pathogen. Field observations and experiments were carried out, determining the potential for hummingbirds to transmit this pathogen between flowers of different plants, however there was no direct sampling of the birds. Hummingbirds did show a foraging preference by visiting more flowers per foraging bout on plants with infected flowers than on healthy plants, with infected plants retaining their flowers for longer than uninfected plants. Kusunoki et al. (1997), documented the discovery of four birds nests which had been constructed with branches infected with thread blight fungus (*Cylindrobasidium argenteum*). In one of the cases the tree which contained the nest also was infected with thread blight fungus. Similarly Peters et al., (2012), documented cases where weaverbirds (*Ploceidae* spp.) constructed their nests with rice leaves infected with Rice Yellow Mottle Virus, which resulted in a localised infection of plants in the field surrounding the tree. These examples do show the potential that birds have as vectors of plant pathogens, but this represents somewhat anecdotal evidence with relatively small sample sizes.

Other work, such as the review by Jackson & Jackson, (2004), points out the relationship between birds and microorganisms such as fungi, which have the potential to act as plant pathogens, including the ability of birds to vector these microorganisms. Jackson & Jackson, (2004), highlighted the ecological association between woodpeckers (*Picidae* spp.) and red heart fungus (*Stereum (Haematostereum) sanguinolentum*), a plant pathogen of trees, and speculated the

potential for woodpeckers to act as vectors for the fungus. Although the authors did not point to direct evidence of woodpeckers acting as plant pathogens, they pointed towards the potential ways woodpeckers could act as vectors for plant pathogens. As woodpeckers excavate cavities in decaying and softened wood, which has often been degraded by wood-decaying fungi, it would be reasonable to expect that the woodpeckers would come into direct contact with the fungi during the creation of their cavities, and thereby transmit the fungus to further trees that they visit. However, as the usual route of dispersal of this fungus is by wind, the authors did speculate that any dispersal carried out by woodpecker vectors could be insignificant due to the narrow and direct route of transmission it would offer. The only way to ascertain a direct link between woodpeckers as vectors of the fungus is to sample from them in the field. Farris et al. (2004) caught woodpeckers in mist nets and swabbed their bills to determine the presence of fungi. They found that woodpeckers carry wood-inhabiting fungi at a greater frequency than would be anticipated by chance, with the highest rates of fungal, yeast and bacterial occurrence being from cavity nesting woodpeckers, indicating some association between the act of nesting in a cavity and carrying fungus. There was a two-way interaction determined at their study site, with the highest levels of wood-inhabiting fungi being isolated from areas of forest which had the highest levels of sapwood decay. This almost suggests a mutualistic relationship between the woodpeckers and the fungi, with higher levels of fungal transmission increasing rates of sapwood (Bashan, 1986) the woodpeckers. Farris et al. (2004), were able to recover fungal species of the genera *Alternaria* and *Fusarium* from woodpeckers sampled. These represent two fungal genera which contain species well known as fungal pathogens, with all species in *Alternaria* identified as major plant pathogens. As identification in their study was carried out morphologically and not by DNA, the fungi were only identified to genus level not species, therefore it cannot accurately be determined that they isolated plant pathogens from woodpeckers bills, as not all species within *Fusarium* are plant pathogens.

Farris et al. (2004), not only isolated fungi from woodpecker bills, but also bacteria. Bacteria include a range of plant pathogens (Mansfield et al., 2012), but as indicated in Table 5 only one paper (Bashan, 1986) has established a link between plant pathogenic bacteria and birds as vectors. Bashan (1986) investigated bacterial transmission of *Pseudomonas syringae* and *Xanthomonas campestris*, alongside the fungus *Alternaria macrospora*. These three pathogens infect a range of plants including economically and agriculturally important crops. Animals, including birds, were trapped opportunistically using bait boxes and samples were taken, in the

case of birds from their feathers. Already this methodology differs from Farris et al.'s (2004) work on woodpeckers, which specifically targeted species for their association with the plant disease of interest. Bashan found bacterial pathogens were only in very low quantities from birds, which were only detected following bacterial enrichment, calling into question the ability for the pathogenic load being vectored by the birds to actually cause plant disease. The birds in this study were also trapped opportunistically and were common bulbul (*Pycnonotus barbatus*), hooded crow (*Corvus cornix*), and starling (*Sturnus vulgaris*) (sample size of individual birds is not clear). Although these birds are common in agricultural fields (Gregory & Marchant, 1996), their association with crops is not as strong as other bird-plant relationships, such as the woodpeckers discussed earlier. By directly targeting birds that have strong relationships with the plants associated with these pathogens, a clearer relationship can be established between vector and plant pathogens. Research into the role of birds as vectors of human pathogenic bacteria has found variations in how long they can survive within bird faeces, and also within the wider environment, depending on the species of bacteria (Smith et al., 2020), therefore it is possible that bacterial pathogens recovered during sampling may not be able to survive in the environment long enough to cause plant disease.

The final, and most recent paper to explore the role of birds as vectors of plant pathogens, focussed on a protist plant pathogen (Menning et al., 2020). By moving away from culturable microorganisms and using molecular methods, a more detailed picture of the role vectors of plant pathogens can be understood. DNA of the protist *Labyrinthula zosterae,* a pathogen of eelgrass, was found in cloacal samples from a range of migratory waterfowl that were collected opportunistically from specimens shot by sport hunters. Of course, recovery of DNA from cloacal samples does not mean that the protist can act as a viable pathogen but does demonstrate the ability of this organism to be carried by a range of waterfowl species.

Overall, the research into the role of birds as vectors of plant pathogens has involved sampling methods which do not necessarily reflect real world conditions and the routes plant pathogens would take if being vectored by birds. Techniques are improving, and the critical review of the papers above has demonstrated that a combination of molecular and morphological identification of pathogens, along with appropriate experimental design, are needed to fully understand the role birds play as vectors of plant pathogens.

To conclude that birds can act as vectors of plant pathogens, studies should contain the following:

1) Pathogens are recovered and identified from wild populations of birds, not birds in artificial settings
2) Recovered stages of the pathogen are infectious and viable
3) Pathogens recovered are sufficient to cause infection (this will differ depending on the pathogen)
4) To conclude that a bird is a plant pathogen vector, more than one occurrence of the pathogen being associated is required, i.e. not just one anecdotal occurrence
5) Transmission can be either biologically, carrying the pathogen internally, or mechanically transmitting pathogens externally through physical contact

2.6.2 - Other vectors of plant pathogens

The results from this literature review confirm that the majority of current research on plant > plant pathogen > vector interactions focus on crops and plants of economic value. The overwhelming majority of research is carried out on systems involving insect vectors and plant viruses, and this is still clear when individual plant > plant pathogen > vector interactions are categorised. When removing agricultural crops, we could still see that most interactions concerned the class Insecta as a vector, and within these Hemiptera were the most common order. The only change was that the dominant pathogen type of interest was fungus followed by bacteria, but this is more of an indication that viruses are likely more common as plant pathogens of crops. What is not clear is whether the research focuses on these systems due to their economic importance and value to humans, or whether hemipteran vectors and viruses are simply the most abundant plant pathogen vector interactions.

When measured in terms of publications (Fig. 8) we can see that there has been an increase in research into all plant pathogen systems in recent decades, but research into crops especially is growing the fastest. Since 2010 the research into tree pathogens has started to increase at a faster rate, potentially indicating a more recent shift in research focus from just crops being affected by plant diseases, to looking at the whole ecosystem or habitat pathology, for example forest pathology. This could be due to increases in funding in these areas, or these systems becoming easier to study due to technological advances. There is the potential for this trend to

continue, or the rate of research on trees and other plants to even increase more steeply as it has done with crops as more papers will inevitably lead to an increase in interest in this field.

This review represents the first comprehensive literature review examining all plant pathogen - vector interactions not just those deemed to be of economic importance. Research into the vectors of pathogens of crops make up two thirds (66%) of the papers on plant pathogen vectors and, with all other plants making up the remaining third of the research. There is a good base of evidence for the potential birds and other animals could play as vectors of plant pathogens in ecosystems not dominated by crops (woodlands for example), and this field should be expanded upon and explored in further detail. As climate change has the potential to disrupt species ranges, and incidences of invasive alien species are increasing (Roy et al., 2017), there is the potential for novel pathogens to invade and become problems, forming even more plant > pathogen > vector systems. By studying a wider range of pathosystems we can understand the complexity of plant pathogen and vector interactions more clearly, which will prepare us for any potential shifts in research due to new and emerging plant diseases.

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2.8 - Supplementary material

Supplementary table 2.1 - Unique plant > pathogen > vector systems categorised from papers recovered in the scoping literature review

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Alfalfa	Fungus	Insecta	Crop	Harper et al., (1984)
Alfalfa	Virus	Insecta	Crop	Ryckebusch et al., (2021) Li et al., (2021) Ryckebusch et al., (2020)
Almond	Bacteria	Insecta	Crop	Daane et al., (2011)
Apple	Bacteria	Insecta	Crop	Candian et al., (2020) Oppedisano et al., (2020) Miñarro et al., (2016)
Arabidopsis thaliana	Virus	Insecta	Flowers	Vega-Arreguín et al., (2007)
Artichoke	Virus	Enoplea	Crop	Brown et al., (1997)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Asian wild rice	Virus	Insecta	Crop	Yan et al., (2023)
Aster yellows	Fungus	Aves	Flowers	Frost et al., (2011)
Avocado	Fungus	Insecta	Crop	Cruz et al., (2019) Menocal et al., (2018) Na et al., (2018)
Banana	Virus	Insecta	Crop	Safari Murhububa et al., (2021) Bressan et al., (2011) Anhalt et al., (2008)
Barley	Virus	Arachnida	Crop	Robertson et al., (1988)
Barley	Virus	Insecta	Crop	Grauby et al., (2022). Cao et al., (2018) Bencharki et al., (2000)
Bean	Virus	Insecta	Crop	Hampton et al., (2005)
Beets	Virus	Insecta	Crop	Brault et al., (1995)
Betelvine	Virus	Insecta	Crop	Lockhart et al., (1997)
Black Pepper	Virus	Insecta	Crop	Lockhart et al., (1997)
Blueberry	Fungus	Insecta	Crop	McArt et al., (2016)
Blueberry	Virus	Insecta	Crop	Bristow et al., (1999)
Broad Bean	Virus	Insecta	Crop	Hodge et al., (2010)
Broad Leaved Trees	Fungus	Aves	Tree	Kusunoki et al., (1997)
Butternut	Bacteria	Insecta	Crop	Stewart et al.,(2004)
Cabbage	Fungus	Insecta	Crop	Dillard et al., (1998)
Cacao	Virus	Insecta	Crop	Wetten et al., (2016)
Cantaloupe	Virus	Insecta	Crop	Carrière et al., (2014) Castle et al., (2017)
Cardamom	Virus	Insecta	Crop	Ghosh et al., (2016)
Carnations	Fungus	Insecta	Flower	Bruns, et al (2019)
Carrot	Bacteria	Insecta	Crop	Keshet-Sitton et al., (2022) Stillson et al., (2020a) Stillson et al., (2020b)
Cassava	Fungus	Insecta	Crop	Fokunang et al., (2000)
Cassava	Virus	Insecta	Crop	Ateka et al., (2017) Jeremiah et al.,(2015) Legg et al., (2014)
Cauliflower	Virus	Insecta	Crop	Palacios et al.,(2002)
Celery	Bacteria	Insecta	Crop	Stillson et al., (2020)
Cereal Crops	Virus	Insecta	Crop	Liu et al., (2018) Manurung et al., (2004)
Chestnut	Fungus	Arachnida	Tree	Simoni et al., (2014)
Chestnut	Fungus	Aves	Tree	Scharf & DePalma (1981)
Chestnut	Fungus	Insecta	Tree	Pakaluk & Anagnostakis (1997)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Chestnut	Fungus	Mammalia	Tree	Scharf & DePalma (1981)
Chilli	Virus	Insecta	Crop	Thesnim et al., (2023) Chakraborty & Ghosh (2022)
Chrysanthemum	Bacteria	Insecta	Flowers	Galetto et al., (2021) Galetto et al., (2018)
Chrysanthemum	Virus	Insecta	Flowers	Nagata & Avila (2000)
Circubit	Bacteria	Insecta	Crop	Shapiro et al., (2014)
Citrus	Virus	Arachnida	Crop	Ferreira et al., (2020) Nunes et al., (2020) Tassi et al., (2017)
Citrus	Bacteria	Insecta	Crop	Hosseinzadeh & Heck (2023) Jiang et al., (2023)
Citrus	Virus	Insecta	Crop	Wu et al., (2021) Shilts et al., (2020) Liu et al., (2019)
Clover	Virus	Insecta	Flowers	Black (1950)
Cocoa	Bacteria	Insecta	Crop	Gassa et al., (2022)
Coconut	Bacteria	Insecta	Crop	Silva et al., (2018)
Coffee	Virus	Arachnida	Crop	Chagas et al., (2003)
Coffee	Bacteria	Insecta	Crop	Silva et al., (2007)
Conifers	Fungus	Insecta	Tree	Whitehill et al., (2014)
Corn	Virus	Arachnida	Crop	Skare et al., (2003)
Corn	Bacteria	Insecta	Crop	Mahmood et al., (1998)
Corn	Virus	Insecta	Crop	Mwando et al., (2018) Barandoc-Alviar et al., (2016) Chen et al., (2015)
Corn salad	Fungus	Insecta	Crop	Stanghellini et al., (1999)
Cotton	Bacteria	Insecta	Crop	Glover et al., (2020) Medrano et al., (2020) Gino Medrano et al., (2009)
Cotton	Virus	Insecta	Crop	Naqvi et al., (2019) Pan et al., (2018) Michelotto et al., (2006)
Cowpea	Virus	Insecta	Crop	Mello et al., (2010)
Cranberries	Bacteria	Insecta	Crop	Pradit et al., (2020)
Creeping Thistle	Fungus	Insecta	Flowers	Kruess (2002) Friedli & Bacher (2001)
Cucumber	Viroid	Insecta	Crop	Walia et al., (2015)
Cucurbits	Bacteria	Arachnida	Crop	Choi et al., (2016)
Cucurbits	Bacteria	Insecta	Crop	Choi et al., (2016) Sasu et al., (2010) Mitchell & Hanks (2009)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Cucurbits	Virus	Insecta	Crop	Schoeny et al., (2020) Lu et al., (2019) Carrière et al., (2017)
Cypress Tree	Fungus	Insecta	Tree	Zocca et al., (2008)
Eastern Black Walnut	Fungus	Insecta	Tree	Juzwik et al., (2021) Mayfield et al., (2014)
Eelgrass	Protist	Aves	Other	Menning, et al (2020).
Elm	Fungus	Insecta	Tree	Moser et al., (2010) McLeod et al., (2005) Prell (1930)
Elm	Bacteria	Insecta	Tree	Rosa et al., (2014) Pooler et al., (1997)
Fig	Fungus	Insecta	Tree	Jiang et al., (2022)
Flowers	Fungus	Insecta	Flowers	Bruns et al., (2017)
Grains	Fungus	Insecta	Crop	Guo et al., (2018)
Grains	Virus	Insecta	Crop	Yang et al., (2022)
Grapevine	Virus	Arachnida	Crop	Morán et al., (2018). Malagnini et al., (2016)
Grapevine	Fungus	Arachnida	Crop	Moyo et al., (2014)
Grapevine	Bacteria	Insecta	Crop	Rodrigues et al., (2023) Lago et al., (2021) Sisterson et al., (2020)
Grapevine	Virus	Insecta	Crop	Hoyle et al., (2022) Uhls et al., (2021) Prator et al., (2020)
Grapevine	Virus	Enoplea	Crop	Garcia et al., (2019) Van Ghelder et al., (2015) Andret-Link et al., (2004)
Grasses	Virus	Insecta	Other	Ren et al., (2014)
Grasses	Fungus	Insecta	Other	Feldman et al., (2008)
Hardwood Trees	Fungus	Insecta	Tree	Linnakoski et al., (2018) Mayorquin et al., (2018)
Horseradish	Bacteria	Insecta	Crop	Yokomi et al., (2020)
Kiwifruit	Bacteria	Insecta	Crop	Pattemore et al., (2014)
Laurels	Fungus	Insecta	Tree	Zhou et al., (2018)
Leek	Virus	Insecta	Crop	Chatzivassiliou et al., (1999)
Lettuce	Virus	Insecta	Crop	Chen et al., (2012) Ng et al., (2011)
Lime	Bacteria	Insecta	Crop	Queiroz et al., (2016) Salehi et al., (2007)
Lodgepole pine	Fungus	Insecta	Crop	DiGuistini et al., (2007)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Maize	Fungus	Insecta	Crop	Feng et al., (2023)
Maize	Bacteria	Insecta	Crop	García González et al., (2016) Mattio et al., (2015)
Maize	Virus	Insecta	Crop	Vilanova et al., (2022) Xu et al., (2021) Moeini et al., (2020)
Mango	Fungus	Arachnida	Crop	Gamliel-Atinsky et al., (2009)
Mango	Fungus	Insecta	Crop	Galdino et al., (2017) Mattio et al., (2015)
Melon	Virus	Insecta	Crop	Castle et al., (2017) Carrière et al., (2014) Kassem et al., (2013)
Mongolia Oak	Fungus	Insecta	Tree	Lee et al., (2011)
Monocot Plants	Virus	Insecta	Other	Erickson et al., (2023)
Montpellier cistus	Bacteria	Insecta	Flowers	Cruaud et al., (2018)
Moussonia deppeana	Fungus	Aves	Flowers	Lara & Ornelas (2003).
Mulberry	Virus	Insecta	Crop	Lu et al., (2022)
Napier Grass	Bacteria	Insecta	Other	Obura et al., (2009)
New Zealand Flax	Bacteria	Insecta	Other	Liefting et al., (1997).
Nightshades	Bacteria	Insecta	Crop	Bourdin et al., (1998)
Nightshades	Virus	Insecta	Crop	Sengoda et al., (2014)
Nutgall Tree	Bacteria	Insecta	Tree	Tanaka et al., (2000)
Oak	Fungus	Insecta	Tree	Jagemann et al., (2018) Suh et al., (2011) Hayslett et al., (2008)
Oak	Bacteria	Insecta	Tree	Suh et al., (2013) Zhang et al., (2011)
Oat	Virus	Insecta	Crop	Torrance (1987)
Okra	Virus	Insecta	Crop	Venkataravanappa et al., (2023) Fargette et al., (1993)
Olive	Bacteria	Insecta	Tree	Formisano et al., (2022) Picciotti et al., (2021) Lago et al., (2021)
Onion	Bacteria	Insecta	Crop	Stumpf et al., (2021) Koinuma et al., (2020) Grode (2019)
Onion	Virus	Insecta	Crop	Muvea et al., (2018) Bag et al., (2014) Srinivasan (2012)
Palm	Bacteria	Insecta	Tree	Mou et al., (2022)
Papaya	Virus	Insecta	Crop	Gadhave et al., (2019) Angelella et al., (2016)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Paspalum grasses	Fungus	Insecta	Other	Feldman et al., (2008)
Passionfruit	Virus	Arachnida	Crop	Kitajima et al., (2003)
Pea	Virus	Insecta	Crop	Clark & Crowder (2021) Clark et al., (2019) Chisholm et al., (2019)
Peach	Virus	Insecta	Tree	Jensen (1959)
Peach	Bacteria	Insecta	Tree	Sabaté et al., (2018) Blomquist & Kirkpatrick (2002)
Peanut	Virus	Insecta	Crop	Arthurs (2018) Fletcher et al., (2016) Pappu et al., (1998)
Pear	Bacteria	Insecta	Tree	Ordax et al., (2015)
Pecan	Bacteria	Insecta	Crop	Sanderlin & Melanson (2010)
Pepper	Bacteria	Insecta	Crop	Swisher et al., (2018)
Pepper	Virus	Insecta	Crop	Ghosh et al., (2019) Widana Gamage et al., (2018) Musser et al., (2014)
Periwinkles	Bacteria	Insecta	Flowers	Chuche et al., (2016) Boutareaud et al., (2004) Fos et al., (1986)
Pine	Fungus	Insecta	Tree	McAllister et al., (2018) McCarthy et al., (2013) Wang et al., (2013)
Pine	Nematode	Insecta	Tree	Chen et al., (2020) Zhang et al., (2017) Alves et al., (2016)
Pine	Bacteria	Insecta	Tree	Ivanauskas et al., (2022)
Pine	Fungus	Mammalia	Tree	Eckhardt et al., (2016)
Pineapple	Virus	Insecta	Crop	Sether et al., (1998)
Plum	Virus	Insecta	Crop	Olmos et al., (2005) Wallis et al., (2005) Isac et al., (1998)
Potato	Bacteria	Insecta	Crop	Dahan et al., (2022) Prager et al., (2022) Reyes et al., (2021)
Potato	Virus	Insecta	Crop	Gamarra et al., (2020) Patton et al., (2020) Khelifa (2019)
Potato	Virus	Enoplea	Crop	Kozyreva & Romanenko (2008)
Prunus	Bacteria	Insecta	Other	Gallinger et al., (2020) Sengoda et al., (2014) Thébaud et al., (2009)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
Pumpkin	Virus	Insecta	Crop	Mauck et al., (2010) Labonne et al., (1992)
Quince	Fungus	Insecta	Crop	Mohammadi & Sharifi (2016)
Radish	Fungus	Clitellata	Crop	Toyota & Kimura (1994)
Raspberry	Virus	Insecta	Crop	McMenemy et al., (2012)
Red Bay	Fungus	Insecta	Tree	Hughes et al., (2017)
Rice	Virus	Aves	Crop	Peters et al., (2012)
Rice	Bacteria	Insecta	Crop	Sun et al., (2016)
Rice	Virus	Insecta	Crop	Zhang et al., (2023) Chang et al., (2023) Kil & Kim (2023)
Scots Pine	Fungus	Insecta	Tree	Davydenko et al., (2014)
Solanaceous crops	Bacteria	Insecta	Crop	Workneh et al., (2018) Ibanez et al., (2014)
Sow thistle	Virus	Insecta	Flowers	O'loughlin & Chambers (1967)
Soybean	Virus	Insecta	Crop	Hameed et al., (2022) Keough et al., (2018) Smith et al., (2017)
Spruce	Fungus	Insecta	Tree	Man (1999) Krokene & Solheim (1998)
Squash	Bacteria	Insecta	Crop	Wayadande et al., (2005)
Squash	Virus	Insecta	Crop	Venkataravanappa et al., (2023) Mauck et al., (2014) Zouba et al., (1998)
Strawberry	Bacteria	Insecta	Crop	Dittmer et al., (2021)
Strawberry	Virus	Insecta	Crop	Lavandero et al., (2012)
String Beans	Virus	Aves	Crop	Peters et al., (2012)
Sugar Beet	Bacteria	Insecta	Crop	Kosovac et al., (2023) Behrmann et al., (2023) Bressan et al., (2011)
Sugar Beet	Virus	Insecta	Crop	Stafford et al., (2009) Dusi et al., (2000) Dusi & Peters (1999)
Sugarcane	Bacteria	Insecta	Crop	Roddee et al., (2021) Bressan et al., (2009) Roddee et al., (2017)
Sugarcane	Virus	Insecta	Crop	Ridley et al., (2008)
Sweet Cherry	Virus	Insecta	Tree	Eastwell & Bernardy (2001)
Sycamore	Fungus	Insecta	Tree	Li et al., (2020)
Takamaka trees	Fungus	Insecta	Tree	Wainhouse et al., (1998)
Thale cress	Bacteria	Secernentea	Other	Karimi et al., (2000)
Tobacco	Virus	Insecta	Crop	He et al., (2015)

Plant	Pathogen	Vector Class	Plant Type	References (3 examples)
				Krenz et al., (2015)
Tobacco	Virus	Enoplea	Crop	Wang & Gererich (1998)
Tomato	Bacteria	Insecta	Crop	Hansen et al., (2008) Akhtar et al., (2018)
Tomato	Virus	Insecta	Crop	Venkataravanappa et al., (2023) McLaughlin et al., (2022) Wang et al., (2022)
Turnip	Virus	Insecta	Crop	Wang et al., (1998)
Urdbean	Virus	Insecta	Crop	Gautam et al., (2016)
Walnut	Fungus	Insecta	Tree	Chahal et al., (2019) Oren et al., (2018) Rugman-Jones et al., (2015)
Watermelon	Virus	Insecta	Crop	Mou et al., (2021) Ghosh et al., (2019) Shrestha et al., (2019)
Wheat	Virus	Arachnida	Crop	Skare et al., (2003) Mahmood et al., (1998) Chen et al., (1998)
Wheat	Bacteria	Insecta	Crop	Mattio et al., (2015)
Wheat	Virus	Insecta	Crop	Hao et al., (2021) Du et al., (2020) Wang et al., (2019)
Zucchini	Virus	Insecta	Crop	Rodríguez et al., (2019) Hadi et al., (2011)

Chapter 3 - The role of woodland birds as vectors of bacteria associated with Acute Oak Decline

3.1 - Abstract

Acute Oak Decline (AOD) is a disease of oak trees which results in rapid decline of tree health in only a few years following infection. The disease symptoms are thought to be caused by three species of pathogenic bacteria; *Brenneria goodwinii, Gibbsiella quercinecans,* and *Rahnella victoriana.* The mechanism behind the spread of these bacteria and AOD is not yet fully understood. These three bacterial species are members of Enterobacteriaceae, a family of bacteria which contains a large proportion of gut associated bacteria, therefore I hypothesised that these bacteria could be spread between trees in the gut of warm-blooded animals. Birds that associate with oak trees were identified as potential vectors, due to the large proportions of Enterobacteriaceae within their guts, and their ability to travel quickly and easily between oak trees therefore giving a potential pathway for the spread of the bacteria. Great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) were used in this study due to their close association to oak for feeding and breeding, their abundance in UK oak dominated woodland, and their ability to take to artificial nest boxes, allowing us to study both adults and nestlings. Body swabs and faecal samples were taken from both adult (n=60) and nestling (n=141) blue tits and great tits across woodland patches with differing levels of AOD. Samples were cultured under conditions that are known to promote the growth of the three AOD associated bacteria, and any bacteria grown were identified to species level using Sanger sequencing. None of the bacteria sequenced were identified as *Brenneria goodwinii* or *Gibbsiella quercinecans,* and three samples were identified as *Rahnella victoriana.* This study indicates that birds have the ability to transmit some bacteria associated with AOD, but not all AOD associated bacteria were present in the samples, at least, in a culturable state. Further analysis of the samples using molecular methods such as Next Generation Sequencing should be used to determine if the bacteria are present in the samples in an unculturable state.

Keywords; Acute Oak Decline, bird vectors, Sanger sequencing, oak woodland.

3.2 - Introduction

Research into the diseases of trees has grown in the past decade, as detailed in section 2.4.3 of this thesis, however the mechanisms behind the spread of many tree diseases remain poorly

understood. Plant diseases are often the result of a variety of factors working in combination to weaken plants through predisposing and inciting factors (Table 1), which weaken the plant enough for contributing factors such as pathogens to cause diseases and death to the plants.

Table 1. Factors influencing plant mortality. Taken from the "plant death spiral" coined by (Manion, 1991)

Predisposing Factors	Inciting Factors
Urbanisation	Defoliating insects
Genetics	Excavation
Poor fertility	Drought
Climatic shifts	Excessive salt
Salt levels	Frost
Age	Air pollution
Soil compaction	
Soil moisture holding capacity	
Poor soil drainage	

Contributing factors to plant disease and death can be caused by different types of pathogens, most commonly viral and bacterial, as indicated in section 2.4.2 of this thesis. Differing pathogen forms will utilise different dispersal mechanisms, for example fungal and oomycete pathogens can use passive environmental dispersal, such as via wind and rain, and these dispersal methods can also vary according to the pathogen's life cycle stage. Phytophthora is a common plant pathogen, which is known for having different dispersal methods depending on the stage of its life cycle (Campbell, 1999). Looking at tree diseases, ash dieback is one example of a disease caused by an ascomycete fungal pathogen - *Hymenoscyphus fraxineus.* This disease has been affecting ash trees in Europe since the early 1990s and was first documented in Britain around 2012 (McMullan et al., 2018). Ash dieback results in necrotic lesions on the leaves, twigs and stems of infected ash trees (Gross et al., 2012, 2014). This pathogen is wind borne, passively

dispersing using ascospores (Timmermann et al., 2011), whereas other tree pathogens such as bacteria are more reliant on direct vectoring.

Declines in tree health can be complex to define as they often have multiple biotic and abiotic causes and varying symptoms. The decline of oak trees in the UK and Europe has been documented since the early 1900s, with syndromes such as Chronic Oak Decline (COD) weakening trees over several decades and leading to death either directly through progressive deterioration of the canopy, or by making the trees susceptible to other secondary factors such as insect damage which leads to weakening and death (Gagen et al., 2019). This has caused large-scale decline and dieback of oak populations across Europe (Denman et al., 2014). Chronic oak decline results in deterioration of the diseased tree over several decades whereas acute oak decline represents a comparatively novel and relatively fast-acting disease, with symptoms first documented in the UK during the 1980s (Gibbs & Greig, 1997). AOD is a bacterial disease of trees that affects the two native British species of oak - pedunculate (common) oak (*Quercus robur*) and sessile oak (*Quercus petraea*) (Denman et al., 2014). Although symptoms were first documented on UK oak in the 1980s, AOD was only distinguished from other more established oak diseases in 2009 (Denman & Webber, 2009), and since then much research has been conducted by tree pathologists into the mechanisms behind the spread of this disease. As this disease is caused by three strains of bacteria, understanding the mechanism of spread of these pathogens is more complex than windborne dispersal utilised by the oomycete and fungal pathogens of some other tree diseases, such as ash dieback.

Most of the reported cases of AOD in the UK have been in southern England or in the English Midlands, but since the disease was first documented at a handful of sites in 2006, it has been documented in a much wider range across the country (Fig. 1). Research carried out by (Brown, et al., 2017b) used citizen science data to track the occurrence of symptomatic trees across the UK and estimated that 38% of England and Wales contains woodland vulnerable to AOD. More recent work has linked the presence of AOD in woodlands to other environmental factors such as low levels of rainfall, higher temperatures and lower elevations (Brown et al., 2018), which is useful in two respects. Firstly, it provides a better insight into which environments are more vulnerable to AOD infection, and secondly it highlights an urgency to policy makers to tackle the problem of AOD in the warming climate before an increasing number of sites become vulnerable to infection with the disease.

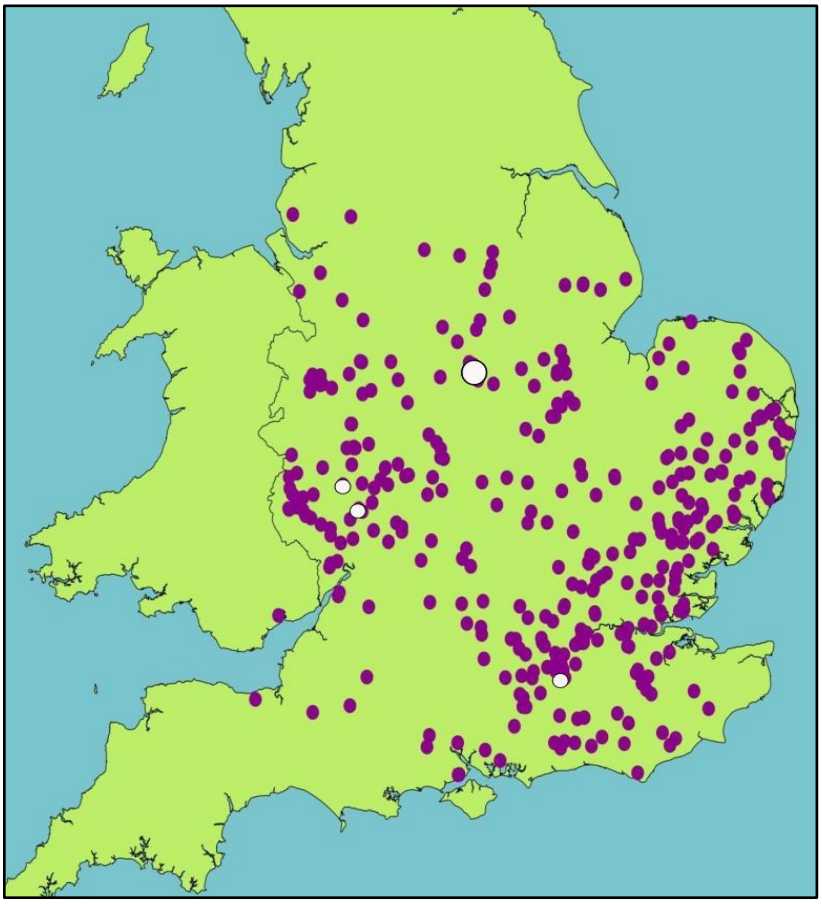


Figure 1. Locations with documented presence of acute oak decline symptoms in the UK between 2006 (white circles) and 2023 (purple circles) - correct as of March 2023 (image adapted from Forestry Commission, 2023).

The presence of AOD in a tree is generally characterised by the following symptoms (Denman et al., 2014):

- vertical weeping or 'bleeding' lesions on the trunk of trees (referred to hereafter as "bleeds";
- black exudate being expelled from cracks in between bark plates
- necrosis of the inner bark layer

AOD symptomatic trees tend to have less canopy foliage, with sparse and thin canopies developing as the tree advances through its infection, alongside a larger proportion of dead or dying branches than asymptomatic trees (Denman, 2010). Trees exhibiting more advanced AOD symptoms and with a greater number of stem bleeds generally have poorer crowns than trees exhibiting mild symptoms (Brown et al., 2016). Symptoms of AOD are generally most visible in

the summer months (Denman et al., 2014), which is also when the extent of a tree's crown foliage could be best assessed as they will be in leaf.

Internal symptoms of AOD can be found under the bark of the tree, and includes bleeds between plates of bark, internal cavities, lesions and beetle larval galleries (Denman et al., 2014) which are not necessarily obvious from external observations of the tree. In the majority of AOD cases, the presence of larval galleries and emergence holes of the oak buprestid beetle (*Agrilus biguttatus*), is associated with stem bleeding (Brown et al., 2017b). This is not surprising as Agrilus species are widely associated with oak trees, with documented occurrences on diseased oaks in the USA and continental Europe (Coleman & Seybold, 2011; Moraal & Hilszczanski, 2000). The presence of *A. biguttatus* larvae in and around symptomatic lesions was initially believed to be another indication of a tree's infection with AOD, however the beetle is not thought to cause AOD directly, but is rather acting opportunistically on trees with weakened immunity, as they serve as easier hosts for providing homes for larvae (Brown et al., 2015; Denman et al., 2014). Table 1 refers to the plant death spiral posed by (Manion, 1991). This model has been updated thirty years on to reflect the current knowledge of the mechanisms behind the causes of disease declines, particularly in reference to tree diseases and declines (Denman et al., 2022). AOD is commonly referred to as a "decline disease" meaning it is the result of a series of factors combining to put pressure on trees over time, making the individual more susceptible to infection with pathogens, rather than being the direct result of that pathogen's infection. Denman's updated version of the disease decline spiral incorporates the importance of microbiomes and microbial imbalances and highlights the importance of bacteria as a plant pathogen, which was overlooked in the original model. It also emphasises the importance of these predisposing and inciting factors working together over time to weaken the tree, making the tree vulnerable to causative agents of disease.

3.2.1 - AOD causative agents

AOD symptoms in the UK are believed to be caused by the interactions of three bacterial strains in the family Enterobacteriaceae: *Gibbsiella quercinecans, Brenneria goodwinii,* and *Rahnella victoriana* (Broberg et al., 2018; Doonan et al., 2019; Kaczmarek et al., 2017). Tissue within the lesions of symptomatic trees have a significantly higher bacterial load than asymptomatic tissue (Denman et al., 2018), with *B. goodwinii* and *G. quercinecans* believed to be the causal agents of tissue deterioration (Broberg et al., 2018). Microbiome studies of AOD lesions from symptomatic

trees have revealed *B. goodwinii* as the dominant bacterium in the AOD bacterial complex, and to a lesser extent *G. quercinecans* and *R. victoriana* (Broberg et al., 2018). The presence of these bacteria within an oak tree acts as a reliable indicator of the extent to which it is affected by AOD. Unfortunately, the most severe symptoms of AOD are only visible at the more advanced stages of infection, with internal symptoms often appearing before the external symptoms such as stem lesions and canopy dieback (Denman et al., 2014). By investigating factors such as root decay as an indication of oak decline (Keča et al., 2016), earlier diagnosis of AOD can be made, although this could involve destructive sampling and would require considerably more resources to identify the quality of each individual tree's roots in an environment dominated by oaks.

AOD is not just a problem in the UK - research has identified these same three bacteria occurring in AOD symptomatic oak trees in Switzerland (Ruffner et al., 2020). Molecular analysis of bacterial communities of symptomatic oak trees in the USA and Spain has identified different species of Enterobacteriaceae to those found in UK trees (Brady et al., 2016), indicating that a range of bacteria can induce the AOD symptoms depending on the environment. AOD mainly impacts trees over 50 years old; however, recent work has found it can affect younger individuals (Denman, 2010; Denman et al., 2014). Experimental work into the causative agents of AOD saw researchers inoculating oak logs with the three AOD associated bacteria, which resulted in development of AOD symptoms (Denman et al., 2018). Similar results have been found by researchers at the University of Reading on oak saplings, indicating that age is not a determining factor in AOD infection (Booth, 2019). Surprisingly there is little in the literature that relates to AOD and tree age. It is possible that increased age may result in a weakened immunity making the tree more susceptible to infection, as has been found in other tree diseases including apple tree valsa canker (Wang et al., 2005) and peach yellow leaf roll (Blomquist & Kirkpatrick, 2002). However, this theory has also been contradicted in other diseases such as ash dieback where younger trees have higher mortality rates (Timmermann et al., 2011). Disease dynamics therefore seem to be specific to the pathogen and its plant hosts, and it is hard to compare between them.

The mechanism by which bacteria are thought to induce the symptoms of AOD has not yet been established (Broberg et al., 2018). Genetic analysis of *B. goodwinii* from seven AOD-affected sites in England did not indicate any geographical patterns of distribution, with genetically similar populations being recovered from similar woodlands in distinct geographical locations (Kaczmarek et al., 2017). The authors suggest that the bacteria could be transported between sites by the movement of infected wood, or by an animal vector, for example, the beetle *A.*

biguttatus was suggested as a possible vector of the bacteria as it enters/exits the tree (Brown et al., 2015). However, no such evidence for this mode of bacterial transfer currently exists.

Recent developments in plant pathology are shifting focus to the plant holobiont and associated hologenome - the host plant and the vast range of microbiota associated with it. This allows focus to be turned to bacteria and fungi which serve functional roles for the host plant, such as stress resistance (Vandenkoornhuyse et al., 2015). Direct studies into the hologenome - the genomes of both the host and its microbial communities - of oak trees inflicted with AOD identified a clear difference between the functional microbiomes in symptomatic and asymptomatic tissue. Further functional bacterial taxa were identified alongside Enterobacteriaceae within symptomatic tissues, namely the genera *Clostridioides* and *Carnobacterium*, which were associated with an increase in virulence activity within the infected lesions (Broberg et al., 2018). This indicated that the microbiomes of symptomatic AOD tissue are more complex than previously thought. Symptomatic tissues demonstrate a shift in the expression of microbial genes away from those associated with general plant metabolism in asymptomatic tissues, to those associated with high levels of virulence and bacterial phytopathogenic activity in symptomatic tissues. Research has also isolated 22 strains of *Pseudomonas* species from the inner bark and sapwood of AOD symptomatic trees (Bueno-Gonzalez et al., 2019). Transcriptomics and proteomics indicated increased expression of virulence activity within the infected lesions, such as the increased production of plant cell wall-degrading enzymes by bacteria (Broberg et al., 2018). Interestingly, metabarcoding studies of AOD symptomatic trees have found the changes in the bacterial community to be characteristic of symptomatic tissue, rather than being a feature of the microbiome of the whole organism (Sapp et al., 2016). This indicates that either the AOD bacteria are present within the trees as part of the natural microbiome of healthy trees, and then become more abundant in symptomatic trees. Another theory is that there could be an external source for these putative pathogens, which will be explored in this chapter.

3.2.2 - Transmission of AOD bacteria

All three putative bacterial pathogens of AOD - *Gibbsiella quercinecans, Brenneria goodwinii,* and *Rahnella victoriana*, are in the family Enterobacteriaceae, which are most often found within the gastrointestinal tract of warm-blooded animals, including birds (Wiley et al., 2017). As such, these bacteria grow and thrive at temperatures between 37 and 42°C, which are generally higher than

those of the surrounding ambient environment. The fact that these enteric bacteria are found to cause tree diseases in UK woodland poses several questions, including whether they grow and thrive at the ambient temperatures at which they are found in the woodland environment, and, if not, how and why do they persist there? These bacteria being found at lower than optimal temperatures will not necessarily lead to their death, moreover they can persist in a dormant state until conditions become optimal for their growth (Price & Sowers, 2004). Consequently, these putative pathogens may be transported between trees within a warm bodied mammal or a bird. Of course, other forms of bacterial transmission are possible, for example through wind or rainwater (Evans et al., 2006) but *G. quercinecans* and *B. goodwinii* are not known to survive in environmental conditions such as rainwater and soil for very long (Pettifor et al., 2020) . Birds are known to act as vectors for human and animal zoonotic bacterial and viral pathogens such as *Vibrio cholerae* and *Mycobacterium tuberculosis* (Tsiodras et al., 2008), which has highlighted their ability to carry and transmit pathogenic bacteria sometimes long distances as is the case with migratory bird vectors.

This study examined whether birds play a role in transmitting the bacteria associated with AOD by examining the different ways in which birds use trees in their ecosystem. Woodland birds use trees for a variety of ecological functions, from great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) preferring to feed and breed on oak trees, to nuthatches (*Sitta* spp.) and woodpeckers (*Picidae* spp.*)* using a variety of tree species for foraging, climbing, roosting and nesting (Nilsson, 1976; Smith, 2007). Great tits and blue tits rely heavily on folivorous moth caterpillars such as the winter moth (*Operophtera brumata*) as a food source during their breeding season, provisioning their young with up to 700 caterpillars each day (Gibb, 1955). The environment surrounding a nest site has a large influence on reproductive success, as observational studies have documented that great tits that use nest boxes surrounded by lower densities of oak have to work harder in order to provide the same quantity of food as birds which are in areas with higher densities of oak which have higher levels of prey availability. These birds in lower density oak woodland therefore expend more energy to rear their young than those pairs surrounded by higher densities of oak (Hinsley et al., 2008). Great tits and blue tits are known to have the highest level of breeding success in mature oak-dominated woodland compared to younger or more fragmented habitats (Hinsley et al., 1999, 2008), making them useful model species with which to examine the multi-trophic effects of woodland tree health and composition.

The implications for birds of breeding in diseased woodlands infected with AOD will be explored in more detail in Chapter 6; however, the interaction between birds and oak trees is important in

studying the hypothesis that birds could be vectors of the bacteria that act as plant pathogens.

The close interaction between birds and oak trees, along with the conditions in which Enterobacteriaceae thrive, all indicate the potential for birds to be vectors of the pathogens responsible for AOD, as has been found for some other plant pathogen systems explored in further detail in Chapter 2 (Malewski et al., 2019; Peters et al., 2012). The extent to which birds may acquire the putative pathogens from the surface of the tree trunk may be negligible, as previous work has demonstrated that higher quantities of bacteria reside in the inner bark than the outer bark (Brady et al., 2017). This, however, is not to say that just because there are greater *quantities* of bacteria in the inner bark, that the bacteria contributing most to AOD are not found on the surface of a tree. Therefore, diseases caused by bacteria that are found on or just below the surface of a tree, have the potential to be spread by those mobile species that frequently visit and use an infected tree.

3.3 - Aims and hypothesis

This chapter aims to investigate the extent to which birds play a role in transmitting the pathogens associated with acute oak decline by sampling two species which are potential candidates as vectors - blue tits and great tits. Samples from oak woodland with differing levels of AOD will be analysed with the aim of detecting the AOD associated bacteria.

I hypothesise that samples from adult and nestling birds in areas with AOD will have a higher incidence of AOD associated bacteria than samples analysed from areas without AOD.

3.4 - Methods

3.4.1 - Fieldwork

3.4.1.1 - Study system

The field site used in this study was Epping Forest (Fig. 2), in Essex, UK (51°64'N, 0°02'W), on the northern edge of the Greater London conurbation. Epping Forest comprises 2,400 ha of woodland and grassland - 1,728 ha of which are designated as Sites of Special Scientific Interest and a Special Area of Conservation (JNCC, 2015). The most abundant type of habitat within Epping Forest is ancient semi-natural woodland, dominated by pedunculate oak (*Quercus robur*) and sessile oak (*Quercus petraea*) alongside common beech (*Fagus sylvatica*), hornbeam (*Carpinus betulus*) and silver birch (*Betula pendula*) (Snow & Medlock, 2008). Blue tits (*Cyanistes*

caeruleus) and great tits (*Parus major*) are common breeding birds in Epping Forest, and are known to have more successful breeding productivity in larger, non-patchy woodlands (Hinsley et al., 1999, 2008), hence the suitability of Epping Forest as a study site. This project collaborated with the Biodiversity team at Epping Forest, who had previously identified symptoms of AOD as part of their long-term monitoring of 600 ancient oak trees (trees over 400 years old), which commenced in 2013. The ancient trees are assessed yearly for signs of disease including symptoms of acute oak decline. Epping Forest is in an area of England that is at a high risk from AOD infection (Fig. 2), there is the potential for this area to become particularly devastated by the disease.

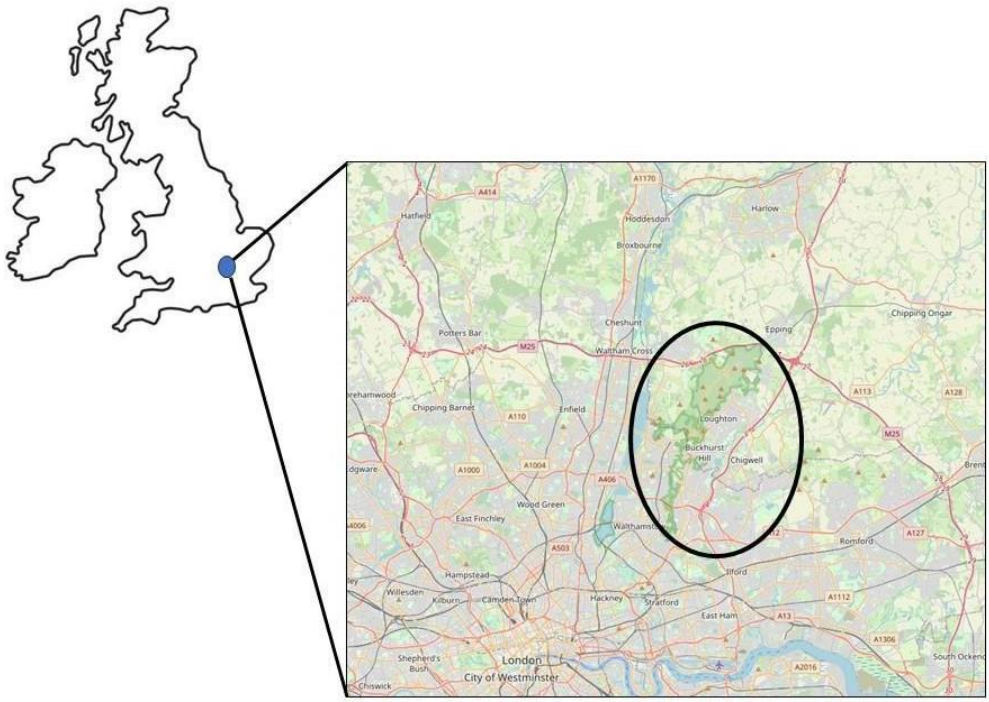


Figure 2. Epping Forest located in relation to the UK and its proximity to London

The primary bird species of interest in this study were great tits and blue tits as they represent ubiquitous, well-studied bird species, which are known to breed well in mature deciduous woodland and have a strong affinity with oak (Hinsley et al., 1999), enabling a simple project design with a high likelihood of good sample sizes. Both bird species readily use nest boxes for breeding, which provides easy access to the nest contents and allows the opportunity to examine the impact of AOD on nestlings, to be examined in detail in Chapter 6.

Overall, 103 nest boxes were installed across the field site between 27th February and 14th March

2020 across 180 hectares of forest (Fig. 3). The nest boxes had 32 mm entrance holes to accommodate both blue and great tits. Great tits have breeding territories that extend to an average radius of approximately 50 m from their nest, although this can extend to 75 m (Hinks *et al*., 2015). Nest boxes were spaced at least 100 m apart to ensure there were no overlapping territories which could influence breeding success. A map of Epping Forest was produced in ArcGIS (v.10.7.1), with particular focus on the area documented as having trees with symptoms of AOD. The area was split into 100x100m cells, with the GPS location of the centroid of each cell being the target point for each nest box. It was not feasible to place each nest box at the centroid of each cell, however, as often this would fall on a path or an open area. When this occurred, the area was searched to identify the nearest suitable target trees. All nest boxes were placed on the trunks of oak trees, at least 2.5 m above the ground and facing away from any public paths or thoroughfares to reduce interference from the public. The aspect in which the nest boxes faced was recorded, as previous studies have found this may have some influence on breeding success (Wilkin et al., 2007).

Figure 3. Locations of nest boxes within Epping Forest. Blue points indicate the nest boxes placed within the forest.

3.4.1.2 - Habitat assessments

It was not possible to assess each nest box tree for the presence of AOD prior to site setup, as boxes were erected during the winter when AOD bleeds are not visible. Habitat and disease assessments were conducted retrospectively in the summers of 2020 and 2021. Due to time constraints, each habitat plot and tree was only surveyed once.

Each oak tree within a 50 m radius of the focal (nest box) tree was recorded, which represented an individual plot. Trees with trunks of >40 cm diameter at breast height (DBH) were assessed for the presence or absence of AOD symptoms, alongside other metrics including height, and canopy density (Table 2). Tree height was measured from the ground using a rangefinder (VYTOOV Laser Rangefinder), and DBH was measured using a Richter Metric Diameter Tape. Each plot was surveyed for the presence of AOD symptoms listed below, and a disease intensity score of each plot was scored from 1-3, where 1 indicated there was no AOD in the plot, 2

indicated moderate severity and 3 advanced. Scoring for AOD severity is listed below, and this was reflected in the AOD scoring of each habitat plot. DBH, height, and canopy density were compared across AOD levels in R using ANOVAs, followed by post-hoc Tukeys tests.

The AOD symptoms surveyed for each tree were: Active stem bleeds, Inactive stem bleeds, *Agrilus biguttatus* emergence holes. Symptoms were determined by visual examination of the stems from the ground, with binoculars used to examine the upper parts of the trunk (>1.5 m) for bleeds. Bleeds on each oak tree were characterised according to the criteria in Table 2, adapted from (Sapp et al., 2016). These criteria were applied to both active and inactive stem bleeds. Active stem bleeds were noted as being wet and "actively" weeping, whereas inactive bleeds represented a stained area of bark where a bleed had once been active but was no longer expelling exudate.

Table 2. AOD symptoms surveyed and the criteria to meet different AOD severity scores.

AOD Symptom	Number of incidences	AOD Severity Classification	AOD Severity Score
Active Stem Bleeds (bleeds actively expelling exudate)	0 bleeds	Healthy (Asymptomatic)	1
	1-10 bleeds	Moderate (early infected)	2
	>10 bleeds	Advanced	3
Inactive Stem Bleeds (old bleeds, not actively expelling exudate)	0 bleeds	Healthy (Asymptomatic)	1
	1-10 bleeds	Moderate (early infected)	2
	>10 bleeds	Advanced	3
Agrilus emergence holes	0 emergence holes	Healthy (Asymptomatic)	1
	1-10 emergence holes	Moderate (early infected)	2
	>10 emergence holes	Advanced	3

The number of *Agrilus biguttatus* emergence holes on each tree were also scored. If there were fewer than 10 emergence holes, then these were individually recorded. Any trees with more than 10 emergence holes were noted as having > 10. The number of bleeds and *Agrilus* emergence holes were combined to create an overall AOD score for each plot - none, moderate and advanced. The density of the canopy of each tree was also scored by visually dividing the canopy into thirds - lower, middle and upper - and scoring the density of each from 0-5 as detailed in

Table 3. This scoring system was created in consultation with the biodiversity team at Epping Forest, and a similar scale has been used in other studies examining the symptoms of AOD (Denman et al., 2014).

Table 3. Tree canopy density scoring system

Density Score	% Live Crown
0	0
1	1-20
2	21-40
3	41-60
4	61-80
5	81-100

Once symptoms outlined in Table 2 were scored for individual trees, the average crown density score was taken across the habitat plot. The classifications of AOD symptoms were combined for each tree in the plot to give a plot AOD severity level, for example where there were multiple trees within a plot that displayed “advanced” symptoms, then the plot was classified as “advanced” for AOD. The proportion of symptomatic trees in each habitat plot was also recorded, along with the number of dead oak trees in the plot.

3.4.1.3 - Sample collection – trees

Between July to September in 2020 and 2021, swab samples were collected both active and inactive bleeds, and from natural bark cracks in asymptomatic control trees. Repeat samples were not taken due to time constraints. A sterile cotton swab (Sterile Applicators, Boettger) was dipped into the buffer phosphate-buffered saline (PBS) solution and then rolled against the uppermost part of the bleed ten times to ensure an even coverage over the swab. The uppermost part of the bleed was swabbed to ensure the highest concentration of bacteria were obtained as possible, as per protocols from other AOD sampling studies (Crampton et al., 2020). Where a tree had multiple bleeds, the bleed closest to head height (approximately 1.6 m above the ground) was sampled due to accessibility and to reduce the likelihood that any bacteria isolated originating from the ground. This process was repeated for the asymptomatic

control oak, where the sample was taken from a random fissure in the bark at approximately head height. The swab was transferred to 1 ml of PBS in a sterile 1.5 ml Eppendorf tube and transported back to the lab at the University of Reading, and stored at 4°C for up to three days prior to plating the samples, the details of which are described below in section 3.4.1.4.

3.4.1.4 - Sample collection - adult birds

Adult birds were sampled by trapping them either in a metal cage trap, or by using mist nets (ranging from 6m-18m in length). Adult sampling took place across the following years and months; 2020 - September and October, 2021- May and December, 2022 - January, June and November, 2023 - March. These timings were chosen due to logistics such as having sufficient time to conduct sampling sessions, and availability of helpers. Cage traps were used for the 2020 sampling sessions due to permit restrictions, and this was changes to mist netting from 2021 onwards. Due to limited resources, birds were only targeted in six plots, three containing oak symptomatic with AOD and three without symptomatic trees. Plots were chosen using a random number generator (calculator.net), ensuring plots were accessible and suitable enough for six mist nests to be erected.

Initial trapping used metal feeder traps (Fig. 5) and later trapping was carried out using mist nets to increase catches. The metal feeder trap operated a trap door system, with a monofilament line attached to a metal pin which held the trap door open until a bird entered the trap to feed. The operator would then quickly pull the monofilament line, lowering the trap door and trapping the bird within. A large, clear plastic bag was placed over the entrance hole and the trap door was propped open. The bird was then encouraged to leave the trap - this worked best by standing to the rear of the trap and tapping on the sides to usher it out of the door. When mist netting, all bird species were extracted from the mist net by hand, and a fresh pair of nitrile gloves were used when extracting target bird species.

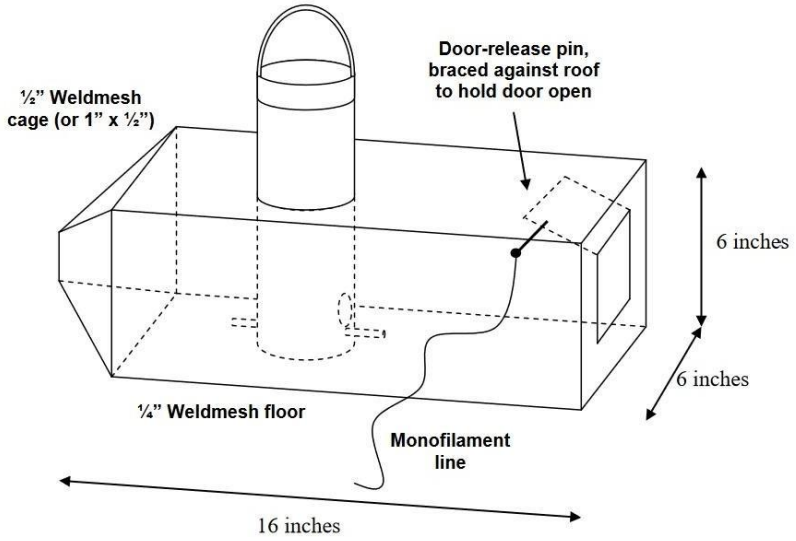
Metal traps allow for targeting of certain individuals, as mist netting can result in many non-target species being caught. The trap or mist net was set up in a suitable area as close to the nest box tree as possible, and always within the experimental plot (i.e. within 50 m of the focal tree). The area was baited with bird feeders containing sunflower seeds and cereals. The traps and feeders were left in situ for at least two weeks prior to sampling to habituate the birds to the equipment. Habituating the birds to the bird feeders was especially important when using the cage trap as birds were especially wary of the equipment to begin with and were not readily exposed to artificial bird feeders. Feeders were refilled at least once per week and monitored for signs of great tits or

blue tits feeding. Once these birds had been seen feeding freely at the feeders, it was replaced with a cage trap (Fig. 4). The habituation process was repeated until the target species had been seen feeding from the feeder within the cage trap. If birds were not observed as visiting the cage trap after 1 month, then it was moved to another area. Where mist nets were used, sampling commenced once birds had been observed visiting the bird feeders, however this habituation process was not as important as the mist nets caught birds flying through the woodland.

After the bird was extracted from the cage trap or mist net it was transferred to a faecal collection bag (Fig. 5). The faecal collection bag consisted of a sterile paper bag where the base was replaced by a sterile PVC viewing window. This minimised the disturbance to the bird when checking if a faecal sample had been produced. The collection bag contained a sterile platform made from wire mesh to ensure that any faecal samples were not contaminated by the bird standing on it.

Each bird was kept in the faecal collection bag for a maximum duration of 15 minutes, after which the bird was removed from the bag to proceed with additional sampling and measurements regardless of whether a faecal sample had been produced. If there was a faecal sample present, this was collected in a 1.5 ml microcentrifuge tube using a sterile cotton swab. The contaminated bag was discarded after each use, and the base and platform were sterilised between each use using 70% ethanol.

Once removed from the bag, the bird was held in the commonly used “ringers grip”, with the neck being held between the middle and index finger in the left hand, as demonstrated in Figure 6. This was done whilst wearing a fresh pair of nitrile gloves to reduce the risk of cross contamination from the hand to the bird. Three swab samples were taken from each bird. A buccal swab was taken using a 2 mm sterile dry cotton swab, rotated gently five times against the bottom of the bird’s buccal cavity (Fig. 7). This method has been successful in retrieving genetic material from birds in the tit family (*Paridae*) (Handel et al., 2006), but this is the first instance where it has been used to retrieve microbial samples.



A

B

Figure 4. A) Schematic of cage trap for catching small passerines, illustrating metal hatched wire surrounding a bird feeder, B) Cage trap in place in the field

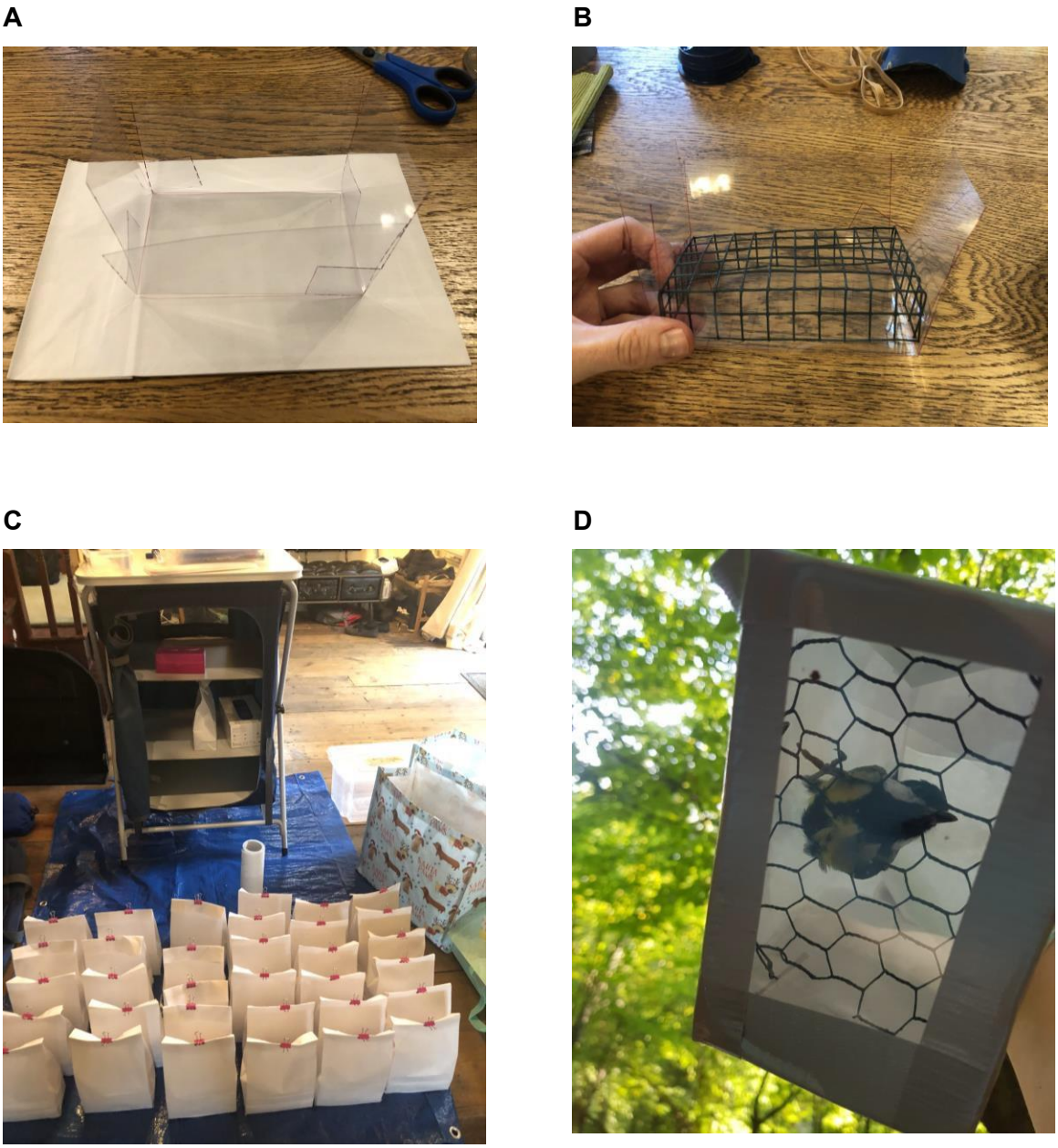


Figure 5. Assembly and use of collection bag used to collect faecal samples from wild birds: A, assembly of PVC base used to replace the base of the paper bag; B, assembly of wire grid used to prevent the bird standing in the faecal sample; C, appearance of fully assembled faecal collection bag; D, bag in use with a great tit inside, with the PVC panel allowing for each viewing to see if a faecal sample had been deposited. (Design and images A-C courtesy of Dr Gabrielle Davidson, University of Cambridge).

Figure 6. Demonstration of the ringer's grip method used to hold small birds whilst processing them, left - great tit (*Parus major*), right - blue tit (*Cyanistes caeruleus*).

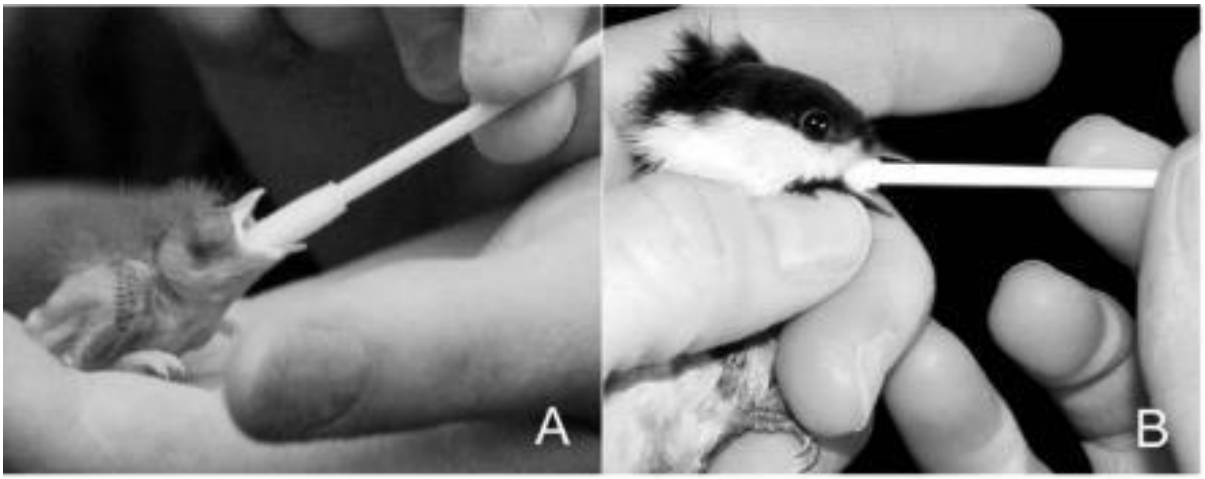


Figure 7. Buccal samples being taken from a black capped chickadee nestling (A) and adult (B) (Figure reproduced with permission from Handel et al., (2006)).

Two samples were taken from the external surface of the bird using sterile 5 mm cotton swabs dipped in PBS. The first swab was rotated around the inner surface of the bird's foot, and the second rotated across the back feathers of the bird. All swab samples were stored in 1 ml PBS and refrigerated for between 0-3 days until being cultured in the laboratory. Each of the four samples serves to determine different modes of transmission that the bird could utilise as a possible vector: faecal samples reflect ingestion and gut survival; buccal samples reflect acquisition via feeding; foot samples reflect transference from the surface of trees; back samples are a control to determine general acquisition from the environment.

After the swabs were collected, each bird was fitted with a BTO (British Trust for Ornithology) metal ring with an individual identifying number and the following biometric data was collected:

species; age (adult or first-year); sex; moult stage; wing length (mm); weight (g). Wing length was measured using a metal wing rule accurate to 1mm, and weight was measured using a portable digital scale accurate to .01g, and recorded to the nearest 0.1g. Both blue tits and great tirs were aged by examining their plumage, and great tits were sexed using this method (Demongin, 2016). Outside of the breeding season it is difficult to sex blue tits as sex specific characteristics overlap, therefore they were not sexed.

A new sampling site was chosen once at least five birds had been sampled from the previous site, or after two unsuccessful catching sessions (at least four hours total attempt).

3.4.1.5 - Sample collection – nestlings

Nest boxes in each plot were monitored during the springs of 2020-2023. Swab samples were taken from nestlings in 2021 and 2022, with faecal samples being taken from nestlings from 2021-2023. Nestlings were assessed and sampled at 11 days old and the same four samples were taken as from adults. Nestlings were handled using sterile nitrile gloves, above a sterile plastic sheet, allowing any opportunistic faecal samples to be collected. Due to the size and vulnerability of nestlings, the faecal collection bag could not be used, therefore any faecal deposited on the sterile plastic sheet were collected into microcentrifuge tubes. Unlike adults, blue tit and great tit nestlings excrete faecal sacs in which the faeces is contained within a clear or white membrane (Ibáñez-Álamo et al., 2017), which aided with faecal collection.

Due to the limited time to process samples during breeding season, only one nestling per nest box was swabbed, however where possible multiple faecal samples were collected. Nestlings within the same brood were not treated as individuals for sampling purposes, as they were assumed to have similar microbial communities within a nest (Lucas & Heeb, 2005). As with the

adults, each chick was weighed and their primary wing-feather length was measured as an index of development, and they were fitted with a metal BTO ring. Nestling monitoring methodology is discussed in further detail in Chapter 6.

3.4.2 - Laboratory Work

3.4.2.1 - Selection for AOD associated bacterial strains

Three of the bacterial strains known to be associated with acute oak decline were put through selection tests prior to any samples being collected. These three initial strains were *Brenneria goodwinii, Gibbsiella quercinecans,* and *Rahnella victoriana,* and samples of these were available at the University of Reading, courtesy of PhD researcher Oliver Booth. These bacteria were grown on Luria Agar (LA) (supplementary material 3.1) at 27°C for 24 hours and their colony morphologies were recorded. Further tolerance tests were used to develop a thorough selection process for field samples.

A thermal tolerance test was conducted where all three strains were incubated for 24h at 27°C, 37°C, 40°C and 42°C. Being members of the *Enterobacteriaceae,* the strains should be able to grow at 37°C, the average internal temperature of the mammalian gut. By determining if they are tolerant of higher temperatures, inferences can be made on their ability to survive in the internal digestive tract of birds, which have a slightly higher internal body temperature of 40°C (Prinzinger et al., 1991). All tested strains were able to grow at 40°C, however none were able to tolerate temperatures of 42°C. By confirming that the AOD associated pathogens can grow at higher temperatures, this represents a good selection pressure for isolating these bacteria from samples in the lab.

Enterobacteriaceae can grow on the selective MacConkey's media (supplementary material 3.1), which is commonly used to inhibit the growth of Gram-positive bacteria through the presence of crystal violet, sodium chloride and 0.15% bile salts, thereby acting as a selective growth medium for Gram-negative bacteria (Lagier et al., 2015).

Antibiotic resistance tests were used as another isolation step to reduce the growth of other environmental bacteria. The test strains from the lab were all subjected to these tests, and it was assumed that wild type bacteria would also react in a similar manner to the presence of environmental pressures. This practice was later deemed as being potentially too harsh and abandoned, as discussed in section 3.4.2.2.1. To establish antibiotic resistance patterns of tested

strains an antibiogram for each was established using a mast ring on a lawn culture plated on 20 ml Luria Agar (LA) for 24 hours at 27°C and 37°C. Each mast ring contained the following six antibiotics: erythromycin (60 ug); rifampicin (15 ug); colistin sulphate (10 ug); penicillin G (Benzylpenicillin) (2 units); kanamycin (100 ug); vancomycin (5 ug). A zone of clearance surrounding the paper disk indicates the bacteria are susceptible to that antibiotic, whereas bacterial growth up to the antibiotic disc infers resistance. Further antibiotics were tested using agar inoculated with antibiotics (concentrations in Table 4) which allowed establishment for resistance to ampicillin and streptomycin. These antibiotics were inoculated in both LA and MacConkey agar with the same results.

Table 4. Antibiotic resistance profiles of the bacteria associated with Acute Oak Decline

	Antibiotic Resistance								
	Mast Ring						**Inoculated media**		
Strain	Erythromycin	Rifampicin	Colistin Sulphate	Penicillin	Kanamycin	Vancomycin	Ampicillin 100ug/ml	Streptomycin 50µg/mL	
Rahnella victoriana	Y	N	N	Y	N	Y	N	N	
Gibbsiella quercinecans	Y	N	N	Y	N	Y	N	N	
Brenneria goodwinii	Y	N	N	Y	N	Y	N	N	

As all AOD bacterial strains showed resistance to erythromycin, penicillin and vancomycin, growth on erythromycin inoculated agar was used as a further selection pressure. The use of erythromycin out of these three antibiotics was due to availability in the lab. Table 5 shows the morphological and physiological characteristics of each of the three AOD associated bacteria.

Table 5. Distinguishing features of the bacteria associated with Acute Oak Decline

Strain	Colony morphology on LA media	Maximum temperature tolerance (°C)	Antibiotic resistance
Gibbsiella quercinecans	Large, raised, circular, white colonies	40	Erythromycin Penicillin Vancomycin
Brenneria goodwinii	Small, flat, circular, grey colonies	40	Erythromycin Penicillin Vancomycin
Rahnella victoriana	Small, raised, circular white colonies	40	Erythromycin Penicillin Vancomycin

3.4.2.2 - Culturing of environmental samples

Samples taken from the field were kept refrigerated for up to three days in 1 ml PBS. Figure 8 summarises the methods used to isolate and identify bacteria of interest from the field samples.

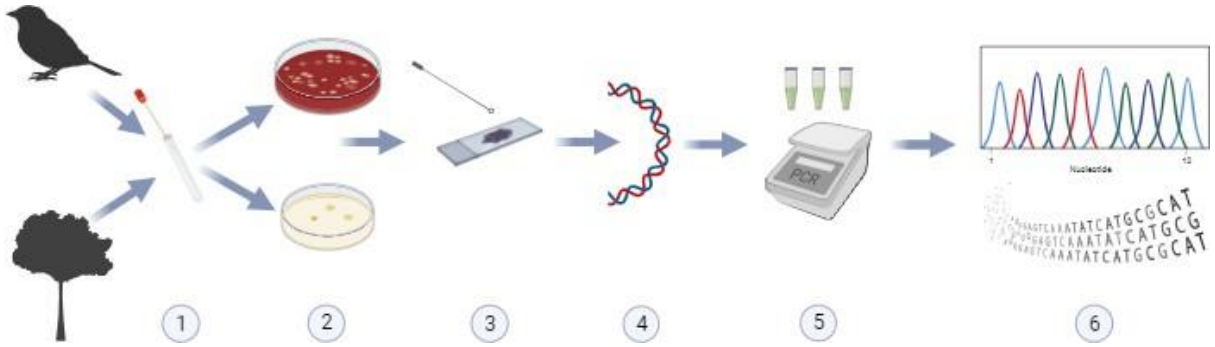


Figure 8. Process of isolating bacteria from field samples. 1 - Sample collection in the field; 2 - Culturing the sample on a variety of media; 3 - KOH test to confirm Gram-negative bacteria; 4 - DNA extraction; 5 - 16S PCR; 6 - Sanger sequencing for species identification. Image created using BioRender.com**.**

Depending on the source of the sample, they were processed as follows.

3.4.2.2.1- Swab samples: bird buccal, foot and back swabs; swabs from trees

Culturing from swab samples from birds was trialled using three methods. Firstly, the tube containing the swab and PBS was vortexed for at least 15 seconds, then 20 μl of the solution was transferred to an agar plate and spread evenly across the plate using a sterile glass spreader. Secondly, the swab and entire PBS it was stored in was transferred to a sterile glass universal tube containing sterile glass beads. The universal was then vortexed for 10 seconds to agitate any bacteria on the swab and release it into the PBS. 20 μl of the PBS solution was pipetted onto the centre of a MacConkey's agar plate and spread evenly across the plate using a sterile glass spreader. The swab was then transferred to a universal tube containing 10 ml Luria broth (LB), and incubated in a shaking incubator at 37°C, 180 rpm for 2-3 hours. 20 μl of the LB culture was then pipetted onto the centre of a MacConkey's agar plate and spread evenly across the plate using a sterile glass spreader. Following incubation, both protocols showed good levels of bacterial growth, therefore the first method was used as it was the most efficient.

3.4.2.2.2 - Faecal samples

The faecal sample was weighed and 10% of the sample was diluted with PBS. The solution was gently homogenised using a combination of an ultrasonic probe, vortex and a plant material homogeniser, for 30 second increments until the sample was liquified. 20 μl of the homogenised samples was pipetted onto the centre of a MacConkey's agar plate and spread evenly across the plate using a sterile glass spreader. Occasionally adult faecal samples were entirely liquid; therefore they were treated in the same way as swab samples, using the cotton swab previously used to collect the faecal sample.

MacConkey's plates were incubated at 37°C for 24 hours. Any bacterial colonies that grew were streaked onto fresh plates containing LA inoculated with erythromycin (200 ug/ml), and again incubated at 37°C for 24 hours. Samples which were resistant were replated onto LA in preparation for further analysis.

Following the initial two years of sample collection, it was determined that the culturing parameters used in this experiment were too strict, as new information on the optimal culturing conditions for field isolates of AOD associated bacteria were understood. From 2022 onwards antibiotic resistance was no longer employed as a marker to isolate any putative AOD pathogens. From this point, samples were incubated for at least 48 h at 27°C, rather than 24 h at 37°C. Research had shown that these conditions were optimal for promoting the growth of AOD bacteria from mixed environmental samples, as these bacteria are slow growing in the laboratory and are often outcompeted by other environmental bacteria (A. Ordonez, personal communication, 2022).

The Gram-negative status of any isolated bacteria was confirmed using 3% potassium hydroxide (KOH). A drop of 3% KOH was added to a microscope slide, into which a bacterial colony was emulsified for 30-60 seconds using a sterile inoculation loop. When the loop is pulled away from the microscope slide, Gram-negative bacteria form a visible mucoid string between the two surfaces. This is due to the potassium hydroxide dissolving the thin peptidoglycan layer in the cell wall of Gram-negative bacteria, which does not occur for Gram-positive bacteria as their layer of peptidoglycan is much thicker (Halebian et al., 1981). The addition of this test allowed an efficient way to confirm the Gram status of a large number of bacterial samples, especially where any bacteria had shown a small amount of growth on MacConkey agar. Any Gram-positive bacteria identified using the KOH test were then excluded from further tests.

3.4.2.3 - PCR protocols

Colony polymerase chain reactions (PCRs) were used to amplify bacterial DNA of isolated bacteria. Colony PCR is a quick method that can be used to identify target bacteria against positive controls without the need for DNA extraction from the bacteria. A small amount of bacteria is placed into the PCR mix In place of DNA. This iscollected from a fresh agar plate by dipping a pipette tip into a bacterial colony. The initial denaturating stage of PCR as detailed in Table 7, is siffucient to break open bacterial cell walls to release the DNA into the PCR mixture. This method can be used for identification against positive controls, but leave a lot of bacterial cell debris and therefore isn't "clean" enough for sequencing. Target-specific primers were used in order to identify potential matches to the AOD bacteria of interest (Crampton et al., 2020). Each sample of isolated bacteria was put through colony PCR in triplicate, with each repeat using a different primer set so all three AOD primer sets were used for each species. Along with the universal bacteria primer, which acted as a control, all samples where put through PCR four times. Details of the primers can be found in Table 6 and the PCR stages in Table 7

PCR products were subjected to gel electrophoresis using 1.5% agarose gel stained with Gel Red (1% w/w), run at 90 v for 45 minutes. Gels were visualised using a GBox gel visualiser and GeneSys software (Syngene) to visualise bands to identify any successfully amplified DNA.

Colony PCR removes the need for prior DNA extraction and is a quick way of identifying the presence or absence of genes of interest - in this case for specific bacteria - however it is not always accurate (Azevedo et al., 2017). The AOD specific primers in Table 6 did not work consistently with amplification of the bacterial DNA not always being successful, therefore colony PCR was abandoned, and an additional step was added to the workflow. Instead, prior to PCR, DNA was extracted from an overnight liquid culture of each bacterial strain isolated, using ThermoScientific GeneJET Genomic DNA purification kit following the Gram-negative protocol. The extracted DNA was amplified by PCR using primers to amplify the full 16S rRNA gene (Clarridge, 2004), which is a highly conserved gene found in all bacterial species, and is most commonly used for sequencing of bacteria. One limitation of 16S PCR is that bacterial species cannot be distinguished from one another using gel electrophoresis, and instead the PCR amplicon needing to be sequenced to generate trace files allowing for more accurate identification using online databases.

Table 6. Details of primers used for PCR.

Species	Target gene	Primer Pair	Sequence (5'-3')	Annealing temperature (°C)	Product Size (bp)	Reference
Brenneria goodwinii	*gyrB*	Bg99F	CTGGCCGAGCC TGGAAAC	50	88	Crampton et al., 2020
		Bg179R	AGTTCAGGAAG GAGAGTTCGC			
Gibbsiella quercinecans	*rpo*	Gq284F	GGCTTTGATAG TGGTGGCC	60	134	Crampton et al., 2020
		Gq418R	CGTTCCGTTAT CACCGTGG			
Rahnella victoriana	*gyrB*	Rv15F	CACCCAGACTT ACGTGCAT	65	119	Crampton et al., 2020
		Rv134R	TCAGTGTGATT GGTGAAGGT			
Universal bacterial primer	16S	27F	AGAGTTTGATC MTGGCTCAG	55	~1400	Liu et al., 2015
		511R	GCGGCTGCTGG CACRKAGT			
Universal bacterial primer	16S	8F	AGAGTTTGATC CTGGCTCAG	55	~1400	Zhang et al., 2020
		1492R	GGTTACCTTGT TACGACTT			

Table 7. Thermal cycler programme for amplification of target genes.

PCR Stage	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	3 min	1
Denaturation	95	40s	30
Annealing (primer specific)	50-65	40s	
Extension	72	40s	
Final Extension	72	7 min	1
End/Hold	4	Hold	-

Initially the 16S primer pairs 27F and 511R (Liu et al., 2015) shown in Table 6 were used, however it was found that this primer pair were producing inconsistent results and were not consistently amplifying the control bacteria, therefore the 16S primer pair 8F and 1492R (Zhang et al., 2020) were used. These primer pairs amplify the same 16S gene, ensuring consistency across samples.

Any samples that were amplified successfully using the 16S primers were cleaned using a GenElute PCR clean up kit (Sigma, USA) to remove residual PCR reagents and any primer dimers. Gel electrophoresis was repeated on the cleaned product and a spectrophotometer (Denovix DS-11 FX +) was used to confirm DNA concentration prior to sequencing. The product to be sequenced was diluted with ddH_2O to between 10-13 ng/μl where necessary. Each sample was sequenced by Sanger sequencing (Source Bioscience, Cambridge), which was used to identify the bacteria to species level, or subsequent lowest taxonomic classification.

3.4.3 – Bioinformatics

Trace files of 16S sequences were visualised using Ugene (v.39) (Okonechnikov et al., 2012) to determine the quality of the sequences using chromatograms. Ugene provides quality scores for each nucleotide base assignment, on a scale of 0-50. If the quality of each sequence was sufficient (> 40) then the primers and sequence ends were trimmed where non-specific binding had occurred. Forward and reverse sequences were aligned using the MUSCLE multiple

alignment package within Ugene. Sequences were uploaded to the NCBI BLAST (Basic Local Alignment Search Tool) 16S ribosomal RNA sequence database (NCBI, 2023) to determine similarity between these bacteria and others and to identify each bacterium to the lowest taxonomic level. This was repeated, firstly using the raw unedited sequence reads received by Source Bioscience. If the similarity to other bacterial sequences was not sufficiently high (< 99.5%) then the sequences were examined in further detail in Ugene for the presence of nucleotide “N” reads, indicating that the sequencing result was not strong enough to confidently categorise this specific nucleotide. Generally, these N reads would be concentrated towards the ends of the sequence, due to reduced binding quality at these areas. Where appropriate, sections of sequences that contained these low-quality reads were trimmed to exclude sections with non-specific binding as indicated in Ugene and rerun through BLAST. If the similarity was too low (< 99.5% confidence), or if the sequences were unable to be aligned for to ambiguous bases, then these sequences were disregarded, and the PCR and sequencing was repeated with fresh DNA extractions.

3.5 - Results

3.5.1 - Disease assessments of trees in sample plots

All oaks within a 50 m radius of each nest box were surveyed for the symptoms and severity of AOD. 103 nest boxes were installed but only plots surrounding 95 nest boxes were surveyed due to eight boxes going missing between installation and the commencement of habitat assessments. A total of 2,623 trees were surveyed, with an average of 28 trees per plot.

In 48 of the 95 plots surveyed there were oaks with symptoms of AOD. The scale of the AOD in the plots varied, with 12 plots having advanced AOD (> 10 bleeds per symptomatic tree), and the remaining symptomatic sites containing trees with only moderate symptoms (< 10 stem bleeds per symptomatic tree). Figure 9 shows the distribution of the diseased plots at the field site.

Height (m), diameter at breast height (DBH), and canopy density were consistent regardless of the AOD status of the plot, however there were slight trends showing that more advanced AOD plots tended to have larger trees, which is consistent with the idea that AOD affects older trees more severely. This trend was reversed for height, with taller trees generally being found in the plots where AOD was absent. 78% trees that were affected with AOD had canopy loss, the most severe of these resulting in partial collapse of the tree. This trend was then reflected in canopy

density, where trees in AOD absent sites had slightly higher canopy density. Anova and post-hoc Tukeys tests revealed that there was a significant difference in DBH and height between AOD advanced and AOD absent sites (DBH p=0.001, height p= 0.009). Canopy density was also significantly greater in AOD absent sites (p=0.004) (Fig. 10). These results are consistent with the literature that AOD typically occurs on larger trees (with a greater DBH), and can reduce the height and canopy density due to crown death.

Agrilus biguttatus emergence holes have been described as a characteristic for trees displaying AOD symptoms and often occur alongside bleeds, however in this study only seven plots contained trees with *Agrilus* emergence holes, therefore they did not contribute much to the AOD scoring. There was only one occasion where these emergence holes did not occur alongside any other AOD symptoms.

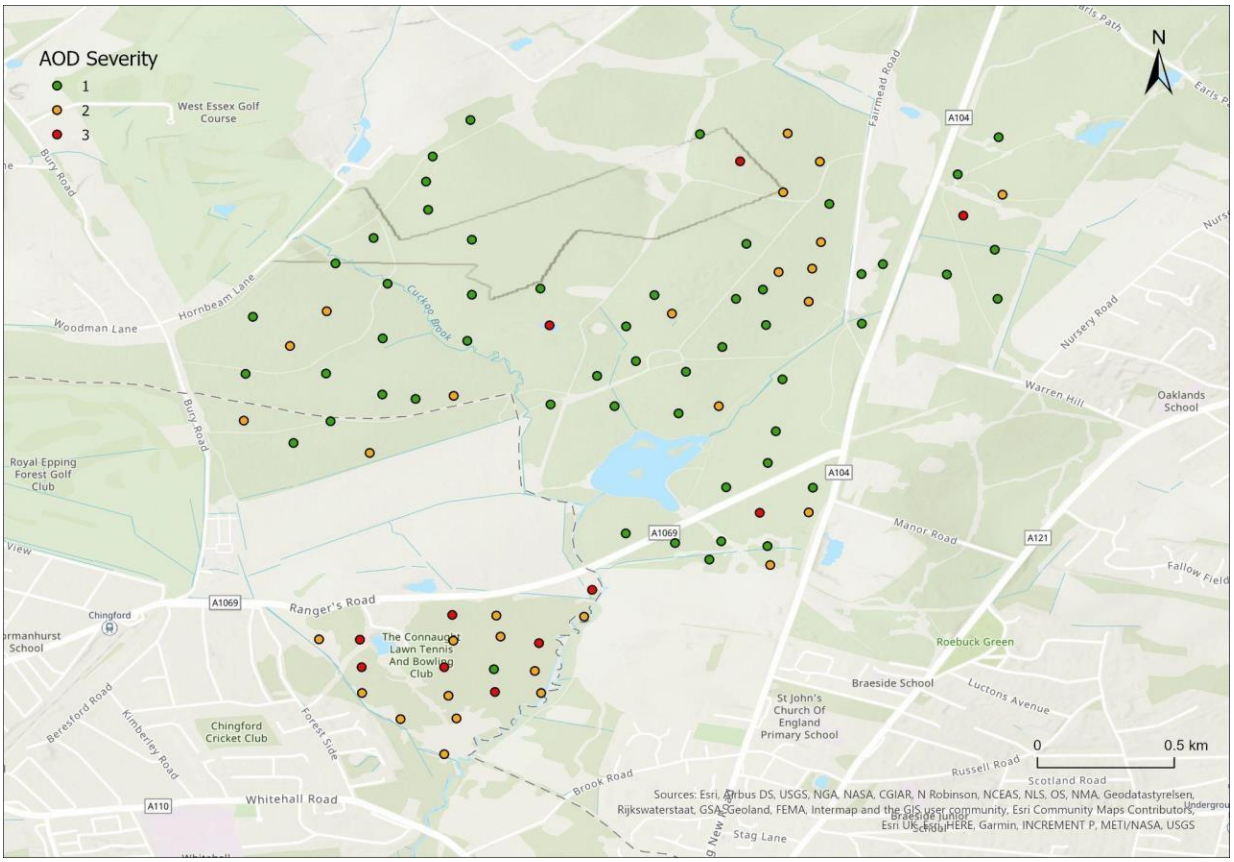
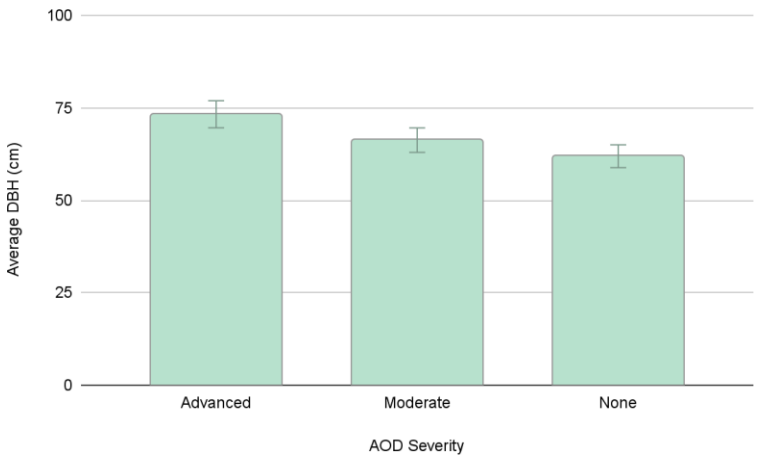
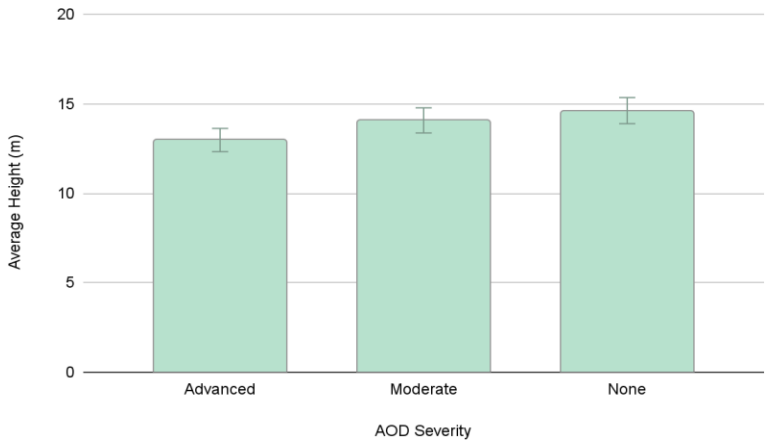


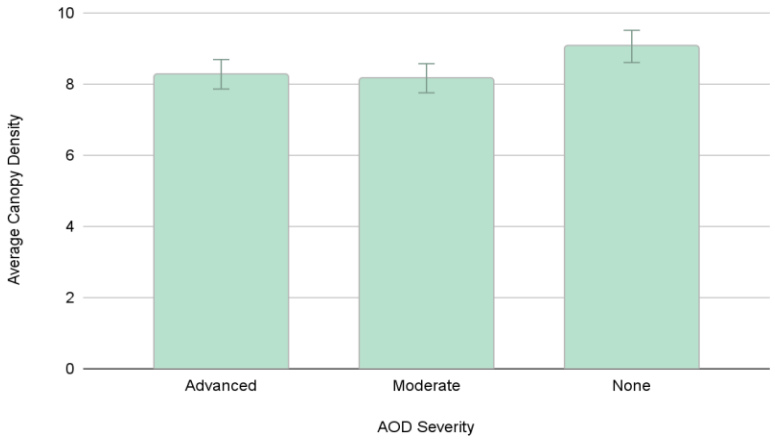
Figure 9. Identification and severity of AOD-affected trees across Epping Forest. Trees were scored on a scale of 1-3, where 1 indicates no AOD symptoms present within the plot, 2 indicates light symptoms and 3 indicates advanced symptoms, as indicated in the image by the different coloured circles.



A



B



C

Figure 10. Average metrics of oak tree health and status within plots with different severities of AOD. A - Average Diameter at Breast Height (DBH); B - Average tree height (m); C - Average canopy density. Error bars indicate 95% Confidence Intervals.

3.5.2 - Identification of bacterial isolates

As detailed in sections 3.4.1.3 - 3.4.1.5, swab samples were taken from 69 symptomatic and 16 asymptomatic oak trees, as well as 99 samples from adults and 277 samples from nestlings. As detailed in section 3.4.2.2.2, the culturing technique was changed in 2022 following developments in the knowledge of culturing these bacteria. The following samples were cultured according to the original culturing parameters; all samples from trees, 37 samples from adult birds, 150 samples from nestlings. The remaining samples were cultured according to the revised parameters.

These samples were all cultured in the lab, and from these, 328 cultured bacteria were successfully sequenced - 117 from nestlings, 120 from adults, and 59 from trees. 61 samples had a 100% match to a sequence on the BLAST database, and 183 had a match of 99.5% similarity or higher, and these samples have been used for interpreting the results.

3.5.2.1 - Bacteria isolated from bird samples

None of the cultured bacteria were identified as being *Brenneria goodwinii* or *Gibbsiella quercinecans*. Three samples were identified as *Rahnella victoriana* with > 99.7% confidence when compared to the NCBI database. These three samples were cultured from adult great tit faecal samples that were caught during the same mist netting session and had been travelling as a flock of young birds in November 2022.

Identified bacteria were classified according to species level where possible, followed by family and genus (Fig. 11). Most of the species detected were from two bacterial families – *Enterobacteriaceae* (>50%) and *Pseudomonadaceae* (>30%). When grouping by genus, over half bacteria identified were from two main genera – *Raoultella* (29%), and *Pseudomonas* (26%).

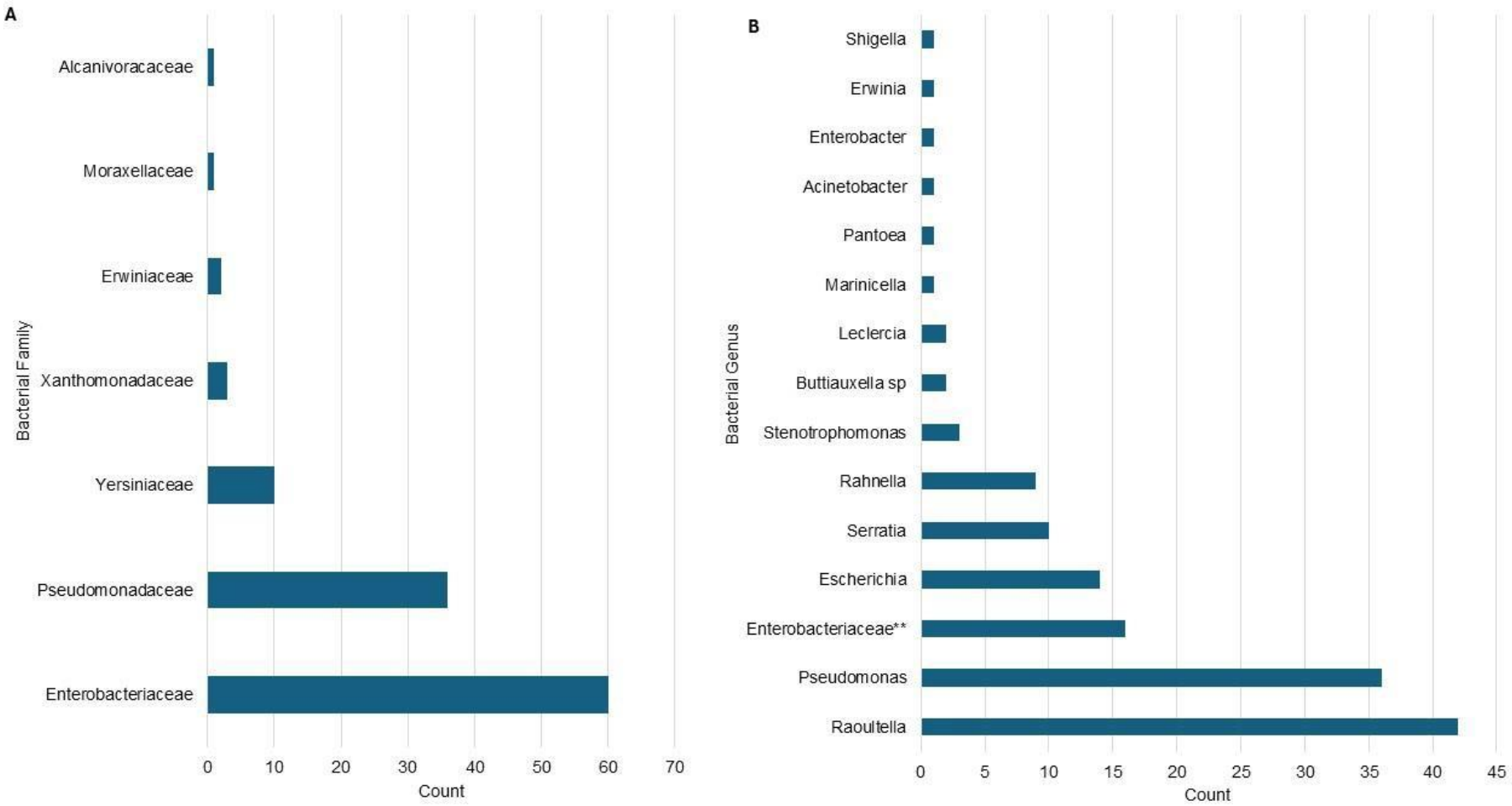


Figure 11. Number of sequences recovered from unique bacterial cultures from bird samples identified as having greater than or equal to 99.5% similarity to the NCBI Blast database. Classified by A) Family, B) Genus - *Enterobacteriaceae*** are bacteria which yielded matches for multiple genera on NCBI Blast, but still within the family Enterobacteriaceae

Table 8 details the number of sequences recovered from different bacterial families from different bird sample types. Species belonging to the *Enterobacteriaceae*, *Pseudomonadaceae* and *Yersiniaceae* bacterial families were found across all bird sample types and were recovered from samples taken from both AOD present and absent sites. Full details of each bacteria species identified by sequencing and the corresponding sample type can be found in supplementary material 3.4.

Table 8. Number of bacterial species categorised by bacterial family, according to what type of bird sample it was cultured from and the AOD status of the plot where the sample was taken.

Bacterial Family	Total species cultured	Age		Sample Type				Species		AOD Status		
		Nestling	Adult	Faecal	Buccal	Foot	Back	Blue Tit	Great Tit	Present	Absent	Unknown
Alcanivoracaceae	**1**	0	1	0	0	1	0	0	0	1	0	0
Enterobacteriaceae	**87**	23	64	16	31	22	18	30	57	53	13	21
Erwiniaceae	**2**	2	0	0	0	2	0	2	0	1	1	0
Moraxellaceae	**1**	0	1	0	0	0	1	0	1	1	0	0
Pseudomonadaceae	**36**	31	5	8	12	9	7	23	13	18	18	0
Xanthomonadaceae	**3**	3	0	3	0	0	0	1	2	2	1	0
Yersiniaceae	**10**	8	2	3	3	2	2	9	1	5	5	0

Some of the bacterial families listed in Table 8 are more surprising than others.

- **Alcanivoracaceae** – there is not much in the literature about this bacterial family, with no mention of their presence in terrestrial environments (Silveria et al., 2014)

- **Enterobacteriaceae** – the isolation of species from this family is not surprising as they are often found in water and soil, and are associated with a variety of plants and animals (Wang. Z et al., 2021). This bacterial family contains many pathogens such as Salmonella and E. coli (Ferreira de Silva et al., 2007).

- **Erwiniaceae –** Thie bacterial family includes a number of plant pathogens (Adeolu et al., 2016).

- **Moraxellaceae** – members of this bacterial family are often found in water and soil, and have been isolated from a range of terrestrial and marine animals (Fernández-

Garayzábal et al., 2015)

- **Pseudomonadaceae** – Members of this bacterial family are widespread in the environment, and can be isolated from many natural niches (Zboralski & Filion, 2023)
- **Xanthomonadaceae** – this family represents one of the largest groups of bacterial phytopathogens (Mhedbi-Hajri et al., 2011).
- **Yersiniaceae** – this family includes a variety of important animal pathogens, members of which have been isolated from a range of ecological niches (Moxley, 2022).

3.5.2.2 - Bacteria isolated from tree samples

Samples were taken from 24 active stem bleeds, 45 inactive stem bleeds, and 16 asymptomatic trees. All samples cultured from trees, regardless of sample type and including from disease lesions, were identified as *Raoultella planticola* (n=38). Figure 12 shows the number of *Raoultella planticola* cultured from different sample types.

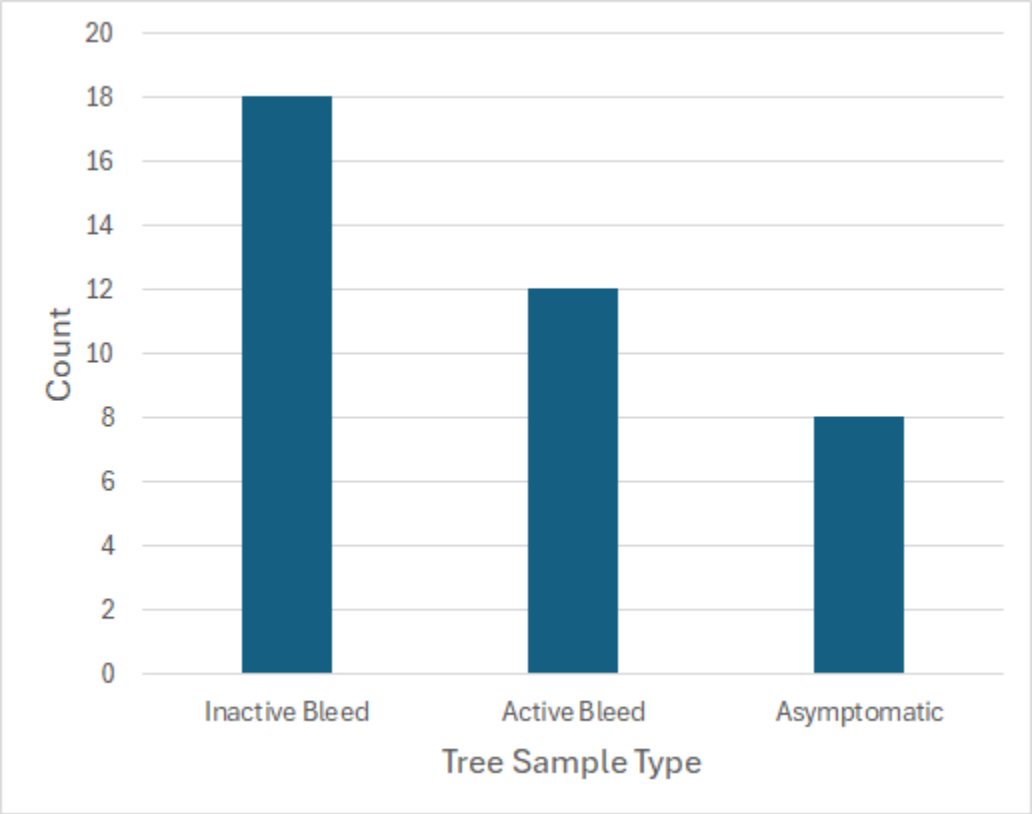


Figure 12. Number of samples from which *Raoultella planticola* was cultured.

3.6 - Discussion

3.6.1 - Disease assessments

The lack of variation in DBH, height and canopy density between the different AOD severity plots was not expected due to the previous work documenting how AOD can lead to canopy thinning and degradation, and that AOD mainly impacts more mature trees (Brown et al., 2016). Symptoms of AOD were found in 48 of the plots and in 12 of these the symptoms of AOD were classified as Advanced according to the criteria in Table 2. The results from the habitat and AOD assessment do not indicate that AOD has a significant detriment on the canopy density of oak trees. The field site of Epping Forest does not have historical data into the occurrence of AOD (personal communication, Jeremy Dagley, Head of Conservation at Epping Forest), therefore it is possible that the disease is not as prevalent as the field sites used by Sapp et al., (2016), whose scoring system was adapted for this project. It is also possible that the areas selected for nest box installation do not accurately reflect the extent and intensity of AOD in the area, as disease assessments could not be carried out until after the field site was set up.

The scoring system used in this research did not consider the density of AOD symptomatic trees within the plot, therefore future work should consider the percentage of symptomatic trees rather than the absolute number. According to the classification used in this work, the presence of just one severe AOD tree in a plot resulted in it being assigned to the “advanced” AOD category. By combining the percentage of symptomatic trees in the plots with the intensity of AOD symptoms and other metrics such as canopy density, we can establish a continuous scale of oak health and AOD status within the plot. This will allow for smaller variations to be considered, rather than having three broad categories for AOD classification. Due to time constraints on this project, this was not investigated but could be a basis for future investigations.

3.6.2 - Bacterial analysis

3.6.2.1 - Bacteria identification

Three isolates identified of *Rahnella victoriana* were successfully cultured, which is the most consistently recovered of the AOD bacteria from environmental samples (Maddock et al., 2023). Interestingly, the three *Rahnella victoriana* samples identified were cultured from great tit faecal samples from three separate birds caught in the same flock of young adult birds. This lends support towards the idea that bacteria could be transmitted between conspecifics, or that

exposure to the same environmental sources can lead to a shared microbial community. Virus transmission between birds that share food and water resources is common, such as with the West Nile Virus and avian influenza (Rappole & Hubálek, 2003, 2006), and recent work into social bird species had found that closely related zebra finches had more similar skin microbiomes, with offspring having the greatest similarities (Engel et al., 2020).

Interestingly, some of the bacteria isolated from the nestling swabs are either plant or human pathogens: *Pantoea conspicua, Pantoea dispersa* and *Serratia fonticola* are important plant pathogens, while *Enterobacter cloacae* is both an important human and plant pathogen (Davin-Regli & Pagès, 2015). *Shigella sonnei,* a significant human pathogen responsible for shigellosis (Kotloff et al., 2018), was recovered from a great tit buccal cavity. Interestingly *Shigella* species are not known to have any animal reservoirs (Shad & Shad, 2021), therefore the detection of this species could be significant for the understanding of this human pathogen, which could have implications for ornithologists working with such birds.

All the sampling equipment used in this study was sterile and aseptic techniques such as wearing gloves, sanitising hands and equipment with alcohol between birds, was ensured, however there is always a possibility that environmental bacteria can contaminate the equipment. The nest boxes used, for example, were not sterile when they were erected, as this was not practical. All nest boxes were made of the same material however and kept in the same indoor storage conditions prior to installation, therefore any contaminating bacteria would likely be consistent across nest boxes. Previous work has indicated variation in the bacterial load of nest boxes across different forested areas, and also higher bacterial loads when nest boxes had been used in the previous nesting season (Zabłotni et al., 2023). The nest boxes used in my study were only replaced when they had been damaged, however all nesting material was cleared out at the end of one breeding season and before the start of the next. Zablotni *et al* sampled different areas of the nest boxes, including the entrance hole. Sampling the entrance hole could be a useful and non-invasive way to collect samples to assess what bacteria are associated with the parents entering and leaving the nest, however as noted by Zablotni (2023), this area of the nest box is likely to be contaminated by the environment. Ideally to fully understand how bacteria vary across different environments, nest boxes should be in the same sterile state at the start of the field season, however this would require substantial additional resources which were not practical for this study.

3.6.2.2 – Recovery of target bacteria

No *Brenneria goodwinii* or *Gibbsiella quercinecans* were detected in any of the samples despite being cultured from samples taken from active bleeds in other studies on AOD (Booth, 2019). The extent to which birds could transmit AOD putative pathogens just from acquiring them from the surface of the tree may be negligible, as previous work has yielded higher quantities of bacteria from the inner bark than the outer bark (Brady et al., 2017). This could limit the potential exposure of birds to AOD pathogens, limiting their ability to act as vectors. Exceptions to this come from other species which utilise oak trees more in their feeding strategies, such as woodpeckers and nuthatches. Nuthatches have been observed to feed on *Agrilus* larvae that are emerging from AOD lesions (R.Jackson, personal communication, 2020), demonstrating direct contact with areas of the tree which have a particularly high bacterial load. It is possible that woodpeckers boring into trees could transmit the bacteria from one oak to another, however it is difficult to imagine that these small unlikely occurrences would contribute to the spread of a disease. In order for effective transmission the bird would have to drill into the tree directly at a bleed site, as previous work has shown that the bacterial load of bleeds stays confined to the symptomatic areas (Brady et al., 2017). Brady et al. (2017), compared AOD-symptomatic and AOD-asymptomatic tissues from the same tree, showed a markedly different bacterial load. Having the pathogens confined to such a small area does have implications for the scale of vectoral transmission.

The lack of AOD putative pathogens detected in either the bird or tree samples does not necessarily indicate that the swabs taken do not contain these pathogens. Previous research has shown that *G. quercinecans* and *B. goodwinii* do not survive in environmental conditions such as rainwater and soil for very long (Pettifor et al., 2020). However, there is little published evidence for the bacteria's survival on trees or bird feathers, or within gastrointestinal tracts. Knowledge on where these bacteria can survive in the environment would be valuable for understanding potential routes of transmission.

Research has also found that Enterobacteriaceae are surprisingly difficult to distinguish from each other, not only on a morphological level but also on a molecular level (Hong Nhung et al., 2007), with even 16S microbial community analysis rarely being able to identify Enterobacteriaceae at the species level. This may explain why a proportion of Sanger sequencing results returned multiple matches for Enterobacteriaceae, which can be seen in Figure 9. The primers used in this study were selected due to their ability to successfully amplify the AOD bacteria of interest, which was confirmed using laboratory strains of the bacteria cultured by previous researchers in the group. Another method which could be used to distinguish different species of Enterobacteriaceae is by

using high melt analysis, which allows single nucleotide polymorphisms (SNPs) to be identified without the need for sequencing (Gundry et al., 2003). This could prove to be a quicker, cheaper and possibly more effective way to detect AOD bacteria within a sample without the need for culturing and Sanger sequencing. As detailed in 3.5.2, just over half of successfully sequenced bacteria had a similarity of 99.5% or greater, and a large amount of samples sent for sequencing had to either be repeated or discarded as the sequencing did not work or the output was too low of a quality to reliably interpret the results. By using this high melt analysis, Brady et al., (2016), were able to distinguish different DNA melt curves for both *B. goodwinii* and *G. quercinecans*, and other commonly isolated bacteria associated with AOD symptomatic oaks (namely *Brenneria rosea* and *Lonsdalea quercina)*. This method was so strong that *G. quercinecans* was detected clearly despite 10,000-fold dilution, meaning that detection in highly contaminated swab samples taken directly from trees may be possible, with no need for bacterial isolation. The samples from the current project could be reanalysed in subsequent projects using high melt analysis, which should remove issues faced with culturing and sequencing.

It is possible that some bacteria present within samples at earlier stages of sample collection were not successfully cultured. As explained in the methods, the initial culturing conditions were more stringent and were later modified to better encourage the growth of AOD bacteria. All samples from AOD symptomatic trees were taken before this change in methodology, and due to time constraints it wasn't feasible to resample the trees. Only after these culturing conditions were modified were the three *Rahnella* samples isolated from bird samples. The tree samples were also taken in a different way to those taken in previous work on isolating AOD bacteria from bleeds. In this study, swabs were taken from the external surface of the tree; however, all other work identifying bacteria associated with AOD bleeds has taken samples destructively from the inner bark (Brady et al., 2010; Broberg et al., 2018; Denman et al., 2018; Kaczmarek et al., 2017; Sapp et al., 2016). Due to the nature of Epping Forest being publicly accessible, and several of the trees surveyed being protected as designated ancient oaks, destructive sampling was not permitted on my research permit. It is possible that if samples had been taken from the inner bark, as they had been with other projects, that AOD bacteria may have been isolated.

The method of sample storage also may not have been optimal for recovery of AOD bacteria from environmental samples. Consistent with other work on AOD, samples were stored either dry or in PBS, at 4°C until they were able to be cultured in the lab (Crampton et al., 2020). There is little consensus in the literature as to how best to store environmental samples for culturing in the lab, as most of the literature focuses on storage for non-culture based techniques such as molecular analyses. Bacteria can enter a state known as VBNC – 'viable but nonculturable',

whereby they cannot be detected by traditional culture-based growth on media but are still present in an alive state (James, 2005). It is possible that the storage and transportation of samples in this project was not optimal to allow for the AOD bacteria to be cultured. Best practice for clinical microbiological samples is to culture them within 24 hours of collection, reducing the risk of cell death or excessive bacterial growth, which would not accurately reflect the conditions under which the swab was taken (Roelofsen et al., 1999). Due to limited time and resources, samples could be left up to 72 hours before culturing in this experiment, which may have impacted bacterial viability.

During the preliminary isolation experiments for optimising AOD bacteria from environmental samples, the bacteria used to refine these methods were long term storage stocks. These bacteria had already been grown, isolated and then stored, making them significantly more concentrated and purer than any environmental samples would be. Environmental samples contain multiple different types of bacteria which could compete for resources, making it difficult for target bacteria to be isolated and grow successfully. The methods used in this chapter assumed that bacteria from the field would behave in the same manner as those isolated from a different project and used for laboratory assays, therefore it is difficult to make comparisons. The bacteria used for the initial isolation trials were isolated using destructive sampling of AOD symptomatic tissue (Booth, 2019) thereby presenting another difference between the pre-sampling trials and the processing of the samples.

The vast majority of bacteria present in the environment persist in an unculturable state, or culturing techniques have not yet been able to isolate them, with a commonly quoted statistic being that less than 2% of environmental bacteria are culturable (Steen et al., 2019; Wade, 2002). Whereas AOD bacteria have been cultured previously, it highlights the difficulties in this as a method for identifying bacteria from environmental samples. Most work looking at microbial diversity therefore focuses more on the whole community of microbiomes using molecular identification techniques (Fricker et al., 2019; Galloway-Peña & Hanson, 2020; Knight et al., 2018; Trinh et al., 2018), which is the focus of Chapter 4 of this thesis.

By improving sample storage and refining culturing practices, alongside targeting different species of bird, a clearer picture of the role birds play in the transmission of emerging tree diseases can be generated. Expanding this work by using a wider microbiome approach, further investigations into any role played by birds as vectors of plant pathogens, which will be the focus of the next chapter of this thesis.

3.7 - References

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3.8 - Supplementary material

Supplementary material 3.1. Recipes used to create different media for bacterial growth

Media	Total quantity	Ingredient	Quantity
Luria Agar (LA)	400ml	Agar	6g
		Tryptone	4g

		NaCL	4g
		Yeast extract	2g
		H20	Up to 400ml
Luria Broth (LB)	400ml	Tryptone	4g
		NaCL	4g
		Yeast extract	2g
		H20	Up to 400ml
MacConkey Agar	1L	Peptone	20g
		Lactose	10g
		Bile Salts No.3	1.5g
		NaCl	5g
		Neutral Red	0.03g
		Crystal Violet	0.001g
		Agar	15g
		H20	Up to 1L

Supplementary material 3.2. A full breakdown of the individual bacteria cultured from different samples, whose Sanger sequences had a match of at least 99.5% when compared to the NCBI BLAST database.

Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
N561	25/05/2021	*Buttiauxella gaviniae*	100	Bird	Nestling	Buccal	Blue Tit	Z822625	0
A483.2	23/12/2021	*Marinicella sediminis*	100	Bird	Adult	Foot	Blue Tit	Z822648	1
A0203.2	24/09/2020	*Acinetobacter lwoffii*	100	Bird	Adult	Back	Great Tit	VZ08606	1
N1013.1	23/05/2025	*Pseudomonas glycinis*	100	Bird	Nestling	Back	Blue Tit	Z822668	1
N261	20/05/2021	*Pseudomonas koreensis*	100	Bird	Nestling	Faecal	Blue Tit	N/A	1
N631	18/05/2021	*Pseudomonas qingdaonensis*	100	Bird	Nestling	Buccal	Great Tit	VZ08617	0
A6006.2	14/11/2022	*Rahnella ecdela/bruchi (Rahnella spp)*	100	Bird	Adult	Foot	Great Tit	VZ08656	0
A0201.2	24/09/2020	*Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08606	1
A0202.1	24/09/2020	*Raoultella planticola*	100	Bird	Adult	Foot	Great Tit	VZ08606	1
A0203.1	24/09/2020	*Raoultella planticola*	100	Bird	Adult	Back	Great Tit	VZ08606	1
A0204	24/09/2020	*Raoultella planticola*	100	Bird	Adult	Faecal	Great Tit	VZ08606	1
A0205	24/09/2020	*Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08607	1
A0206.2	24/09/2020	*Raoultella planticola*	100	Bird	Adult	Foot	Great Tit	VZ08607	1

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Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
A0213	08/05/2021	*Raoultella planticola*	100	Bird	Adult	Foot	Blue Tit	Z822602	1
A0215	08/05/2021	*Raoultella planticola*	100	Bird	Adult	Faecal	Blue Tit	Z822602	1
A0216	08/05/2021	*Raoultella planticola*	100	Bird	Adult	Buccal	Blue Tit	Z822603	1
A0217	08/05/2021	*Raoultella planticola*	100	Bird	Adult	Foot	Blue Tit	Z822603	1
A0218	08/05/2021	*Raoultella planticola*	100	Bird	Adult	Back	Blue Tit	Z822603	1
A1704_1	15/09/2020	*Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08602	1
A1704_2	15/09/2020	*Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08602	1
A1705_2	15/09/2020	*Raoultella planticola*	100	Bird	Adult	Foot	Great Tit	VZ08602	1
A1706	15/09/2020	*Raoultella planticola*	100	Bird	Adult	Back	Great Tit	VZ08602	1
A1710	15/09/2020	*Raoultella planticola*	100	Bird	Adult	Back	Great Tit	VZ08603	1
A1712	16/09/2020	*Raoultella planticola*	100	Bird	Adult	Foot	Great Tit	VZ08604	1
N053	14/05/2021	*Raoultella planticola*	100	Bird	Nestling	Back	Great Tit	VZ08610	1
N233	14/05/2021	*Raoultella planticola*	100	Bird	Nestling	Back	Great Tit	VZ08609	1
N381	20/05/2021	*Raoultella planticola*	100	Bird	Nestling	Buccal	Blue Tit	Z822617	0
N622	18/05/2021	*Raoultella planticola*	100	Bird	Nestling	Foot	Blue Tit	Z822610	1
N623	18/05/2021	*Raoultella planticola*	100	Bird	Nestling	Back	Blue Tit	Z822610	1

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Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
N731	15/05/2021	*Raoultella planticola*	100	Bird	Nestling	Buccal	Blue Tit	Z822605	0
N732	15/05/2021	*Raoultella planticola*	100	Bird	Nestling	Foot	Blue Tit	Z822605	0
N801	18/05/2021	*Raoultella planticola*	100	Bird	Nestling	Buccal	Blue Tit	Z822611	0
N803	18/05/2021	*Raoultella planticola*	100	Bird	Nestling	Back	Blue Tit	Z822611	0
A0201.1	24/09/2020	*Raoultella terrigena/Raoultella ornithinolytica/Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08606	1
A0211.1	01/10/2020	*Raoultella ornithinolytica/Raoultella planticola*	100	Bird	Adult	Back	Blue Tit	Z822601	1
A0201.3	24/09/2020	*Klebsiella aerogenes/Raoultella ornitholytica/Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08606	1
A1709_2	15/09/2020	*Klebsiella aerogenes/Raoultella ornitholytica/Raoultella planticola*	100	Bird	Adult	Foot	Great Tit	VZ08603	1
A1711	16/09/2020	*Klebsiella aerogenes/Raoultella ornitholytica/Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08604	1
A1715	16/09/2020	*Klebsiella aerogenes/Raoultella ornitholytica/Raoultella planticola*	100	Bird	Adult	Buccal	Great Tit	VZ08605	1
A1717	16/09/2020	*Klebsiella aerogenes/Raoultella ornitholytica/Raoultella planticola*	100	Bird	Adult	Back	Great Tit	VZ08605	1

Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
A1718	16/09/2020	*Klebsiella aerogenes/Raoultella ornitholytica/Raoultella planticola*	100	Bird	Adult	Faecal	Great Tit	VZ08605	1
A481	08/06/2022	*Escherichia fergusonii/Shigella flexneri*	99.91	Bird	Adult	Buccal	Blue Tit	Z822648	1
A483	08/06/2022	*Escherichia fergusonii/Shigella flexneri*	99.91	Bird	Adult	Foot	Blue Tit	Z822648	1
A493	08/06/2023	*Shigella flexneri/Escherichia fergusonii*	99.91	Bird	Adult	Foot	Blue Tit	Z822649	1
A521	08/06/2022	*Escherichia coli/Shigella flexneri*	99.91	Bird	Adult	Buccal	Great Tit	VZ08652	1
A6014.1	14/11/2022	*Shigella flexneri/Escherichia fergusonii*	99.91	Bird	Adult	Foot	Great Tit	VZ08658	0
A862.2	08/06/2022	*Escherichia fergusonii/Shigella flexneri*	99.91	Bird	Adult	Back	Blue Tit	Z822686	1
N341	11/05/2021	*Escherichia fergusonii/Shigella flexneri*	99.91	Bird	Nestling	Buccal	Great Tit	N/A	0
A501	08/06/2022	*Pseudomonas granadensis*	99.91	Bird	Adult	Buccal	Great Tit	VZ08650	1
N921.3	23/05/2021	*Pseudomonas paraversuta*	99.91	Bird	Nestling	Buccal	Blue Tit	Z822676	1
N341	11/05/2021	*Pseudomonas qingdaonensis*	99.91	Bird	Nestling	Buccal	Great Tit	VZ08608	0
A1702	15/09/2020	*Raoultella planticola*	99.91	Bird	Adult	Foot	Great Tit	VZ08601	1
N075	23/05/2022	*Stenotrophomonas rhizophila*	99.91	Bird	Nestling	Faecal	Blue Tit	Z822682	1
N857	15/06/2021	*Buttiauxella agrestis*	99.9	Bird	Nestling	Faecal	Blue Tit	Z822640	1

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Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
A433.2	23/12/2021	*Shigella flexneri/Escherichia fergusonii*	99.9	Bird	Adult	Foot	Blue Tit	Z822643	1
A862.1	08/06/2022	*Shigella flexneri/Escherichia fergusonii*	99.9	Bird	Adult	Back	Blue Tit	Z822686	1
A3605	04/11/2022	*Escherichia coli*	99.9	Bird	Adult	Buccal	Great Tit	VZ08654	0
A6011	14/11/2022	*Escherichia coli*	99.9	Bird	Adult	Back	Great Tit	VZ08657	0
N994	22/05/2021	*Pseudomonas psychrophila*	99.9	Bird	Nestling	Faecal	Blue Tit	Z822618	1
N995	22/05/2021	*Pseudomonas psychrophila*	99.9	Bird	Nestling	Faecal	Blue Tit	Z822618	1
A6012.1	14/1/2022	*Pseudomonas putida*	99.9	Bird	Adult	Faecal	Great Tit	VZ08657	0
N875	18/05/2022	*Rahnella ecdela/Rahnella bruchi*	99.9	Bird	Nestling	Faecal	Blue Tit	Z822670	0
A0207	24/09/2020	*Raoultella planticola*	99.9	Bird	Adult	Back	Great Tit	VZ08607	1
N231	14/05/2021	*Raoultella planticola*	99.9	Bird	Nestling	Buccal	Great Tit	VZ08609	1
N501	18/05/2021	*Raoultella planticola*	99.9	Bird	Nestling	Buccal	Great Tit	VZ08645	0
N892	17/05/2021	*Raoultella planticola*	99.9	Bird	Nestling	Foot	Blue Tit	Z822608	1
N317.1	19/05/2021	*Stenotrophomonas rhizophila*	99.9	Bird	Nestling	Faecal	Great Tit	VZ08644	0
N445	23/05/2021	*Pseudomonas fragi*	99.89	Bird	Nestling	Faecal	Blue Tit	Z822621	0
A501.2	08/06/2022	*Escherichia coli*	99.88	Bird	Adult	Buccal	Great Tit	VZ08650	1

Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
A512	08/06/2022	*Escherichia coli*	99.88	Bird	Adult	Back	Great Tit	VZ08651	1
N371	22/06/2021	*Shigella sonnei*	99.88	Bird	Nestling	Buccal	Great Tit	VZ08623	0
N793	01/06/2021	*Pantoea conspicua*	99.84	Bird	Nestling	Faecal	Blue Tit	Z822636	0
A6001.1	14/11/2022	*Shigella flexneri/Escherichia fergusonii*	99.83	Bird	Adult	Buccal	Great Tit	VZ08655	0
A523.2	08/06/2022	*Escherichia fergusonii/Shigella flexneri*	99.82	Bird	Adult	Foot	Great Tit	VZ08652	1
A6001.2	14/11/2022	*Shigella flexneri/Escherichia fergusonii*	99.82	Bird	Adult	Buccal	Great Tit	VZ08655	0
N561A	25/05/2021	*Pseudomonas koreensis*	99.82	Bird	Nestling	Buccal	Blue Tit	Z822667	0
N463.2	16/05/2021	*Pseudomonas mucidolens/Pseudomonas synxantha/Pseudomonas gessardii*	99.82	Bird	Nestling	Back	Blue Tit	Z822606	0
A6008.1	14/11/2022	*Rahnella victoriana*	99.82	Bird	Adult	Faecal	Great Tit	VZ08656	0
A6016	14/11/2022	*Rahnella victoriana*	99.82	Bird	Adult	Faecal	Great Tit	VZ08658	0
N232	14/05/2021	*Raoultella planticola*	99.82	Bird	Nestling	Foot	Great Tit	VZ08609	1
N361.2	21/05/2021	*Serratia fonticola*	99.82	Bird	Nestling	Buccal	Blue Tit	Z822664	0
A3602	04/11/2022	*Shigella flexneri/Escherichia fergusonii*	99.81	Bird	Adult	Foot	Great Tit	VZ08653	0
A431	23/12/2021	*Shigella flexneri/Escherichia fergusonii*	99.81	Bird	Adult	Buccal	Blue Tit	Z822643	1
A6024.2	14/11/2022	*Shigella flexneri/Escherichia fergusonii*	99.81	Bird	Adult	Faecal	Great Tit	VZ08660	0

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Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
A442.1	23/12/2021	*Escherichia coli*	99.81	Bird	Adult	Faecal	Blue Tit	Z822644	1
N641.1	18/05/2022	*Pseudomonas caspiana*	99.81	Bird	Nestling	Buccal	Blue Tit	Z822674	0
A502	08/06/2022	*Pseudomonas granadensis*	99.81	Bird	Adult	Back	Great Tit	VZ08650	1
N562.1	17/05/2022	*Pseudomonas koreensis*	99.81	Bird	Nestling	Foot	Blue Tit	Z822667	0
N892B	18/05/2022	*Pseudomonas koreensis*	99.81	Bird	Nestling	Foot	Blue Tit	Z822671	1
N1014	17/05/2022	*Serratia fonticola*	99.81	Bird	Nestling	Faecal	Blue Tit	Z822668	1
A6009	14/11/2022	*Escherichia coli*	99.8	Bird	Adult	Buccal	Great Tit	VZ08657	0
A6024.1	14/11/2022	*Escherichia coli*	99.8	Bird	Adult	Faecal	Great Tit	VZ08660	0
N611	01/06/2021	*Pseudomonas fragi*	99.8	Bird	Nestling	Buccal	Great Tit	VZ08621	0
A1701	15/09/2021	*Raoultella planticola*	99.8	Bird	Adult	Buccal	Great Tit	VZ08601	1
N621	18/05/2021	*Pseudomonas fragi/Pseudomonas psychrophila*	99.79	Bird	Nestling	Buccal	Blue Tit	Z822610	1
N682	31/05/2021	*Serratia fonticola*	99.79	Bird	Nestling	Faecal	Blue Tit	Z822632	0
N922	23/05/2021	*Pseudomonas fragi*	99.78	Bird	Nestling	Foot	Blue Tit	Z822676	1
N941	23/05/2021	*Serratia fonticola*	99.78	Bird	Nestling	Buccal	Blue Tit	Z822619	0
A3604	04/11/2022	*Pseudomonas helleri*	99.77	Bird	Adult	Faecal	Great Tit	VZ08653	0
A3601	04/11/2022	*Shigella flexneri/Escherichia fergusonii*	99.74	Bird	Adult	Buccal	Great Tit	VZ08653	0

Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
N982	20/05/2022	*Erwinia billingiae*	99.74	Bird	Nestling	Foot	Blue Tit	Z822677	1
N592	10/05/2022	*Pseudomonas koreensis*	99.74	Bird	Nestling	Back	Blue Tit	Z822609	0
N282.2	09/05/2022	*Pseudomonas koreensis*	99.73	Bird	Nestling	Foot	Great Tit	VZ08632	0
N462.2	07/05/2022	*Pseudomonas koreensis*	99.73	Bird	Nestling	Foot	Blue Tit	Z822606	0
N463.1	07/05/2022	*Pseudomonas koreensis*	99.73	Bird	Nestling	Back	Blue Tit	Z822606	0
N531	19/05/2022	*Pseudomonas yamanorum*	99.73	Bird	Nestling	Buccal	Blue Tit	Z822675	0
A6021.2	14/11/2022	*Rahnella ecdela/Rahnella bruchi*	99.73	Bird	Adult	Buccal	Great Tit	VZ08660	0
N354	27/05/2021	*Leclercia adecarboxylata*	99.72	Bird	Nestling	Faecal	Great Tit	VZ08620	1
A6020.1	14/11/2022	*Rahnella victoriana*	99.72	Bird	Adult	Faecal	Great Tit	VZ08659	0
A423.1	23/12/2021	*Serratia fonticola*	99.72	Bird	Adult	Foot	Blue Tit	Z822642	1
A3606	04/11/2022	*Escherichia coli*	99.71	Bird	Adult	Foot	Great Tit	VZ08654	0
A3608.1	04/11/2022	*Escherichia coli*	99.71	Bird	Adult	Faecal	Great Tit	VZ08654	0
N1022.2	10/05/2022	*Pseudomonas glycinis*	99.69	Bird	Nestling	Foot	Great Tit	VZ08633	0
N512	29/05/2021	*Pseudomonas koreensis*	99.69	Bird	Nestling	Foot	Blue Tit	Z822631	1
N652	20/05/2021	*Pseudomonas brenneri/Pseudomonas proteolytica*	99.68	Bird	Nestling	Foot	Great Tit	VZ08618	1
N344.1	11/05/2021	*Pseudomonas koreensis*	99.66	Bird	Nestling	Foot	Great Tit	VZ08608	0

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Sample	Date Collected	Bacteria Identified	% Identity	Sample Type				Bird Ring Number	AOD Status 1=Present, 0=Absent
N071	19/05/2021	*Pseudomonas paracarnis*	99.66	Bird	Nestling	Buccal	Blue Tit	Z822614	1
N076	23/05/2022	*Pseudomonas yamanorum*	99.65	Bird	Nestling	Faecal	Blue Tit	Z822682	1
N1012.1	17/05/2022	*Serratia fonticola*	99.65	Bird	Nestling	Foot	Blue Tit	Z822668	1
N054.5	14/05/2021	*Stenotrophomonas chelatiphaga*	99.65	Bird	Nestling	Faecal	Great Tit	VZ08610	1
N411	02/06/2021	*Pseudomonas koreensis*	99.64	Bird	Nestling	Buccal	Blue Tit	Z822637	1
A6020.2	14/11/2022	*Pseudomonas neuropathica*	99.64	Bird	Adult	Faecal	Great Tit	VZ08659	0
N431	20/05/2022	*Pseudomonas hunanensis/Pseudomonas promysalinigenes*	99.64	Bird	Nestling	Buccal	Blue Tit	Z822680	0
A6024.3	14/11/2022	*Rahnella variigena*	99.64	Bird	Adult	Faecal	Great Tit	VZ08660	0
N641.4	18/05/2022	*Rahnella ecdela/Rahnella bruchi*	99.63	Bird	Nestling	Buccal	Blue Tit	Z822674	0
A522	08/06/2022	*Escherichia coli*	99.62	Bird	Adult	Back	Great Tit	VZ08652	1
N941A	17/05/2022	*Serratia fonticola*	99.61	Bird	Nestling	Buccal	Great Tit	VZ08642	0
N832	01/06/2021	*Enterobacter kobei*	99.6	Bird	Nestling	Faecal	Blue Tit	Z822635	1
A862	08//06/2022	*Escherichia coli*	99.6	Bird	Adult	Back	Blue Tit	Z822686	1
N023	29/05/2021	*Pseudomonas koreensis*	99.6	Bird	Nestling	Back	Blue Tit	Z822629	1
N162	06/06/2021	*Leclercia adecarboxylata*	99.57	Bird	Nestling	Foot	Great Tit	VZ08622	0

Sample	Date Collected	Bacteria Identified	% Identity	Sample Type			Bird Ring Number	AOD Status 1=Present, 0=Absent
A1709_1	15/09/2020	*Raoultella ornithinolytica*	99.57	Bird	Adult	Foot	Great Tit	VZ08603	1
N563.3	25/05/2021	*Serratia fonticola*	99.57	Bird	Nestling	Back	Blue Tit	Z822667	0
N591	17/05/2021	*Escherichia marmotae*	99.56	Bird	Nestling	Buccal	Blue Tit	Z822609	0
A3607	04/11/2022	*Escherichia coli*	99.54	Bird	Adult	Back	Great Tit	VZ08654	0
N983	20/05/2022	*Serratia quinivorans*	99.53	Bird	Nestling	Back	Blue Tit	Z822677	1
N511	29/05/2021	*Pseudomonas koreensis*	99.52	Bird	Nestling	Buccal	Blue Tit	Z822631	1
A6012.2	14/11/2022	*Rahnella laticis*	99.52	Bird	Adult	Faecal	Great Tit	VZ08657	0
A6013	14/11/2022	*Escherichia coli*	99.51	Bird	Adult	Buccal	Great Tit	VZ08658	0
A454.3	23/12/2021	*Serratia fonticola*	99.51	Bird	Adult	Faecal	Blue Tit	Z822645	1
N653	20/05/2021	*Pseudomonas brenneri/Pseudomonas proteolytica*	99.5	Bird	Nestling	Back	Great Tit	VZ08618	1
T063	14/07/2020	*Raoultella planticola*	100	Tree	Active bleed				1
T1003	09/09/2020	*Raoultella planticola*	100	Tree	Inactive bleed				1
T113_1	05/08/2020	*Raoultella planticola*	100	Tree	Inactive bleed				1
T115_2	05/08/2020	*Raoultella planticola*	100	Tree	Inactive bleed				1
T183	12/08/2020	*Raoultella planticola*	100	Tree	Inactive bleed				1
T192	12/08/2020	*Raoultella planticola*	100	Tree	Inactive bleed				1

Sample	Date Collected	Bacteria Identified	% Identity		Sample Type	Bird Ring Number	AOD Status 1=Present, 0=Absent
T261_2	06/09/2020	*Raoultella planticola*	100	Tree	Inactive bleed		1
T271	29/08/2020	*Raoultella planticola*	100	Tree	Active bleed		1
T274	29/08/2020	*Raoultella planticola*	100	Tree	Inactive bleed		1
T351	06/09/2020	*Raoultella planticola*	100	Tree	Asymptomatic		1
T41_2	14/07/2020	*Raoultella planticola*	100	Tree	Active bleed		1
T42_2	14/07/2020	*Raoultella planticola*	100	Tree	Inactive bleed		1
T52_1	14/07/2020	*Raoultella planticola*	100	Tree	Inactive bleed		1
T571_1	09/09/2020	*Raoultella planticola*	100	Tree	Active bleed		1

Chapter 4 – The impact of Acute Oak Decline on avian gut microbiomes

4.1 - Abstract

Gut microbiomes can vary with age, health, diet and due to habitat and environmental variability. By analysing gut microbiomes, we can better understand microbial community composition, and in particular gain insights into which bacteria may be present within a sample in an unculturable state. Most of the work examining avian gut microbiomes has focussed on poultry, with work on wild birds only recently developing. In this chapter I aimed to examine how gut microbiomes of great tits and blue tits varied across habitats with differing levels of acute oak decline (AOD). Additionally, I aimed to examine the unculturable microbiome and determine if the AOD causative pathogens are present within the samples in an unculturable state. Faecal samples were collected from nestling and adult birds in areas with differing levels of AOD, and all DNA was extracted from these samples. Illumina Next Generation Sequencing was carried out using the 16S bacterial rRNA gene. Sequences were compared to the Silva database to assign taxonomy and identify any key bacterial species and genera. No further AOD pathogenic bacteria were identified within the samples following the culture-based analysis in Chapter 3. The taxonomic composition of these samples was analysed and compared across bird age, species, and the AOD status of the area where the sample had been taken, alongside alpha and beta diversity of the samples. Taxonomically the samples were very similar, however samples from advanced AOD sites had differing proportions of bacterial taxa than those from AOD negative and AOD absent sites, for example they had much higher proportions of Enterobacteriaceae than sites with none or moderate levels of AOD. Alpha diversity did not vary significantly across differing AOD statuses, however within adult samples beta diversity was significantly different. AOD status contributed very little to this result however, so it is likely there are unrecorded variables influencing this. The results indicate that the presence and severity of AOD does have a slight impact on avian gut microbial composition, but further work on is needed incorporating a wider set of environmental variables over a larger range of habitats and AOD scales.

Keywords: microbiome, Acute Oak Decline, Next Generation Sequencing, blue tit, great tit

4.2 - Introduction

The term microbiome encompasses the entire microbial community of a given environment, characterised by bacteria, fungi and viruses (the microbiota). Advances in culture-independent molecular sequencing technologies, such as Illumina Next Generation Sequencing (NGS) in the early 2000s, have allowed for more in-depth analysis of whole microbial communities. As such, microbiome research has grown rapidly in the past few decades, with an initial focus on human gut microbiomes for medical research, followed by more recent interest in other species (Grond et al., 2018).

The rate of research into avian microbiomes, specifically gut and cloacal, has been much slower compared to some other taxa. A review by Waite & Taylor (2015) found that, at that in 2015 there were only 23 papers which examined the role of biological and non-biological factors in shaping avian gut microbiomes, and by 2018 studies of gut microbiota in mammals were ten times that of birds (Grond et al., 2018). Wild bird microbiome research has increased in recent years, with a more up to date review indicating that between 2017 and 2020 over 100 papers were published on non-poultry avian microbiomes (Bodawatta et al., 2022). Research into microbial community assembly of birds is important in answering questions of how a bird's external environment can impact their microbiome, and what impacts this has on individual health. Of the research into avian gut microbiota, the overwhelming majority has focussed on poultry and other domestic birds (Grond et al., 2018), however these are not necessarily applicable to wild birds due to a range of differences in ecology and lifestyle, from the environments they inhabit to their inherent behaviours such as migratory species. The increasing research into avian microbiomes largely focuses on how a bird's environment can impact the diversity and composition of their microbiomes, and subsequent knock-on effects on host health and condition.

Gut microbial composition is known to be impacted by a variety of internal and external factors, including diet, genetics, lifestyle and environment (Bahrndorff et al., 2016; Debelius et al., 2016), and the composition of gut microbiomes have implications for development and health of their hosts (Peixoto et al., 2021). A link between gut microbiota and host health has already been established in mammals (Maurice et al., 2015), and shifts in relative abundance of certain bacterial taxa have been correlated with variance in body condition of birds such as house sparrows (*Passer domesticus*) (Teyssier et al., 2018). Microbiomes can influence and be influenced by the characteristics of the host, including the individual's behaviour and physiology

(Neuman et al., 2015; Williams et al., 2020), for example age has been identified as a driver of cloacal microbiome composition in female tree swallows (*Tachycineta bicolor*) (Hernandez et al., 2021).

4.2.1 - Nestling Microbial Acquisition

Nestlings acquire their gut microbiomes through a variety of mechanisms. Microbes can be acquired both vertically from parents (Colston, 2017; Renelies-Hamilton et al., 2021), and also horizontally from environmental sources (Cohen et al., 2020; Kwan et al., 2017). The initial gut microbiome of chicks is thought to be acquired either via transfer of microbiota from the mother's reproductive tract to the developing egg (Darolová et al., 2018; Dietz et al., 2020; Thiagarajan et al., 1994), or via trans-shell migration of eggshell and environmental microbiota after laying (Cook et al., 2003; Maki et al., 2020), or a combination of the two (Pedroso et al., 2016). Parents are also able to transmit pathogens to their progeny by contaminating the eggs with faecal bacteria during the nesting process, as has been documented in poultry (Cox et al., 2012). Little is known about the role of eggshell microbiome in birds, with conflicting research showing positive, negative and no effect of microbiome on hatching success (Hansen et al., 2015; Martín-Vivaldi et al., 2014; Schmitt et al., 2017). However, parental incubation behaviour and microbiome appear to be closely linked in some species within the *Paridae* family. Increased parental incubation of oriental tit (*Parus minor*) eggs increases the abundance of bacterial taxa that are known to produce antibiotics, which have the potential to reduce the growth of pathogenic bacteria on the eggshells and thereby can protect the hatchlings (Song et al., 2023), demonstrating a direct influence of parental investment and provisioning on their chicks' microbiomes.

4.2.2 - Environmental impacts on gut microbiomes

A review by (Waite & Taylor, 2015) determined that the environment plays a larger role in shaping the avian gut microbial composition more than any individual variation, and biological variables such as diet have less of an impact on the microbiota than the bird's environment. More recent reviews have indicated that avian gut microbiomes appear to be unique when compared to other vertebrates (Bodawatta et al., 2022). Compared to flightless birds and non-flying mammals, birds with flight have much more variation in their gut microbiomes, which is possibly due to the ability to exploit a much wider range of environments. Interestingly, a comparison of 900 vertebrate gut microbiomes found a convergence in the microbiomes of birds and bats, with many microbial

species being shared across taxa as opposed to being species specific as can be seen with non-flying mammals. Compared to other vertebrates, diet and body condition (determined by host body mass) does not seem to have a significant influence on the gut microbiomes of bats and birds (Song et al., 2020). Microbiomes of flightless and weak flying birds were more similar to those of non-flying mammals out of all the bird species analysed, demonstrating that flight appears to be a major driver of gut microbial composition.

A bird's environment plays a significant role in the formation of cloacal bacterial assemblages. Cross fostering experiments of great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) have demonstrated that environment is a stronger driving factor than relatedness in determining similarities in cloacal microbial communities across nestlings (Lucas & Heeb, 2005), with similar trends of stronger environmental influences being found in gut microbiomes of brood-parasitic brown-headed cowbird (*Molothrus ater*) nestlings (Hird et al., 2014). Migrating birds have been documented as having differing gut microbiota depending on where they are sampled during different times of their migration, and these changes in microbiota were attributed to the change in environment (Lewis et al., 2016). It is not only gut microbiomes that are influenced by location; cloacal, feather, skin and nest microbiomes of woodlarks (*Lullula arborea*) and skylarks (*Alauda arvensis*) have been found to be shaped by the local environment (Van Veelen et al., 2017).

Microbial composition of environments themselves, such as soil microbial communities, can be impacted and shaped by environmental pressures. Changes in temperature, acidification etc. which are linked to climate change can affect the microbes which are available in the environment (Fields et al., 2005; Zogg et al., 1997). Environmental changes and pressures such as urbanisation (Gadau et al., 2019; Murray et al., 2020), captivity (Alba et al., 2023; Grieves et al., 2022; Kelly et al., 2022; West et al., 2022), and environmental pollutants and contaminants such as antibiotics can alter avian gut microbiomes (Comizzoli et al., 2021; Ruuskanen et al., 2020). Habitat quality can influence stress levels in some species of bird (Cīrule et al., 2017; Marra & Holberton, 1998), and stress levels can impact microbial composition (Noguera et al., 2018) and reproductive success (Vitousek et al., 2014). By experimentally increasing stress levels in wild yellow-legged gull (*Larus michahellis*) chicks, researchers were able to alter their gut microbiome composition, showing that increased stress levels can impact microbial diversity in this species.

It is possible that smaller, more localised pressures on the environment such as the presence of tree diseases including the bacterial induced syndrome, Acute Oak Decline (AOD), can shape microbial communities in such a way that there are knock-on effects on the organisms inhabiting these environments. AOD is caused by three species of bacteria within the order Enterobacterales - *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Rahnella victoriana.* AOD can alter oak dominated habitats by changing the woodland composition through die back of oak trees, which could have knock-on effects for birds and other organisms that commonly associate with oak. So far there is no documented evidence of the role of AOD in influencing the microbiome of the wider environment, and this is likely the first study that combines tree diseases with other microbiomes of other organisms.

4.3 - Aims and hypotheses

This chapter aims to

1. Identify the three bacteria associated with acute oak decline within faecal samples of birds collected across a woodland with different levels of AOD, using culture-independent methods
2. Assess the role that AOD has on shaping the taxonomic composition and microbial diversity on avian gut microbiomes.

It is hypothesised that the AOD associated bacteria will be present within samples taken from areas containing AOD, and that samples from these areas will have a higher proportion of bacteria within the order Enterobacterales than areas without the disease. Furthermore, I hypothesise that the microbiomes of the guts of birds inhabiting woodlands containing AOD will differ significantly to those that inhabit areas free of any symptoms of this tree disease.

4.4 - Methods

Faecal samples collected from nestlings and adult birds as detailed in Chapter 2 were used for Illumina Next Generation Sequencing to identify microbial communities.

4.4.1 - DNA extraction

DNA was extracted from faecal samples using the Qiagen QIAamp DNA Stool Mini Kit following manufacturer's protocols. Between 180-220 mg of faecal sample was used per extraction if there was sufficient sample. DNA was extracted from 95 faecal samples, with one negative control.

4.4.2 - PCR1

Extracted DNA was subjected to PCR (referred to as PCR1) amplifying the v3-v4 region of the 16S rRNA gene, using tailed Illumina sequencing primer 515F and 806R, details of which can be found below. A negative sample was used in each PCR run, which included water in place of extracted DNA. PCR stages are outlined in Table 1 and primer details in Table 2. Sample preparation was carried out in a PCR hood that had been bleached and sterilised using UV to minimise environmental contamination.

Table 1. Stages of PCR1

PCR Stage	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	15 min	1
Denaturation	94	30s	30
Annealing	58	90s	
Extension	72	2 min	
Final Extension	72	10 min	1
End/Hold	4	Hold	-

Negative controls containing sterile water in place of DNA were incorporated from PCR1 onwards to identify any bacteria that could contaminate the kit and were not from the samples.

PCR products were visualised using gel electrophoresis on a 1% agarose gel stained with Ethidium Bromide (1% w/w), ran at 90 v for 45 minutes. Gels were visualised using a gel visualiser to identify any successfully amplified DNA.

The product from PCR1 was cleaned using Promega Pronex magnetic beads following manufacturer protocols to remove any reagents left over from PCR1.

Table 2. Details of primers used in PCR1.

Forward Primer	Primer Name	515F_tag
	Sequence 5'-3'	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA
Reverse Primer	Primer Name	806R_tag
	Sequence 5'-3'	GTGACTGGAGTTGACACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT

4.4.3 - PCR2

Cleaned PCR product from PCR1 was used for PCR2 using tailed dual-plexed primers Fi5 and Ri7 which provide each sample with a unique combination barcode. Full details of these primers can be seen below, along with an example of a unique barcode identifier. 96 unique combinations were used as 96 samples were sequenced. A negative sample was used in each PCR run, which included water in place of extracted DNA. The stages of PCR2 can be seen in Table 3 and primer details in Table 4.

Following PCR2, samples were subjected to automated electrophoresis using an Agilent TapeStation to quantify the product size and confirm the unique identifiers had been added successfully. The concentration of each PCR2 product was determined using a BioTek Fluorometer, which allowed samples to be diluted accordingly when pooling, with a final DNA quantity of 40 ng per sample.

Samples were combined into four pools, with 24 samples in each. These pools were cleaned as above, using Promega Pronex magnetic beads following manufacturer protocols to remove any leftover PCR reagents.

Table 3. Stages of PCR2

PCR Stage	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	15 min	1
Denaturation	98	10s	12
Annealing	65	30s	
Extension	72	30s	
Final Extension	72	5 min	1
End/Hold	4	Hold	-

Table 4. Details of primers used in PCR2. An example of a unique barcode identifier is highlighted in red. 96 distinct barcodes were used to distinguish samples from one another.

Forward Primer	Primer Name	Fi5_01
	Sequence 5'-3'	CAAGCAGAAGACGGCATACGAGATTATCTTCTCGGTGAC TGGAGTTCAGACGTGTGCTCTTCCGATC*T
Reverse Primer	Primer Name	Ri7_01
	Sequence 5'-3'	AATGATACGGCGACCACCGAGATCTACACCGTCGCCTA TACATCTTTCCCTACACGACGCTCTTCCGATC*T

4.4.4 - qPCR

Each pool was subjected to qPCR to quantify the DNA (Table 5). Serial dilutions were made for each library; 100, 1000 and 10000 – fold. This was repeated twice for each pool, which gave three independent dilutions of the library. By using repeats and running a series of serial dilutions concurrently, the concentration of DNA in each pool was determined. These pools were subsequently pooled in equimolar amounts into one tube with a final concentration of 4 nM.

Table 5. Stages of qPCR

PCR Stage	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	30 s	12
Annealing	60	45 s	
Extension	72	30 s	
Final Extension	72	5 min	1
End/Hold	4	Hold	-

4.4.5 - Sequencing

The final pool was sequenced using Illumina Next Generation Sequencing (NEOF, University of Liverpool).

4.4.6 - Bioinformatics

Raw, paired-end DNA sequences were quality filtered and adapters removed using TrimGalore (https://github.com/FelixKrueger/TrimGalore). Cutadapt (Martin, 2011) was used to remove primer sequences. Sequences were analysed using the DADA2 package (v.1.8) (Callahan et al., 2016) in R, which generated an amplicon sequence variant (ASV) table listing each unique sequence. This was compared to the SILVA database (v.138.1) (Quast et al., 2013) to assign each ASV to taxonomic levels from kingdom to genus. The SILVA database also categorises ASVs which have been recovered from mitochondria and chloroplasts, therefore any of these matches were removed. Each sample was compared to the unique ASVs to determine how many of each were detected in each sample, generating an ASV count table for each sample. ASVs that were grouped within the same genus as bacteria associated with AOD (*Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*) were compared to the NCBI BLAST (Basic Local Alignment Search Tool) 16S ribosomal RNA sequence database (NCBI, 2023) to identify to species level where possible.

Samples were rarefied to a minimum read depth of 50,000 reads to ensure consistency across the samples and avoid skew by samples with very high or very low numbers of reads. This resulted in two samples being removed due to low read numbers.

4.4.7 - Statistics

Bacterial community composition was visualised across sample types across different taxonomic levels, from phylum to genus. Taxa relative abundance analysis was carried out in R using the phyloseq (v.1.46) and microbiome (v.1.23.1) packages, first transforming the ASV tables into relative abundance tables, which provides the fractional relative abundances for each ASV compared to 1. Relative abundances could be visualised using ggplot2 (v.3.4.4) in R. To reduce the amount of noise in the taxa plots, rare taxa were aggregated into an “other” category using the microbiome package. This included ASVs that represented less than 0.01 relative abundance of the sample, in fewer than 5% of the samples. Differences in taxonomy relative abundances were analysed using Kruskal-Wallis test, in R package rstatix (v. 0.7.2). Alpha diversity was plotted overall and by three variables; AOD status (absent or present from the plot where the faecal sample was collected), species of bird (blue tit or great tit), and age of the bird (adult or nestling).

Alpha and beta diversity analyses were carried out using the phyloseq and microbiome packages. The distribution of ASVs according to the following alpha diversity metrics were plotted using ggplot2 - Observed, Chao1, and Shannon diversity. Pairwise wilcoxon rank sum tests were carried out using all three alpha diversity metrics across the three variables, AOD status, age and species. Beta diversity was assessed using the weighted unifrac distance metric in phyloseq, and visualised using multidimensional scaling using ggplot2. To assess differences in beta diversity, PERMANOVA analysis was carried out using the vegan (v.2.6.4) package, using both weighted UniFrac and Bray-Curtis dissimilarity matrices. Both alpha and beta diversity were plotted according to all samples, and by three variables; AOD score (absent, moderate or advanced within the plot where the faecal sample was collected), species of bird (blue tit or great tit), and age of the bird (adult or nestling).

Negative controls were analysed alongside the samples to determine if there had been cross contamination during the sample preparation and sequencing process. Any microbial communities detected in negative samples were compared across the test samples, and where appropriate they were disregarded from analysis.

4.5 - Results

A summary of the metadata for the successfully sequenced samples can be seen in Table 6.

Table 6. A breakdown of the metadata for the successfully sequenced samples and their counts.

Species	Age	AOD Status	Count
Blue Tit	Adult	None	5
Blue Tit	Adult	Moderate	9
Blue Tit	Adult	Advanced	0
Blue Tit	Juvenile	None	19
Blue Tit	Juvenile	Moderate	16
Blue Tit	Juvenile	Advanced	3
Great Tit	Adult	None	17
Great Tit	Adult	Moderate	3
Great Tit	Adult	Advanced	5
Great Tit	Juvenile	None	8
Great Tit	Juvenile	Moderate	4
Great Tit	Juvenile	Advanced	3

A total of 10,318,936 reads within 8,611 unique ASVs were detected from all the samples, with an average of 112,162 reads per sample (min. 2,735 and max. 256,278). When mitochondria and chloroplasts were removed, these numbers reduced to 10,083,813 reads within 8,354 ASVs with an average of 109,606 sequences per sample (min. 2,735 and max. 251,358).

Samples were rarefied to 50,000 reads to normalise the data, which excluded two samples from the dataset which had a small number of reads.

Taxa classified as ‘Unknown’ indicates ASVs that were not able to be classified to that taxonomic level, and ‘Other’ indicates ASVs that were present in small quantities (detected as < 1% abundance in < 5% of the samples) that have been grouped.

4.5.1 – Taxonomy

In all AOD conditions, over half of the bacterial phyla represented Proteobacteria, and over half of the bacterial classes were in Gammaproteobacteria. The five most abundant taxa in each taxonomic level across different AOD sites are shown in Table 67, and a comparison of these according to differing levels of AOD can be seen in Figure 1. There were no significant

differences in relative bacterial abundance across AOD sites at any taxonomic level. A full breakdown of all taxonomic classes can be found in supplementary material 4.1.

Table 7. Average percentage abundance of five most common bacterial taxa in samples collected from sites with differing levels of Acute Oak Decline (AOD). P-values are representative of the Kruskal-Wallis test, which compares if the relative abundance of bacterial taxa differed across AOD categories.

	Average Percentage Abundance ± Standard Error			
	No AOD	**Moderate AOD**	**Advanced AOD**	**p-value**
Phylum				
Proteobacteria	60.01 ± 4.29	57.50 ± 5.71	57.79 ± 7.49	0.923
Firmicutes	33.21 ± 4.14	35.89 ± 5.59	33.23 ± 7.41	0.909
Actinobacteriota	5.95 ± 1.19	6.22 ± 2.21	5.55 ± 1.74	0.899
Other	0.44 ± 0.14	0.25 ± 0.08	0.18 ± 0.08	0.910
Bacteroidota	0.39 ± 0.18	0.18 ± 0.07	3.24 ± 2.51	0.421
Class				
Gammaproteobacteria	59.22 ± 4.32	56.94 ± 5.72	57.26 ± 7.45	0.919
Bacilli	25.76 ± 3.07	29.29 ± 3.07	16.94 ± 3.36	0.428
Clostridia	7.44 ± 2.04	6.59 ± 2.27	16.28 ± 5.85	0.300
Actinobacteria	5.79 ± 1.15	6.12 ± 2.21	5.46 ± 1.72	0.882
Alphaproteobacteria	0.75 ± 0.22	0.53 ± 0.17	0.51 ± 0.22	0.649
Order				
Enterobacterales	33.61 ± 3.83	23.46 ± 3.39	32.93 ± 9.03	0.376
Pseudomonadales	23.94 ± 3.27	32.94 ± 5.45	21.76 ± 4.75	0.555
Lactobacillales	11.87 ± 2.55	12.88 ± 3.54	5.14 ± 2.06	0.645
Bacillales	8.71 ± 2.05	11.75 ± 3.51	5.64 ± 2.23	0.926
Micrococcales	5.38 ± 1.07	5.58 ± 2.18	5.28 ± 1.69	0.722
Family				
Pseudomonadaceae	21.19 ± 3.28	28.19 ± 5.75	18.78 ± 4.56	0.953
Unknown	15.82 ± 2.99	15.49 ± 3.68	5.95 ± 2.37	0.195
Carnobacteriaceae	9.32 ± 2.24	6.42 ± 2.03	4.05 ± 1.89	0.480
Enterobacteriaceae	8.97 ± 2.38	7.96 ± 1.81	22.76 ± 9.56	0.474
Planococcaceae	7.60 ± 1.77	10.10 ± 3.02	4.50 ± 2.03	0.879

	Average Percentage Abundance ± Standard Error			
	No AOD	**Moderate AOD**	**Advanced AOD**	**p-value**
Genus				
Pseudomonas	21.18 ± 3.28	28.18 ± 5.75	18.78 ± 4.56	0.953
Unknown	20.78 ± 3.14	20.90 ± 3.74	11.97 ± 3.23	0.363
Carnobacterium	9.31 ± 2.24	6.40 ± 2.03	4.03 ± 1.89	0.473
Other	6.71 ± 1.52	3.87 ± 0.97	9.55 ± 4.04	0.238
Klebsiella	4.40 ± 1.20	4.83 ± 1.43	7.72 ± 5.32	0.727

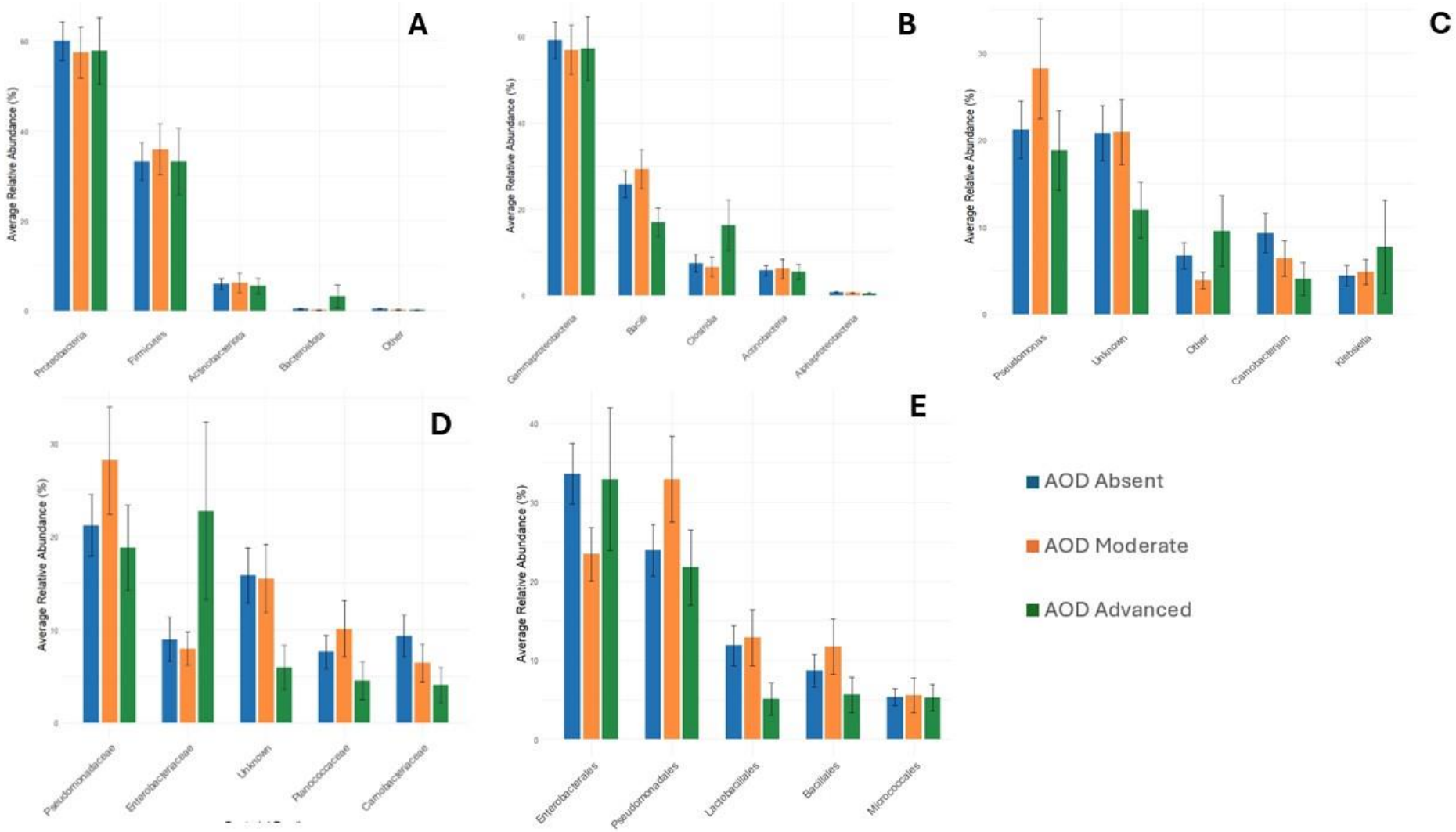

Figure 1. Average relative abundances of the five most commonly occurring bacterial taxa, by A) Phylum, B) Class, C) Order, D) Family, E) Genus.

At a phylum and class level, the two most abundant taxa were consistent across all levels of AOD, however there was some variation at order level, with Enterobacterales being the most abundant order in samples taken from areas with no AOD and advanced AOD, however in sites where there was moderate AOD, Enterobacterales were the second most abundant order after Pseudomonadales. This was the only instance where there was variation in the two most abundant taxa within a taxonomic level. There was not much variation in the relative abundance of taxa depending on AOD level, and the largest variability in relative abundance between different AOD levels was 15% (Fig. 2). Figure 2 shows that sites with moderate scores for AOD had higher proportions of the bacterial family *Psuedomonadaceae*, whereas sites with advanced AOD had higher proportions of *Enterobacteriaceae.* A full breakdown of the relative abundance of taxa across AOD levels can be found in supplementary material 4.2 - 4.6.

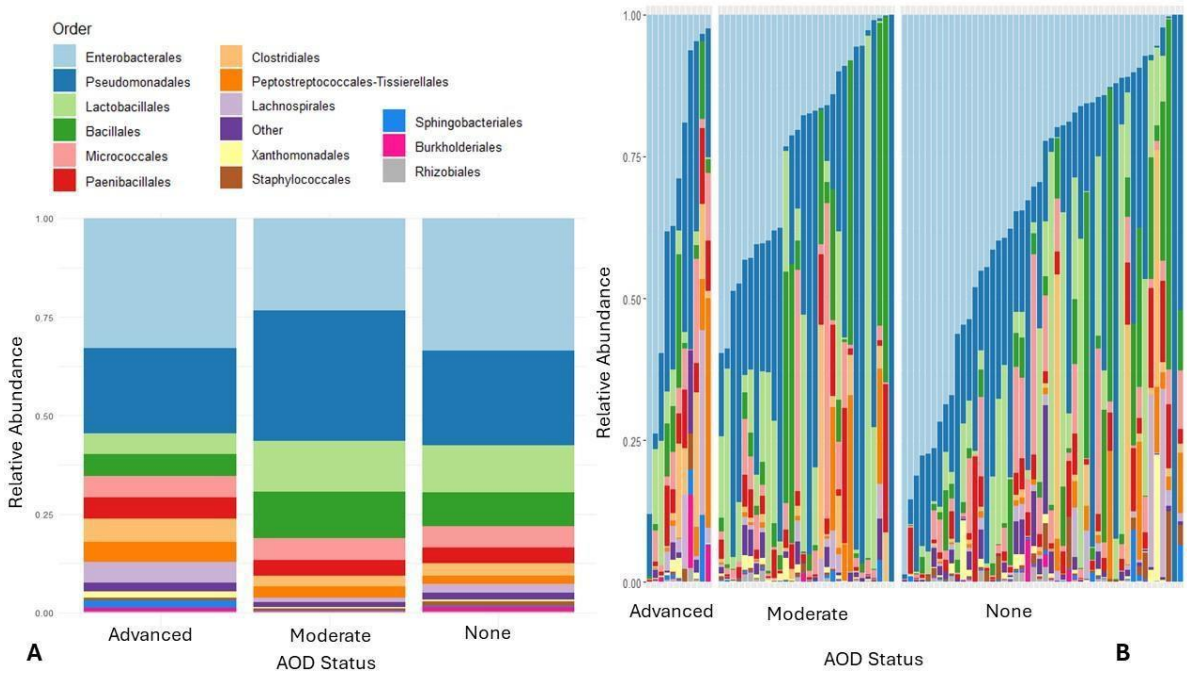

Figure 2. Relative abundance of bacterial orders across samples collected from areas with differing levels of AOD. A) Average relative abundance across samples, B) Relative bacterial orders abundance across all samples.

All AOD bacteria belong to the order Enterobacterales, which was the most abundant order in sites without AOD, and in sites with "Advanced" AOD. In sites which were scored as "Moderate" for AOD, the most abundant order was Pseudomonadales. In all samples over 50% of bacteria belong to the orders Enterobacterales and Pseudomonadales.

The relative abundance of family and genera within Enterobacterales can be seen in Table 8 and Figure 3. These results show shifts in relative abundance of bacterial taxa in AOD advanced sites compared to sites with none and moderate AOD. Notably bacteria within the family *Enterobacteriaceae* represents over half of the bacterial community in AOD advanced plots, which is of particular interest as this is the family that the AOD pathogenic bacteria are part of.

Table 8. Average percentage abundance of bacterial taxa within the order Enterobacterales, in samples collected from sites with differing levels of Acute Oak Decline (AOD).

	Average Percentage Abundance ± Standard Error			
	No AOD	Moderate AOD	Advanced AOD	p-value
Family				
Unknown	45.15 ± 5.39	45.25 ± 8.03	30.17 ± 14.64	0.244
Enterobacteriaceae	29.89 ± 4.88	32.56 ± 6.21	54.60 ± 15.65	0.159
Erwiniaceae	16.74 ± 4.30	16.01 ± 5.86	2.81 ± 1.64	0.762
Yersiniaceae	7.14 ± 3.20	5.54 ± 4.38	0.56 ± 0.32	0.375
Hafniaceae	0.65 ± 0.38	0.43 ± 0.25	11.80 ± 11.44	0.235
Other	0.43 ± 0.18	0.20 ± 0.09	0.06 ± 0.06	0.605
Genus				
Unknown	49.40 ± 5.13	49.31 ± 7.58	33.62 ± 14.32	0.290
Klebsiella	15.43 ± 3.08	19.25 ± 5.25	20.75 ± 11.64	0.647
Buttiauxella	10.42 ± 3.29	7.95 ± 3.36	29.48 ± 15.04	0.157
Pantoea	8.68 ± 2.98	5.35 ± 3.05	1.46 ± 0.79	0.771
Erwinia	7.30 ± 2.70	10.24 ± 5.33	1.16 ± 0.77	0.729
Yersinia	7.04 ± 3.20	5.36 ± 4.39	0.53 ± 0.30	0.422
Other	0.84 ± 0.32	1.45 ± 1.11	1.19 ± 1.01	0.992
Hafnia-Obesumbacterium	0.65 ± 0.38	0.43 ± 0.25	11.80 ± 11.44	0.235
Franconibacter	0.24 ± 0.12	0.65 ± 0.63	0.03 ± 0.03	0.988

There were no specific matches for the three AOD associated bacteria in the ASVs identified. No ASVs for the genus *Brenneria* were detected. Two bacteria within the *Gibbsiella* genus were found but these were not identified as *Gibbsiella quercinecans*. Six ASVs in the *Rahnella* genus were found, but none of these were a match to *Rahnella victoriana*. Two of these ASVs

matched against *Rahnella victoriana* using the NCBI BLAST database, but these ASVs also yielded matches for 27 other *Rahnella* species.

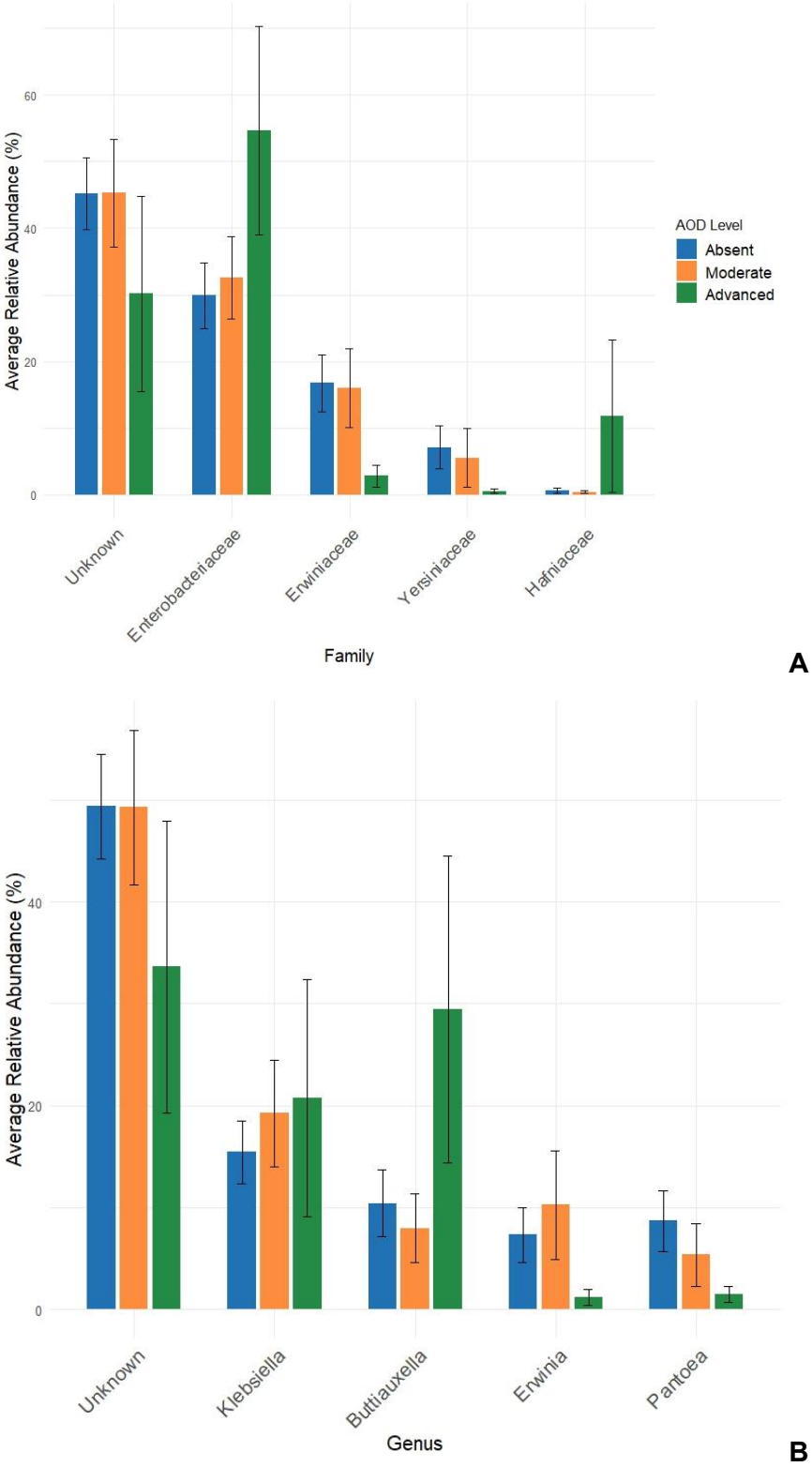

Figure 3. Average relative abundances of the five most commonly occurring bacterial taxa within the order Enterobacterales, by A) Family, B) Genus.

4.5.2 - Alpha diversity

Alpha diversity measures how diverse the samples are within themselves, for example how many ASVs are present within the sample. Alpha diversity was analysed using pairwise comparisons using Wilcoxon rank sum test across three measures of alpha diversity - Observed, Chao1 and Shannon (Table 9). None of the diversity metrics indicated that there was a difference between alpha diversity in any of the AOD levels, however the difference in alpha diversity was much smaller in the AOD positive and negative samples than when compared to the negative controls.

Table 9. Statistical output of pairwise Wilcoxon rank sum test based on different diversity measures. Numbers represent the p-values of the associated pairwise comparisons. Values <0.05 represent a significant difference between the alpha diversity in samples from sites with differing levels of AOD. Three different measures of alpha diversity are used – Observed diversity, Chao1, and Shannon diversity.

Diversity Index	Pairwise Wilcoxon rank sum test output
Observed diversity	Advanced Moderate Moderate 0.023 - None 0.023 0.881
Chao1	Advanced Moderate Moderate 0.461 - None 0.424 0.463
Shannon diversity	Advanced Moderate Moderate 0.497 - None 0.401 0.494

Average alpha diversity across the different sample types can be seen in Figure 4, where there does not appear to be a large difference in diversity between samples from sites with differing levels of AOD. Data were also grouped and analysed according to the age of the bird sampled (adult or nestling), and by species (great tit or blue tit), but no differences in alpha diversity were found (see Supplementary Material 4.7 and 4.8).

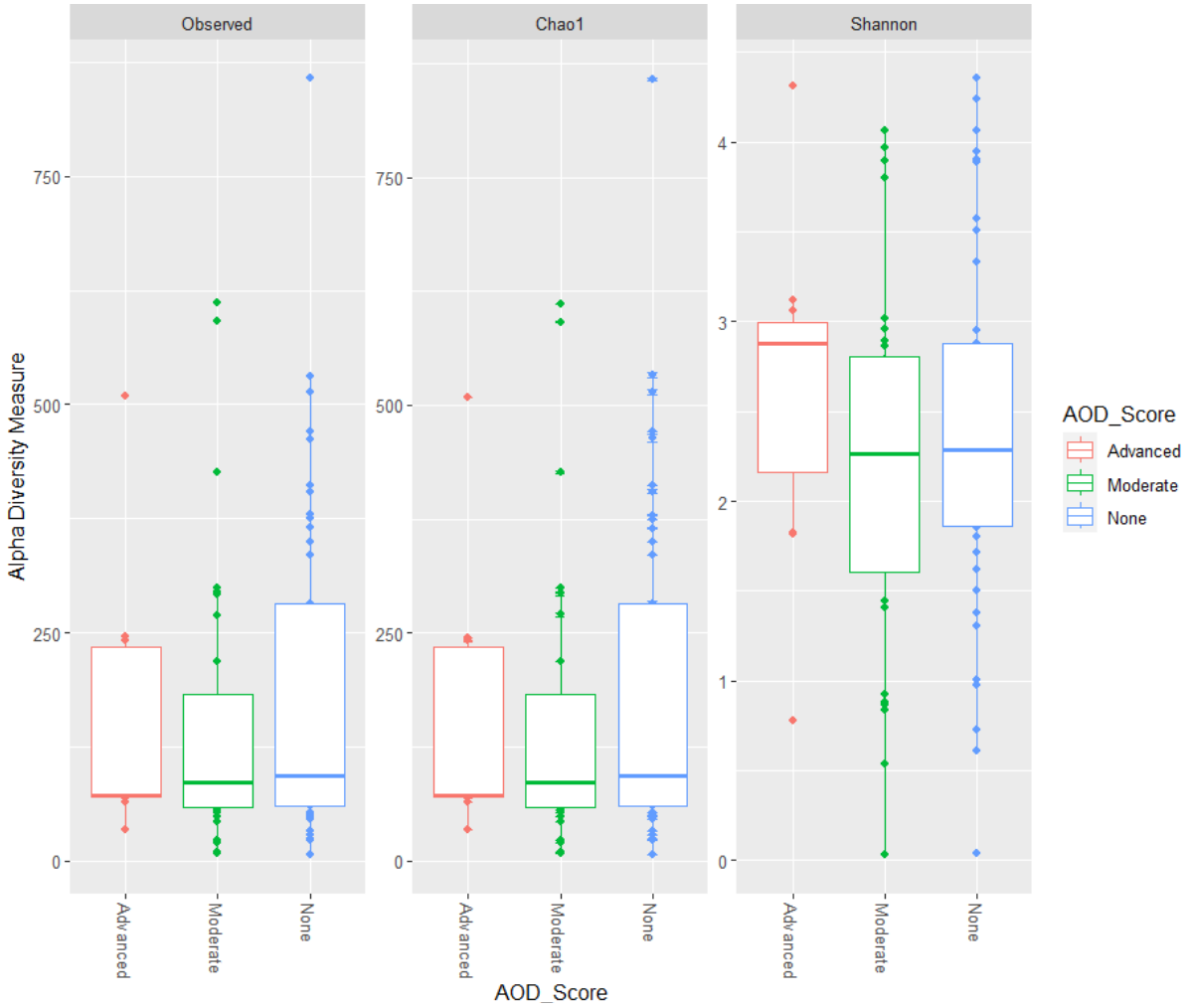

Figure 4. Alpha diversity of samples collected from areas with differing levels of AOD

4.5.3 - Beta diversity

Beta diversity is a measure of how bacterial diversity varies between different samples. By grouping these samples, we can investigate how similar microbial diversity is between samples taken from sites with differing AOD statuses.

Analysis of beta diversity using PERMANOVA showed that the samples did not significantly differ from each other - Weighted UniFrac (DF = 2, F = 1.1577, R^2 = 0.02622, p = 0.242), Bray Curtis (DF = 2, F = 1.0425, R^2 = 0.02367, p = 0.3864) indicating there likely is not an effect of AOD status on avian gut microbial composition. Figure 5 shows no evidence of microbial community clustering due to AOD status in the samples, as all sample groups overlap.

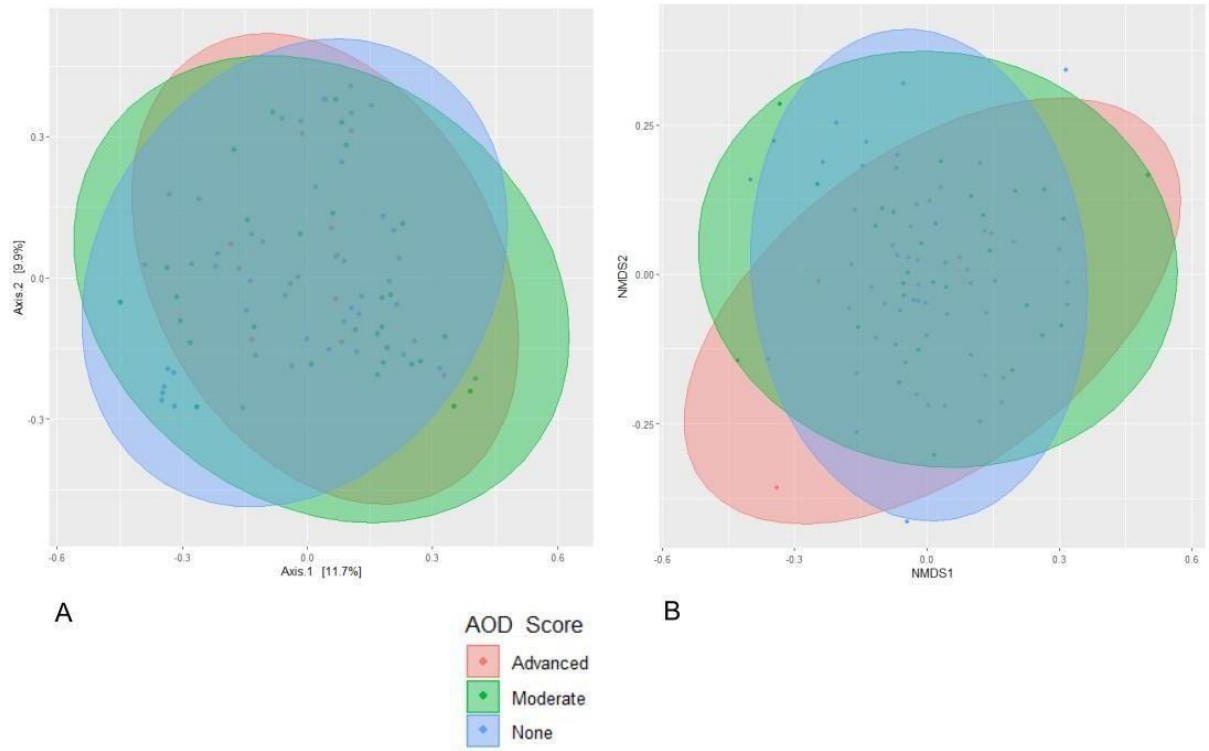

Figure 5. Beta diversity of samples from areas with differing levels of AOD, represented by A) Non-metric multidimensional scaling (NMDS) from weighted-unifrac dissimilarity scores B) Bray-Curtis dissimilarity indices

Samples were grouped into their unique species and age classifications and the effect of AOD Score on Beta diversity was analysed again using PERMANOVA. The only significant effect of AOD score on beta microbial diversity was within the adults, Weighted UniFrac - (DF = 2, F = 1.9698, R^2= 0.02622, p = 0.006), Bray Curtis - (DF = 2, F = 1.8359, R^2 = 0.10013, p = 0.005). The results indicate that while bird age had a significant effect on bacterial community structure, the effect size (R^2) is small at 2-10%. Figure 6 shows the community composition of adult faecal samples taken from differing levels of AOD, however no distinct clustering patterns can be seen, with substantial overlap for all AOD levels examined using both NMDS and Bray-Curtis dissimilarity.

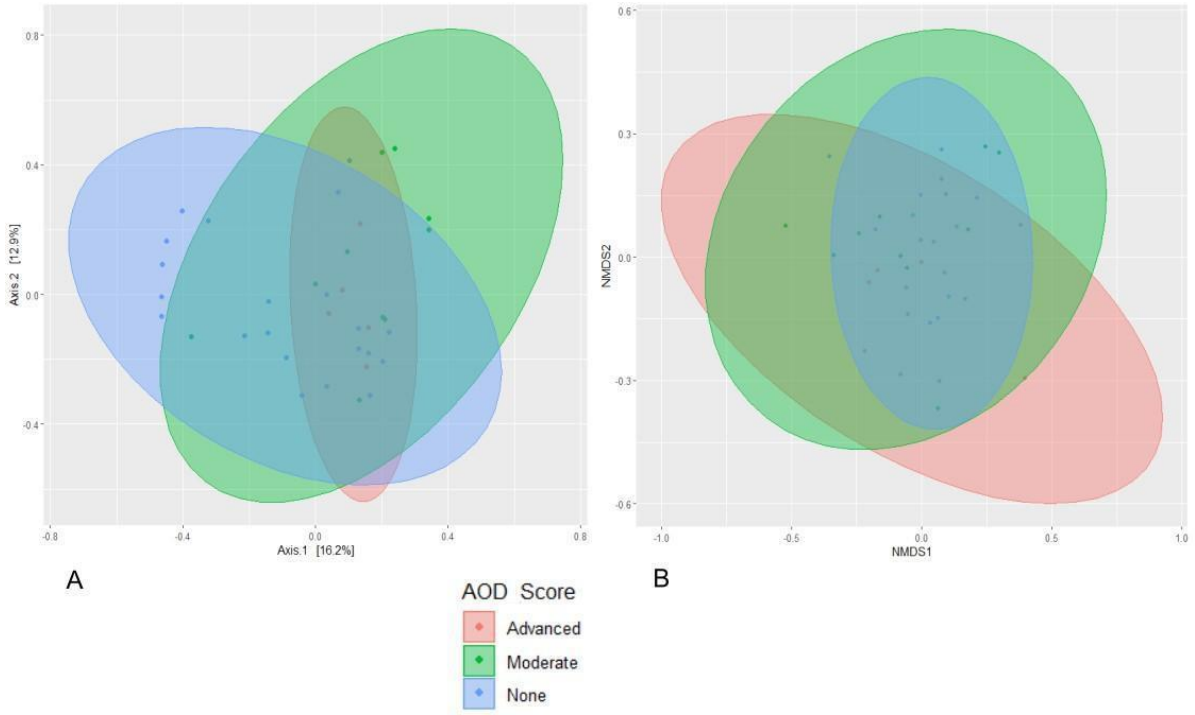

Figure 6. Beta diversity of adult samples from areas with differing levels of AOD, represented by A) Non-metric multidimensional scaling (NMDS) from weighted-unifrac dissimilarity scores B) Bray-Curtis dissimilarity indices

Beta diversity was analysed for adults and nestlings separately, as well as for blue tits and great tits separately, with no significant clustering. Diversity matrices for these analyses can be seen in Supplementary material 4.8 – 4.10, however not enough datapoints were available to confidently group the AOD Advanced sampled in the blue tit data (n=3).

4.4 - Discussion

This chapter aimed to determine if the bacteria associated with acute oak decline - *Brenneria goodwinii, Gibbsiella quercinecans* and *Rahnella victoriana* could be detected within avian faecal samples, and to determine how the presence of AOD within bird's habitat shapes its taxonomic diversity.

4.6.1 - Taxonomic diversity

Relative taxonomic abundance of microbial communities was examined in relation to differing levels of acute oak decline. The results from the taxonomic assessments are consistent with other research into avian gut microbiomes that indicate the shared core microbiota of wild birds is dominated by members of four major phyla - Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria (Grond et al., 2018). The relative abundances of these phyla is consistent

with some studies of blue tit and great tit gut microbiomes (Drobniak et al., 2022; Kropáčková et al., 2017), but also differs from other studies (Davidson et al., 2021; Dion-Phénix et al., 2021), indicating that these communities are not necessarily species specific and there are other influences on gut microbial composition. Grond et al., (2018) also compared bacterial taxonomic composition at a class level across a range of bird species, which highlighted substantial inter and intraspecific variation. Currently there are few comprehensive studies to determine a true core microbiome for species such as blue tits and great tits.

Firmicutes and Proteobacteria are the two most abundant phyla across all samples, making up over 90% of the bacteria isolated. These phyla contain different species of pathogenic bacteria which have been isolated from wild birds (Benskin et al., 2009). Pseudomonadaceae being the most abundant bacterial family across samples is consistent with findings from other studies examining microbial communities of nests and eggs of blue tits and great tits (Goodenough & Stallwood, 2010), and *Pseudomonas* is known to be a commonly occurring bacterial genus found in blue tits (Drobniak et al., 2022).

4.6.2 - Presence of AOD associated bacteria

I hypothesised that putative AOD pathogens would be present within samples taken from areas with higher levels of AOD, and that if these bacteria were found they would be present at higher levels compared to non-AOD sites. As discussed in section 3.6.2.1 of this thesis, three *Rahnella victoriana* species were isolated from great tit faeces, but the additional culture-independent analysis carried out in this chapter did not provide evidence suggesting presence of any of the AOD pathogens.

The v3-v4 region of the bacterial 16S rRNA gene was used in this work for Illumina Next Generation Sequencing. This represents an important hypervariable region, making it useful for distinguishing between bacterial taxa, although the region itself is small at approximately 460 bp whereas the full 16S gene is approximately 1500 bp. The hypervariability and short length of the v3-v4 region makes it a useful region to examine when distinguishing a large number of different bacterial genera within a sample, however it is not able to differentiate bacteria at a species level (Johnson et al., 2019). Recent work has identified other variable regions of the 16S gene as being able to discriminate between bacterial species than the v3-v4 region (Hiergeist et al., 2023). Despite this, no bacteria of the genus *Brenneria* were detected, and those detected from the genera *Gibbsiella* and *Rahnella* were not reliably matched to the AOD associated bacteria. By sequencing longer fragments of the bacterial genome, a higher sequencing depth could be achieved, thereby making species identification more reliable, however this was not possible for this research due to time and

funding constraints.

Techniques such as Next Generation Sequencing (NGS) are useful for determining presence/absence of certain taxa within a sample, however the overall abundance of these taxa within a sample cannot be determined this way and would require more in-depth analysis. Arguably a more important question in this research than presence/absence analysis would be how abundance and concentration of the same bacterial species (determined by their unique ASVs) vary according to AOD status. One could examine if there is a relationship between the strength of AOD within a site, and the overall abundance of the AOD associated bacteria. Combining sequencing technologies such as NGS with other methods such as flow cytometry could allow for abundance measurements of bacterial species of interest to be analysed (Heyse et al., 2021) alongside diversity metrics.

4.6.3 – Alpha and beta diversity

The results of this analysis have not detected any statistically significant differences in alpha of the microbial communities as caused by differences in AOD status of the woodland the samples were taken from. Alpha diversity is an indication of species richness, therefore the results indicated samples taken from sites both with and without AOD had no significant difference in the number of bacterial species present within the microbiome.

When analysing beta diversity, there was a significant difference between different diversity in AOD samples when analysed just within the adult samples. Beta diversity is a measure of how microbial communities differ in relation to one another, so there was no difference in the microbial composition of samples from AOD positive sites compared to those from AOD negative sites. Beta diversity was found to be significantly different between differing AOD sites when just the adult samples were analysed, however the effect size was very small (between 2-10% of variability was attributed to the AOD level). It is worth noting that these differences may be influenced by asymmetrical design in the sample collection, as there was not an equal number of samples taken across the differing levels of AOD due to difficulties in catching wild birds. There could also be additional environmental variables aside from AOD which contribute to the significance values seen here.

One reason for the absence of variation in alpha and beta diversity across AOD levels could be that the sites classified as AOD positive and negative were not distinct enough from each other. Chapter 3, Figure 9, shows the distribution of plots which contained AOD. Care was taken to ensure that plots were not overlapping, however the plots were set up in such a way that breeding territories would not overlap. These plots were then used throughout the study as discrete experimental plots, therefore the minimum distance two plots could be from each other would be 100 m, despite the fact that adults and fledglings may move much further

distances outside of breeding season. It is likely that this may be too fine of a scale, as post fledging great tits have been known to disperse over 1 km away from where they hatched (Dhondt, 1979; van Overveld et al., 2011), the distance of which can be impacted by parental behaviour (Dingemanse et al., 2003; Matthysen et al., 2010). Future studies should take care to ensure the scales at which they set the parameters for their sampling sites accurately reflect real world conditions and should possibly use geographically distinct sites for comparisons. For this project, several attempts were made to sample from birds at a control site at the University of Reading which had no symptoms of AOD, however sufficient sampling was unsuccessful due to a low catch rate of the target species. Furthermore, as discussed in chapter 3, the disease assessments carried out for each of the plots could be improved, therefore by analysing the microbiomes in light of different measures of AOD status of each plot, different results may be found.

Gut microbiome studies can produce interesting results when the same individuals are sampled over time due to ontogenetic shifts in microbiome composition during development (Teyssier et al., 2018) and can be altered due to life history traits and various external factors such as food availability (Davidson et al., 2021). By monitoring microbial community changes over time, a better idea of a core microbiome can be established and variations away from this can be examined in light of short and long term environmental changes (Escallón et al., 2019; Grond et al., 2019). Establishing a core microbiome for birds is, however, challenging due to the broad range of ecological niches, adaptations and morphologies of birds, and there is huge variation in the abundance and diversity found within microbiomes of different species. It would be interesting to see how a bird's microbiome varies and develops across woodlands with different levels of acute oak decline, and even other tree diseases and woodland qualities. Of course, in wild populations with varying recruitment levels, repeated monitoring is especially difficult, particularly once the nestlings have fledged. Monitoring gut microbial variance across time in a wild population would require significant sampling effort and resources to ensure a good sample size. By fitting PIT trackers onto birds and capturing them for subsequent samples could allow for repeated gut microbiome sampling to be combined with longevity data, however this would require a substantial amount of resources as cavity nesting birds have been documented as having 50% mortality of fledglings within the first few weeks out of the nest (Naef-Daenzer et al., 2001). The samples analysed in this chapter contained faecal samples from both adult and nestling birds, and were analysed both together and separately, however these microbiomes cannot be treated as the same due to variances in the gut microbiome with age. Nestlings show substantial shifts in their gut microbiome during their time in the nest, therefore variability of microbiomes in early life is characteristic of birds (Teyssier et al., 2018).

4.6.4 – Future Work

Faecal samples have widely been used as a proxy for sampling intestinal microbiota, as they can be obtained without killing the individual and yield large amounts of bacterial DNA compared to swab samples, however it is easy for faecal samples to be contaminated by the environment or even other chemicals excreted by the cloaca (Crouch et al., 2020), which could affect the stability of the gut microbial community. A bird's cloaca serves as a singular external orifice for the rectum, urinary and genital ducts, making them difficult to examine in relation to gut microbiomes. Buccal and body swabs were taken for culture-based analysis in Chapter 3, however these were not assessed for their microbial composition due to limited resources. By examining how microbiomes vary depending on where they have been sampled on a bird, for example buccal swabs compared to faecal samples, further inferences can be made about the mechanisms of bacterial acquisition and the potential avenues for pathogen spread. Research has indicated differences in the microbial communities recovered from different sections of the gastrointestinal tract of birds (Grond et al., 2018), and also significantly different microbial diversity when comparing buccal and cloacal diversity (Herder et al., 2023), thereby examining a range of sample types could provide further insights into microbiome variations. Further work could also consider the egg microbiome. Much work has pointed to the eggshell microbiome as having an important role in establishing the initial chick microbiome and can also be influenced by a wide variety of external and environmental conditions such as temperature and humidity, nest structure, nest reuse, nesting environment/habitat, nest-lining materials, and ectoparasite presence (Bakermans et al., 2019; Basso et al., 2022; Darolová et al., 2018; Peralta-Sánchez et al., 2014; Ruiz-Castellano et al., 2016; Ruiz-de-Castañeda et al., 2011; Tomás et al., 2018).

The results of this work, and indeed most work on animal microbiomes and environmental variability, is difficult to consistently determine. It is difficult to say, for example, that stressors such as acute oak decline will have a direct impact on defining the microbiome in a particular and predictable way, for example we cannot say that a disruption in microbiomes caused by x will result in y changing in every microbiome. Instead, it is more commonly thought that disruptions to microbiomes as an effect of external stressors will lead to stochastic changes which may not be detectable across all microbiomes, even within the same species. This is known as the Anna Karenina Principle of microbiome variability, where "all healthy microbiomes are alike, whereas each disease associated microbiome is 'sick' in its own way", and may be one of the reasons research into microbiomes (especially wild animal microbiomes) is still in its infancy (Zaneveld et al,., 2017). Instead of looking for directional effects of stressors, it is somewhat easier to ascertain the makeup of a core microbiome and detect deviations from this.

Although this chapter only considers external factors comparing avian gut microbiomes, it is also important to consider how intrinsic factors can work alongside environmental factors.

Altricial birds such as passerines are reliant on their parents for food as they do not leave the nest or forage for themselves for at least several weeks after hatching. Conversely, precocial birds such as waders and waterfowl do not rely as heavily on parental provisioning of food and forage independently shortly after hatching. These two different lifestyles of birds point towards different ways they could acquire their microbiomes, with potentially a higher level of influence by the parents in the development of the chick's microbiome in altricial birds. Although research on avian microbiomes is currently limited, a few studies have found that the microbiomes of altricial birds are more dynamic and less stable in early life compared to those of some precocial birds (Grond et al., 2018). This highlights the importance of considering both intrinsic and extrinsic factors when assessing microbiomes.

4.7 - References

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4.6 - Supplementary material

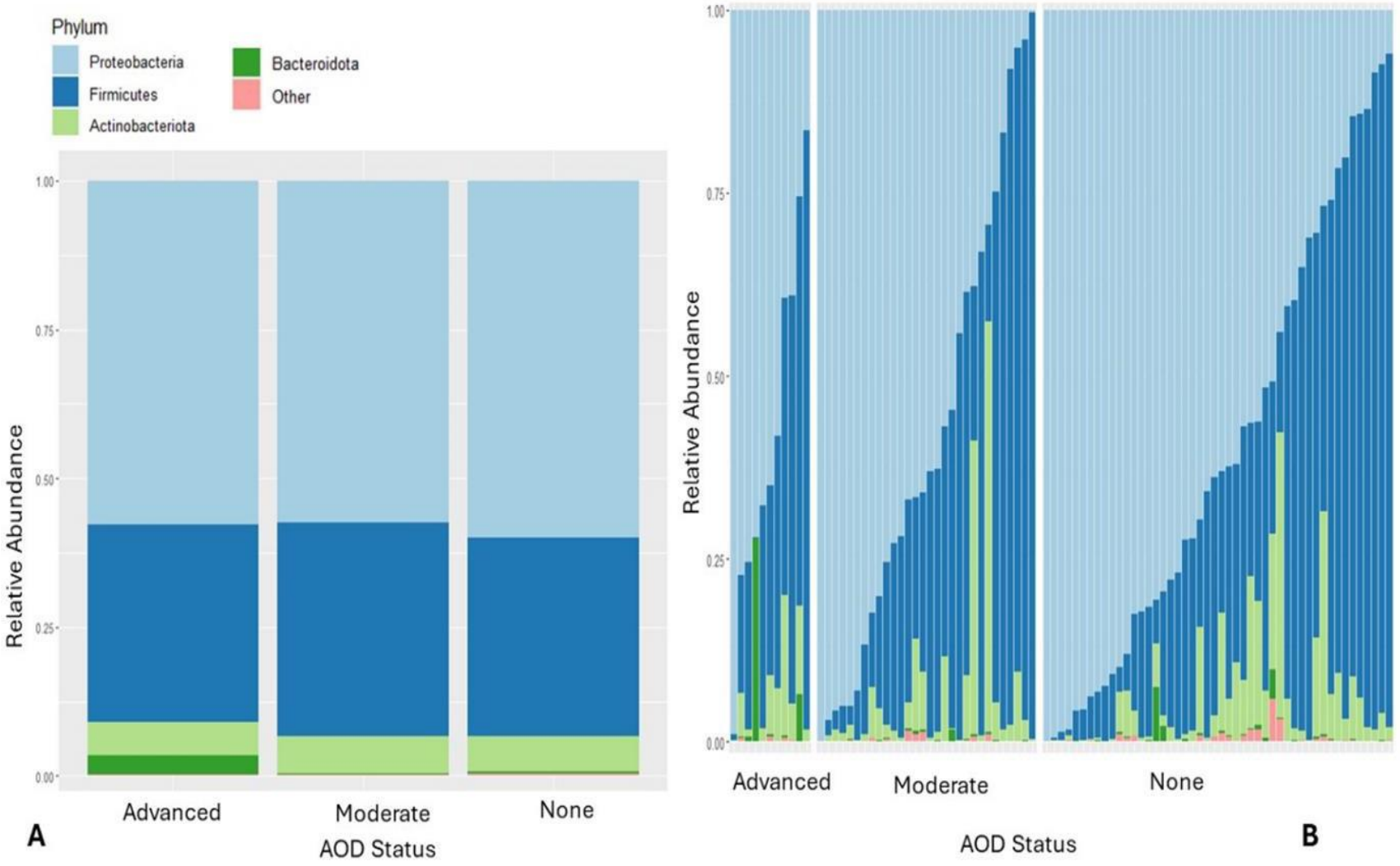
Supplementary material 4.1. Average percentage abundance of bacterial taxa in samples collected from sites with differing levels of Acute Oak Decline (AOD).

	Average Percentage Abundance ± Standard Error			
	No AOD	**Moderate AOD**	**Advanced AOD**	**p-value**
Phylum				
Proteobacteria	60.01 ± 4.29	57.50 ± 5.71	57.79 ± 7.49	0.923
Firmicutes	33.21 ± 4.14	35.89 ± 5.59	33.23 ± 7.41	0.909
Actinobacteriota	5.95 ± 1.19	6.22 ± 2.21	5.55 ± 1.74	0.899
Other	0.44 ± 0.14	0.25 ± 0.08	0.18 ± 0.08	0.910
Bacteroidota	0.39 ± 0.18	0.18 ± 0.07	3.24 ± 2.51	0.421
Class				
Gammaproteobacteria	59.22 ± 4.32	56.94 ± 5.72	57.26 ± 7.45	0.919
Bacilli	25.76 ± 3.07	29.29 ± 3.07	16.94 ± 3.36	0.428
Clostridia	7.44 ± 2.04	6.59 ± 2.27	16.28 ± 5.85	0.300
Actinobacteria	5.79 ± 1.15	6.12 ± 2.21	5.46 ± 1.72	0.882
Alphaproteobacteria	0.75 ± 0.22	0.53 ± 0.17	0.51 ± 0.22	0.649
Other	0.64 ±0.19	0.39 ± 0.12	0.30 ± 0.14	0.841
Bacteroidia	0.39 ± 0.18	0.15 ± 0.05	3.24 ± 2.51	0.404
Order				
Enterobacterales	33.61 ± 3.83	23.46 ± 3.39	32.93 ± 9.03	0.376
Pseudomonadales	23.94 ± 3.27	32.94 ± 5.45	21.76 ± 4.75	0.555
Lactobacillales	11.87 ± 2.55	12.88 ± 3.54	5.14 ± 2.06	0.645
Bacillales	8.71 ± 2.05	11.75 ± 3.51	5.64 ± 2.23	0.926
Micrococcales	5.38 ± 1.07	5.58 ± 2.18	5.28 ± 1.69	0.722
Paenibacillales	3.96 ± 0.70	4.05 ± 1.19	5.45 ± 1.44	0.450
Clostridiales	3.24 ± 1.53	2.70 ± 1.41	5.88 ± 2.50	0.177
Lachnospirales	2.20 ± 0.94	1.02 ± 0.34	5.27 ± 2.95	0.196
Peptostreptococcales-Tissierellales	1.99 ± 0.70	2.87 ± 1.38	5.13 ± 3.63	0.733
Other	1.73 ± 0.46	1.33 ± 0.36	2.07 ± 1.30	0.670
Staphylococcales	1.12 ± 0.51	0.57 ± 0.17	0.69 ± 0.33	0.835
Burkholderiales	1.09 ± 0.35	0.21 ± 0.06	0.87 ± 0.57	0.303
Xanthomonadales	0.54 ± 0.23	0.31 ± 0.14	1.68 ± 1.08	0.360
Rhizobiales	0.34 ± 0.09	0.26 ± 0.09	0.37 ± 0.21	0.870
Sphingobacteriales	0.29 ± 0.16	0.08 ± 0.05	1.85 ± 1.25	0.310

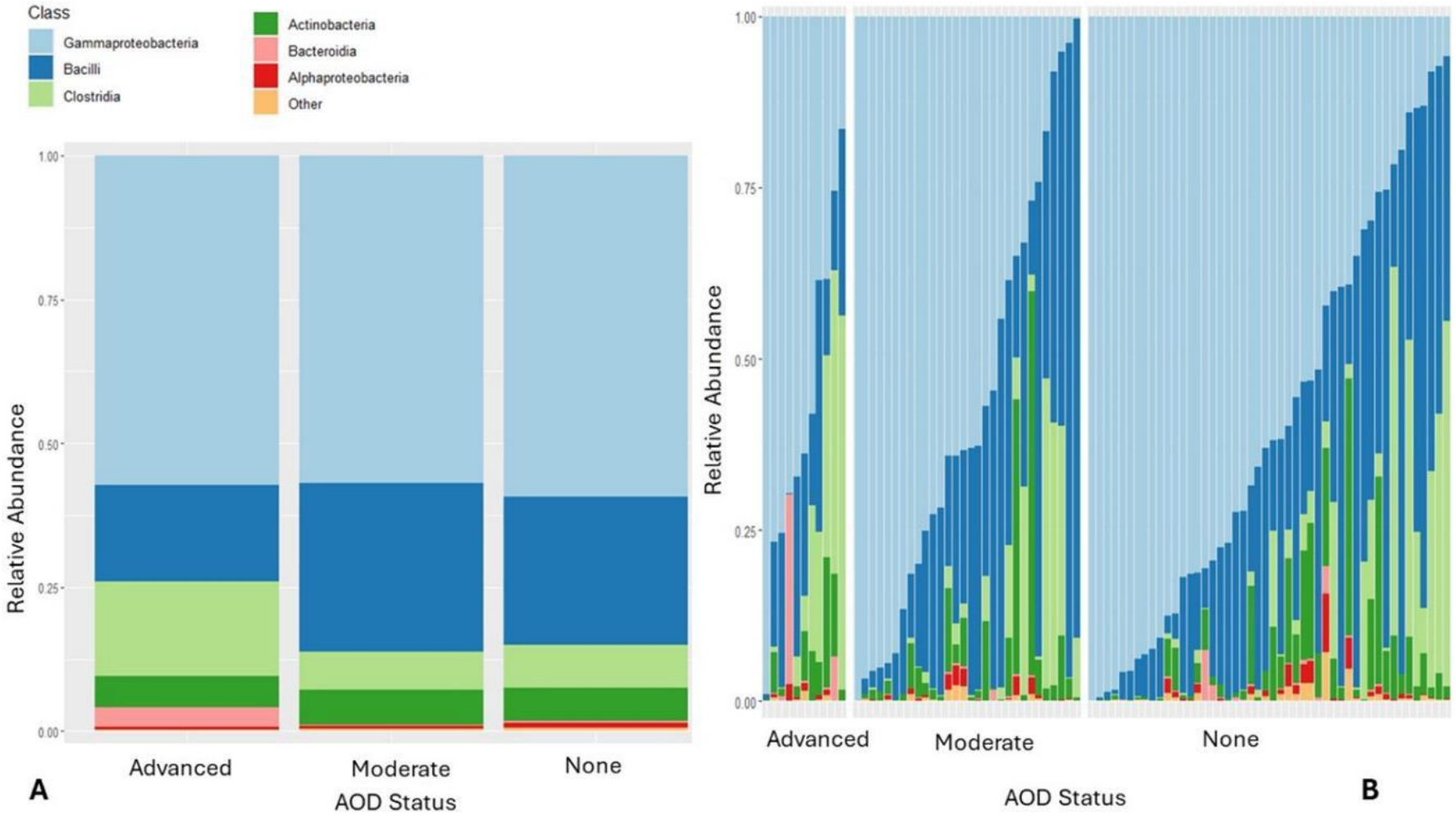
	Average Percentage Abundance ± Standard Error			
	No AOD	**Moderate AOD**	**Advanced AOD**	**p-value**
Family				
Pseudomonadaceae	21.19 ± 3.28	28.19 ± 5.75	18.78 ± 4.56	0.953
Unknown	15.82 ± 2.99	15.49 ± 3.68	5.95 ± 2.37	0.195
Carnobacteriaceae	9.32 ± 2.24	6.42 ± 2.03	4.05 ± 1.89	0.480
Enterobacteriaceae	8.97 ± 2.38	7.96 ± 1.81	22.76 ± 9.56	0.474
Planococcaceae	7.60 ± 1.77	10.10 ± 3.02	4.50 ± 2.03	0.879
Erwiniaceae	7.00 ± 2.28	3.80 ± 1.52	1.11 ± 0.42	0.821
Other	4.69 ± 1.47	2.91 ± 0.81	6.73 ± 3.03	0.642
Paenibacillaceae	3.96 ± 0.70	4.05 ± 1.19	5.45 ± 1.44	0.450
Dermabacteraceae	3.71 ± 0.94	4.37 ± 2.12	2.62 ± 1.14	0.883
Clostridiaceae	3.23 ± 1.52	2.69 ± 1.41	5.86 ± 2.49	0.177
Moraxellaceae	2.74 ± 0.95	4.75 ± 1.69	2.98 ± 1.75	0.465
Lachnospiraceae	2.20 ± 0.94	1.02 ± 0.34	5.27 ± 2.95	0.196
Yersiniaceae	1.86 ± 0.92	0.74 ± 0.48	0.37 ± 0.22	0.783
Staphylococcaceae	1.12 ± 0.51	0.57 ± 0.17	0.69 ± 0.33	0.831
Bacillaceae	1.11 ± 0.55	1.65 ± 1.20	1.13 ± 0.88	0.771
Sanguibacteraceae	1.05 ± 0.35	0.64 ± 0.38	2.20 ± 1.11	0.074
Enterococcaceae	0.93 ± 0.44	1.02 ± 0.88	0.09 ± 0.04	0.998
Peptostreptococcaceae	0.72 ± 0.29	0.29 ± 0.22	1.11 ± 0.74	0.676
Family XI	0.71 ± 0.36	1.46 ± 0.81	0.92 ± 0.71	0.299
Gottschalkia	0.56 ± 0.35	1.11 ± 0.60	3.10 ± 2.94	0.847
Xanthomonadaceae	0.53 ± 0.23	0.29 ± 0.14	1.68 ± 1.08	0.266
Alcaligenaceae	0.40 ± 0.26	0.04 ± 0.02	0.56 ± 0.52	0.286
Micrococcaceae	0.32 ± 0.06	0.36 ± 0.11	0.23 ± 0.09	0.884
Sphingobacteriaceae	0.29 ± 0.16	0.08 ± 0.05	1.85 ± 1.25	0.324
Genus				
Pseudomonas	21.18 ± 3.28	28.18 ± 5.75	18.78 ± 4.56	0.953
Unknown	20.78 ± 3.14	20.90 ± 3.74	11.97 ± 3.23	0.363
Carnobacterium	9.31 ± 2.24	6.40 ± 2.03	4.03 ± 1.89	0.473
Other	6.71 ± 1.52	3.87 ± 0.97	9.55 ± 4.04	0.238
Klebsiella	4.40 ± 1.20	4.83 ± 1.43	7.72 ± 5.32	0.727
Pseudomonas	21.18 ± 3.28	28.18 ± 5.75	18.78 ± 4.56	0.953
Unknown	20.78 ± 3.14	20.90 ± 3.74	11.97 ± 3.23	0.363
Carnobacterium	9.31 ± 2.24	6.40 ± 2.03	4.03 ± 1.89	0.473

	Average Percentage Abundance ± Standard Error			
	No AOD	**Moderate AOD**	**Advanced AOD**	**p-value**
Other	6.71 ± 1.52	3.87 ± 0.97	9.55 ± 4.04	0.238
Klebsiella	4.40 ± 1.20	4.83 ± 1.43	7.72 ± 5.32	0.727
Paenibacillus	3.95 ± 0.70	4.04 ± 1.19	5.45 ± 1.44	0.464
Brachybacterium	3.71 ± 0.94	4.37 ± 2.12	2.62 ± 1.14	0.884
Pantoea	3.47 ± 1.33	1.92 ± 1.19	0.60 ± 0.24	0.827
Erwinia	3.27 ± 1.44	1.82 ± 0.99	0.46 ± 0.25	0.961
Clostridium sensu stricto 1	2.95 ± 1.46	2.27 ± 1.15	4.95 ± 2.11	0.068
Paenisporosarcina	2.48 ± 0.76	1.76 ± 0.60	1.50 ± 0.66	0.464
Buttiauxella	2.16 ± 0.61	2.01 ± 0.88	13.72 ± 8.14	0.161
Acinetobacter	2.09 ± 0.92	3.88 ± 1.67	2.71 ± 1.71	0.220
Yersinia	1.83 ± 0.92	0.71 ± 0.48	0.36 ± 0.22	0.734
Lysinibacillus	1.75 ± 1.06	0.31 ± 0.19	0.27 ± 0.19	0.807
Caryophanon	1.71 ± 0.82	4.18 ± 1.88	1.61 ± 1.22	0.996
Anaerocolumna	1.33 ± 0.71	0.87 ± 0.31	4.84 ± 2.97	0.120
Staphylococcus	1.08 ± 0.50	0.55 ± 0.16	0.66 ± 0.33	0.892
Sanguibacter-Flavimobilis	1.05 ± 0.35	0.64 ± 0.38	2.20 ± 1.11	0.074
Enterococcus	0.93 ± 0.44	1.02 ± 0.88	0.09 ± 0.04	0.998
Tissierella	0.68 ± 0.37	1.42 ± 0.81	0.90 ± 0.71	0.648
Psychrobacter	0.56 ± 0.27	0.74 ± 0.43	0.24 ± 0.18	0.909
Psychrobacillus	0.55 ± 0.36	0.94 ± 0.74	0.24 ± 0.11	0.762
Sporosarcina	0.53 ± 0.23	0.92 ± 0.46	0.04 ± 0.02	0.878
Stenotrophomonas	0.52 ± 0.23	0.28 ± 0.14	1.67 ± 1.08	0.301
Clostridioides	0.47 ± 0.25	0.28 ± 0.22	0.78 ± 0.70	0.530
Sphingobacterium	0.23 ± 0.16	0.07 ± 0.05	0.81 ± 0.60	0.402
Bacillus	0.18 ± 0.06	0.13 ± 0.06	0.03 ± 0.02	0.152
Viridibacillus	0.08 ± 0.02	0.37 ± 0.15	0.49 ± 0.39	0.968
Clostridium sensu stricto 13	0.06 ± 0.03	0.30 ± 0.27	0.71 ± 0.42	0.063

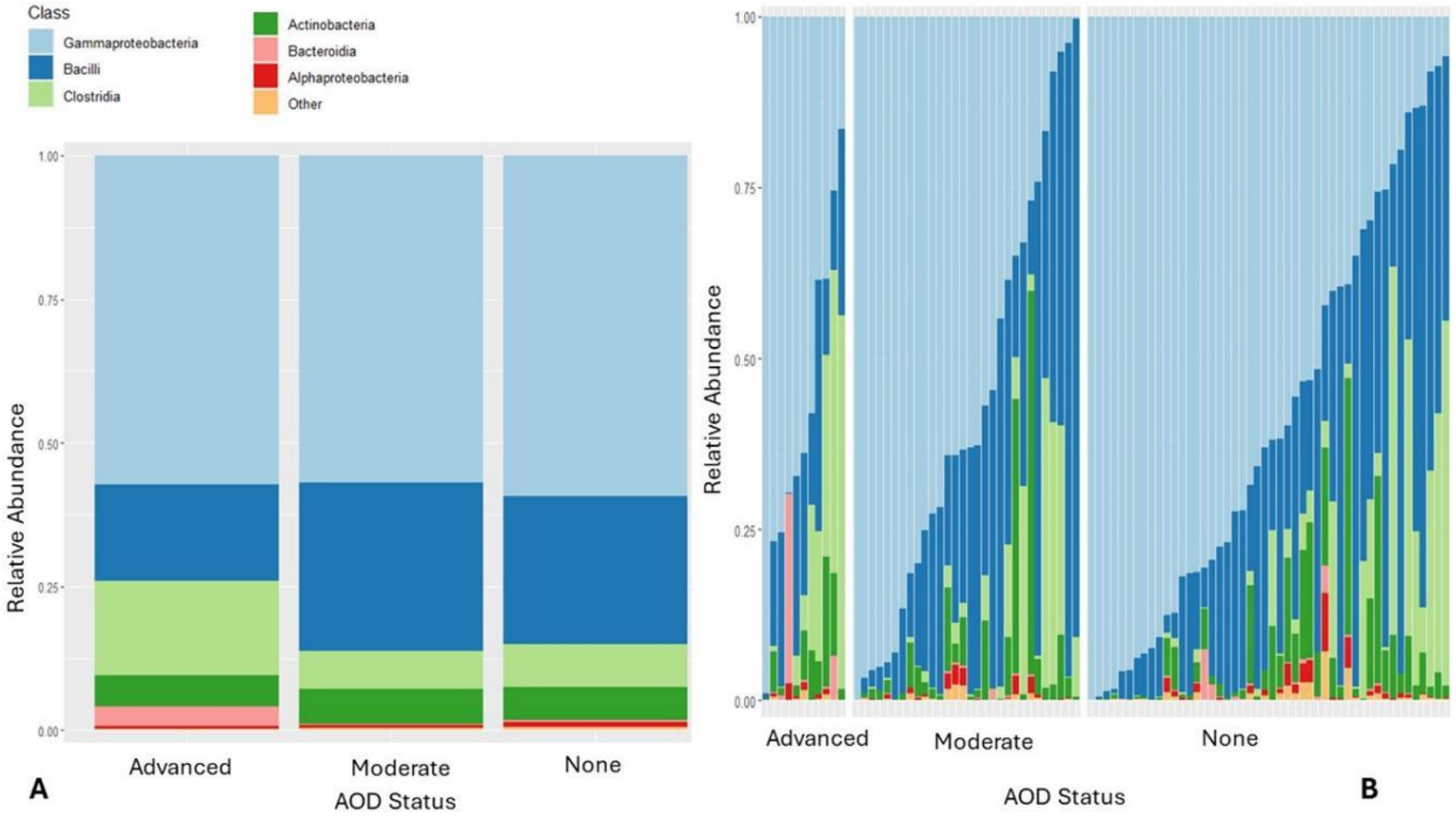
Supplementary material 4.2. Relative abundance of bacterial phyla across samples collected from areas with differing levels of AOD. A) Average relative bacterial phylum abundance across samples, B) Relative bacterial phylum abundance across all samples.



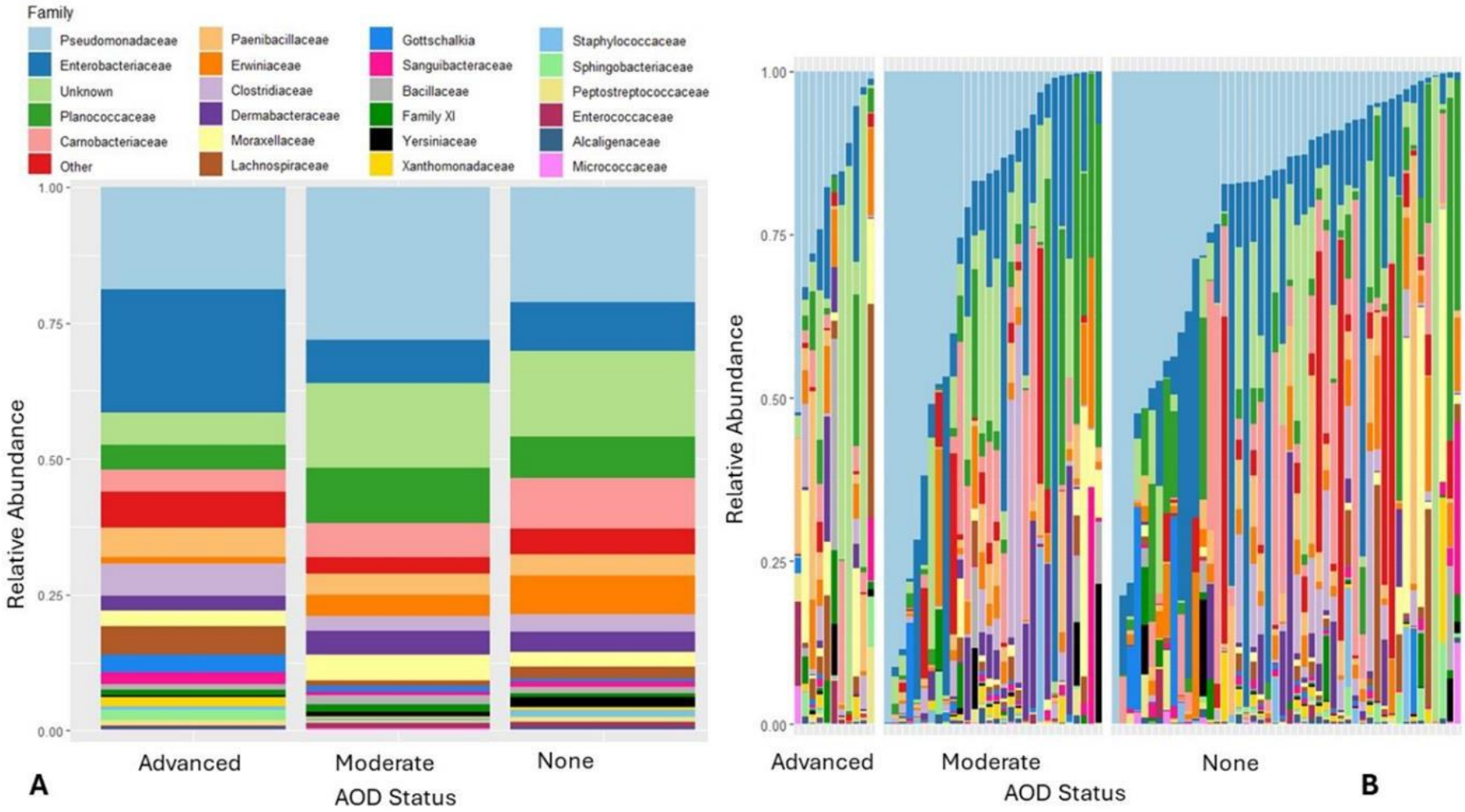
Supplementary material 4.3. Relative abundance of bacterial classes across samples collected from areas with differing levels of AOD. A) Average relative bacterial class abundance across samples, B) Relative bacterial class abundance across all samples.



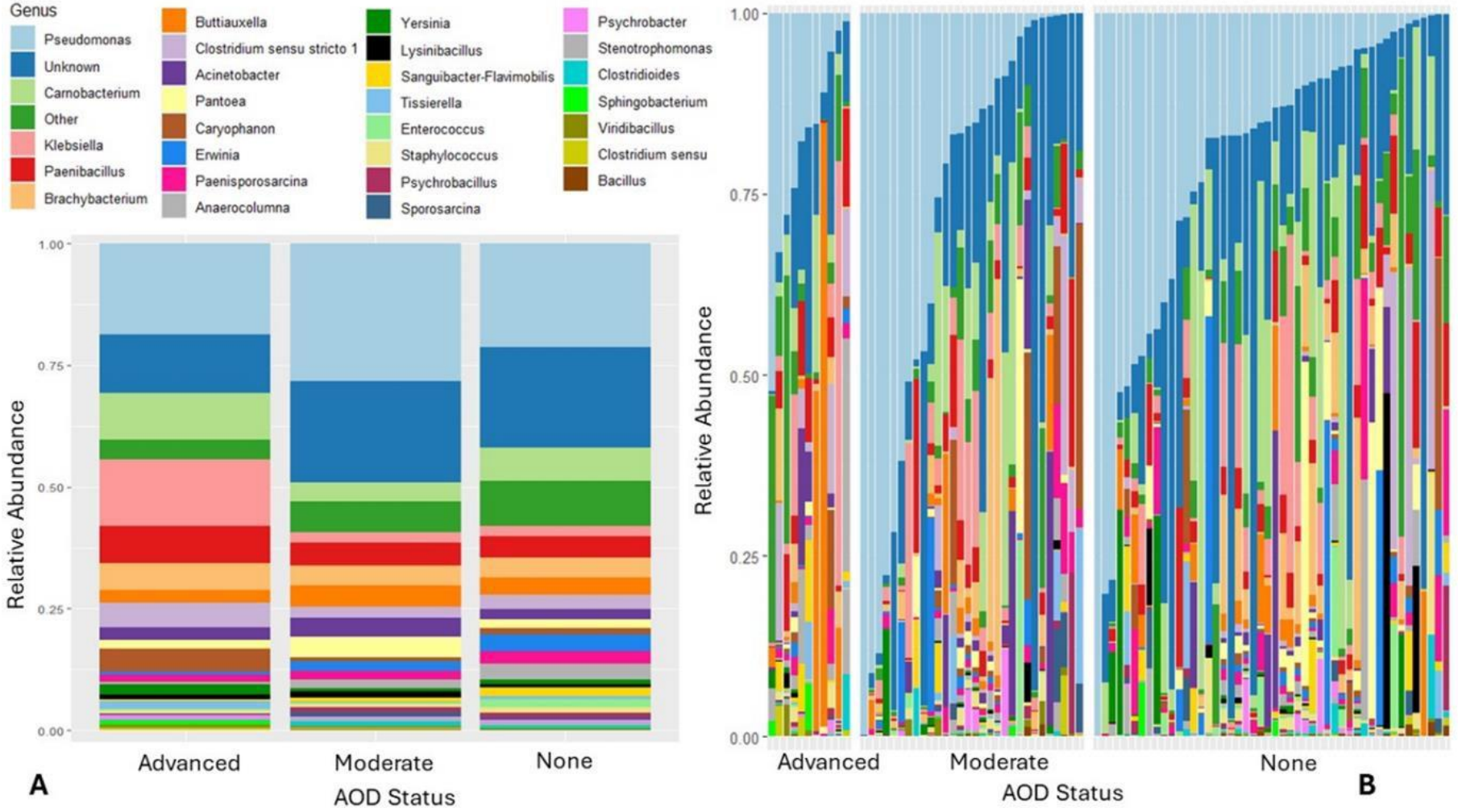
Supplementary material 4.4. Relative abundance of bacterial classes across samples collected from areas with differing levels of AOD. A) Average relative bacterial class abundance across samples, B) Relative bacterial class abundance across all samples.



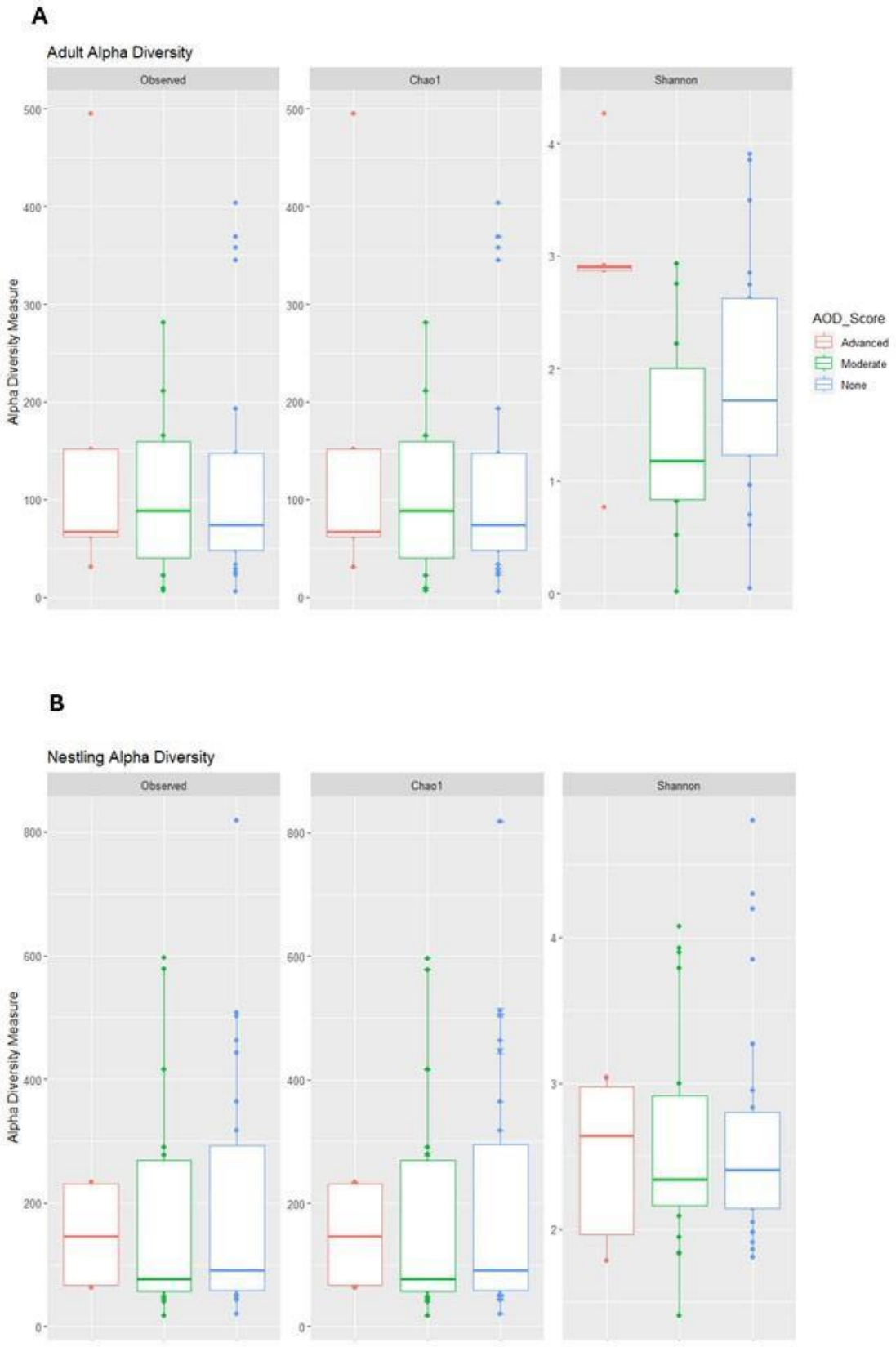
Supplementary material 4.5. Relative abundance of bacterial families across samples collected from areas with differing levels of AOD. A) Average relative bacterial family abundance across samples, B) Relative bacterial family abundance across all samples.



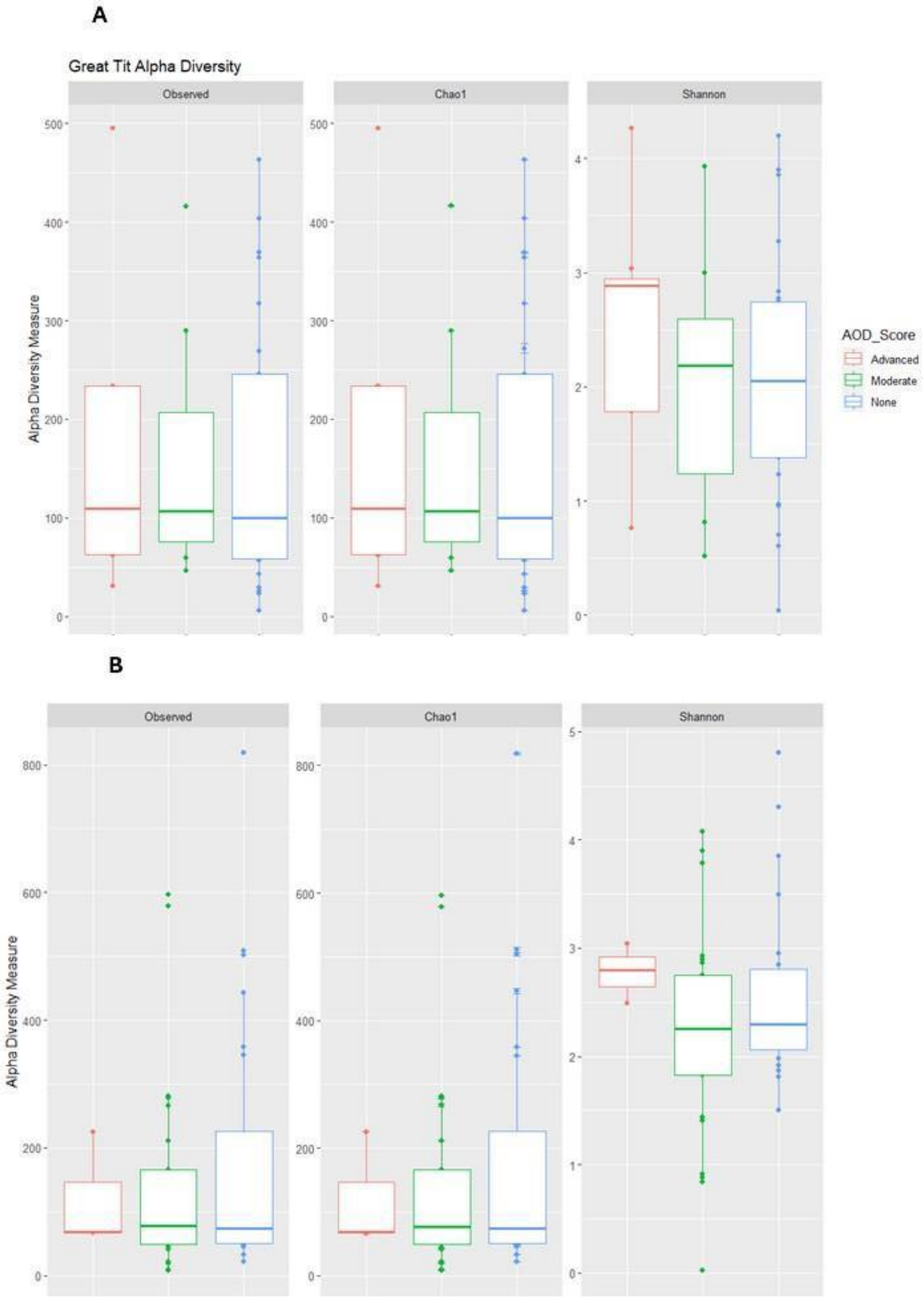
Supplementary material 4.6. Relative abundance of bacterial genera across samples collected from areas with differing levels of AOD. A) Average relative bacterial genus abundance across samples, B) Relative bacterial genus abundance across all samples.



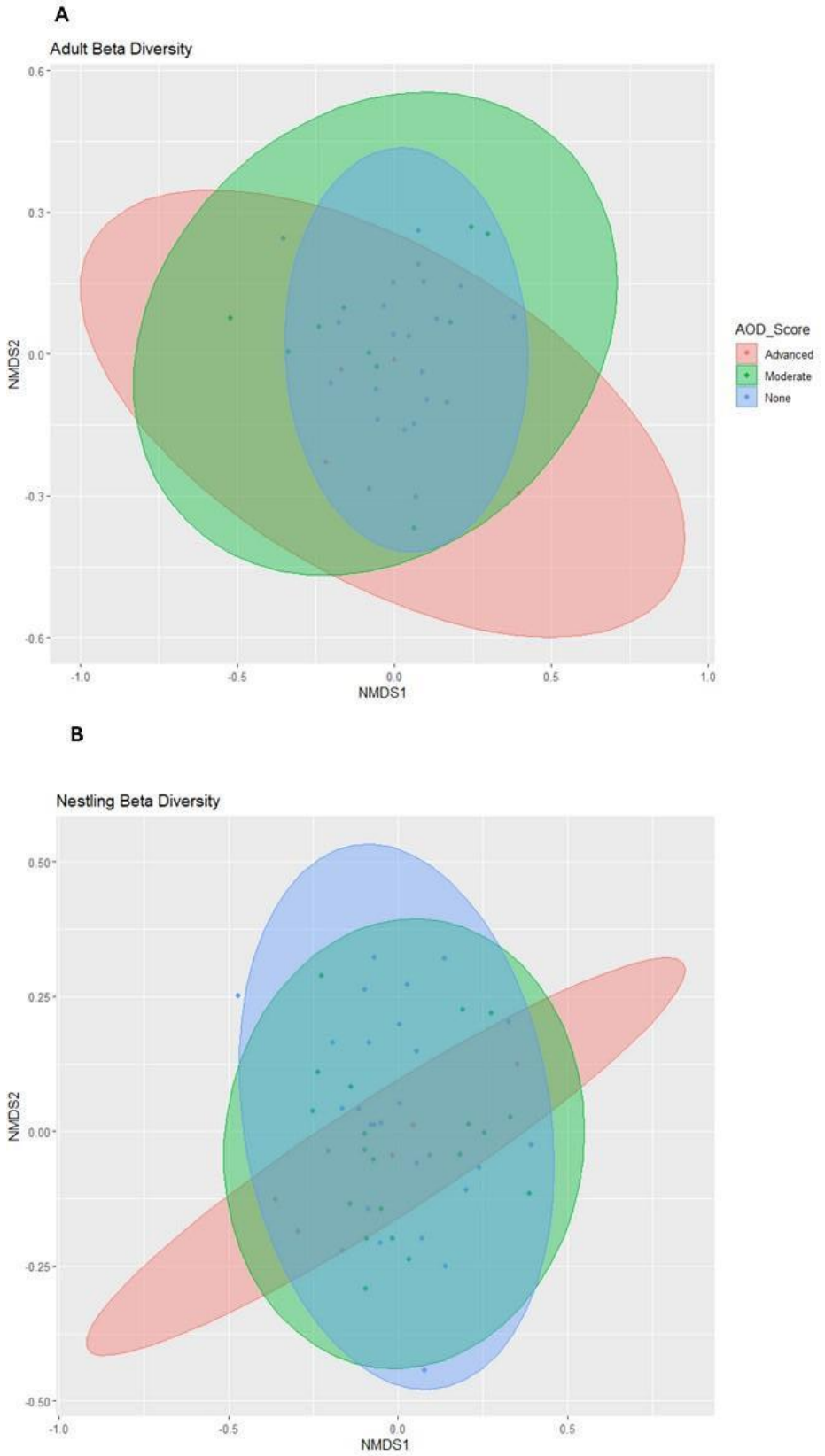
Supplementary material 4.7. Alpha diversity of faecal samples across differing levels of AOD, grouped by A) adult bird samples, B) nestling samples.



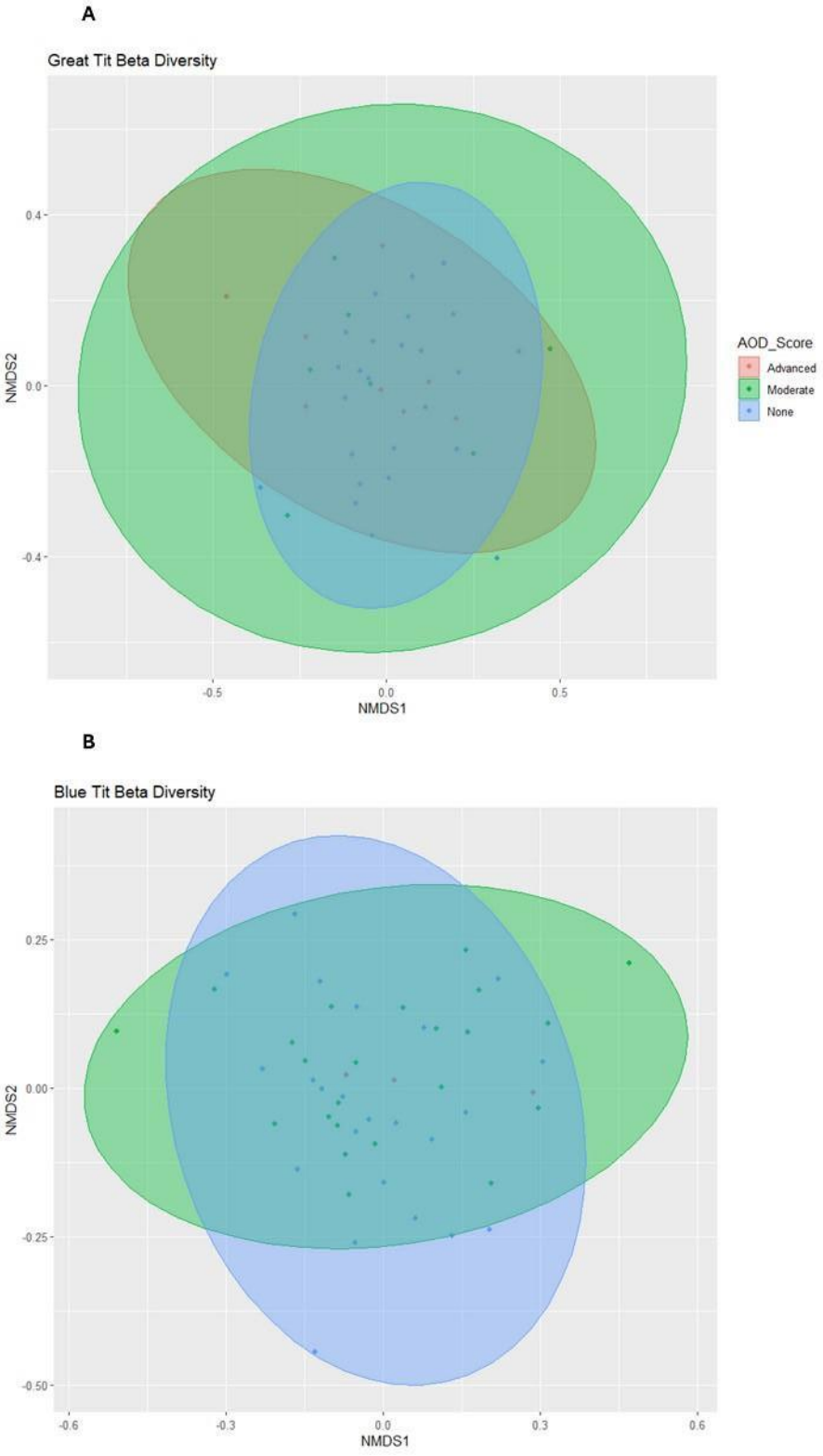
Supplementary material 4.8. Alpha diversity of faecal samples across differing levels of AOD, grouped by A) great tit samples, B) blue tit samples.



Supplementary material 4.9. Beta diversity of faecal samples across differing levels of AOD, grouped by A) adult bird samples, B) nestling samples.



Supplementary material 4.10. Beta diversity of faecal samples across differing levels of AOD, grouped by A) great tit samples, B) blue tit samples. Not enough data points were available to confidently group the AOD Advanced samples in the blue tit data.



Chapter 5 - The impact of Acute Oak Decline on oak insect herbivory damage

5.1 - Abstract

Trees face a range of pressures from natural enemies, including pathogens, parasites and herbivores. Plant pathogens can interact with insect herbivores, acting synergistically to increase damage on their hosts, but in some cases the presence of pathogens can deter herbivores and reduce their rates of damage. The strength and direction of these interactions vary depending on the system. This chapter examines how herbivory rates vary between trees that are symptomatic and asymptomatic for AOD. Branches were removed from oak trees both with and without AOD, and leaves were examined to compare herbivory levels. Insect herbivory was found to be over three times higher on AOD symptomatic trees than on asymptomatic trees. This is the first study to demonstrate the impact of bacterial tree pathogens on insect herbivory rates and calls for further study into the mechanisms behind this interaction. Shifts in herbivory rates within an ecosystem can have localised effects on the host tree, and more widespread cascading effects on the community of herbivores and, potentially, to impact herbivores' predators.

Keywords; folivorous herbivore, Acute Oak Decline, oak herbivory

5.2 - Introduction

Diseases such as Acute Oak Decline (AOD) can result in damage, deteriorating condition and ultimately to the death of trees, as discussed in section 1.4. AOD is a bacterial syndrome of oak trees, which reduces canopy density through branch die off and canopy thinning (Denman et al., 2014). Other pressures faced by trees, such as herbivory, reduce the area of leaves and can disrupt processes involved in photosynthesis (Zangerl et al., 2002), thereby potentially impacting plant growth. Herbivory is carried out by a range of species, however for the purpose of this work I will be focussing on insect folivores. To counteract herbivore damage, plants are able produce defences that may make them less appealing to further herbivores, thereby reducing herbivory rates (Karban & Myers, 1989). Such defences can be chemical, for example increasing the production of secondary metabolites such as tannins and phenolics (Schuldt et al., 2017), and altering the nutritional quality of their leaves (Wetzel et al., 2016), or physical through the production of tougher leaf material (Carmona et al., 2011). Defences can be part of the plant's phenotype, for example thorns, spines and tough surfaces (Halpern

et al., 2007), or induced by the action of herbivory, which can be localised to the damaged area resulting in defence mechanisms such as apoptosis (Pegadaraju et al., 2005). The production of volatile compounds from herbivore damaged trees can also trigger nearby plants to prime or produce induced defence (Paré & Tumlinson, 1999).

Plant herbivore defences can result in longer and poorer, or indeed unsuccessful, development of insect herbivores such as caterpillars (Coley & Barone, 1996). Insects can react to plant defences through the production of their own defences, which can result in antagonistic interactions, with both parties developing morphological and biochemical defensive traits to counteract the defences of the other (War et al., 2018). This system can be disrupted when further pressures are introduced, such as plant pathogens and their associated disease symptoms. Plant diseases and invertebrate herbivores can work simultaneously and additively to put physiological pressure on plants. Increased insect herbivory can provide easier access for plant pathogens to cause infection (Gossner et al., 2021; Schausberger, 2018; Simon & Hilker, 2003), and herbivore damage levels have been positively associated with increased levels of pathogen damage (Schuldt et al., 2017). It is important to study the variety of stressors upon trees and their interactions, in this case with regards to how the presence of tree diseases and levels of insect herbivory are connected.

As established in Chapter 2 of this thesis, insects make up the majority of documented plant pathogen vectors. The relationship between plant pathogens and their vectors, and the mechanisms by which insect herbivore vectors provide a route of access for plant pathogens is well researched (Eigenbrode et al., 2018). Herbivory provides a route for pathogens to enter a plant, suggesting that any increases in herbivory could potentially increase the abundance and diversity of pathogens within a plant (Chisholm et al., 2019). This is not just restricted to vectoring herbivores, however, as (Gossner et al., 2021) demonstrated that increased herbivory of beech (*Fagus sylvatica*) by the beech leaf-miner beetle (*Orchestes fagi*), increased facilitation of the plant pathogenic fungus *Petrakia liobae* into the tissue of these trees, despite the beetle not being a known vector for this pathogen. This study exemplifies how plant pathogens can benefit from herbivore activity by gaining access to the plant.

AOD is an oak tree decline disease caused by bacterial plant pathogens. The disease is often associated with the presence of the herbivorous Oak Buprestid Beetle *Agrilus biguttatus*, which is able to weaken oak trees through its larvae feeding within the inner bark (Denman et al., 2014) - a potential mechanism for the pathogenic bacteria to infect the oak trees (Brown et al., 2015). Adult *A. biguttatus* beetles feed on oak leaves, but it is unlikely that a folivorous

herbivore would transmit pathogens to leaves as AOD symptoms are most often observed on the trunk of oaks. Despite its association with AOD, this beetle has yet to be established as a vector for the disease's bacterial pathogens (Brown et al., 2017).

Insect herbivores are an integral link in woodland trophic interactions and provide a large quantity of food for young birds in the nest. The winter moth caterpillar (*Operophtera brumata*) is a herbivore that is associated with many plants, including oak (*Quercus* spp.) trees (Van Dongen et al., 1997), and forms part of the widely studied tri-trophic interaction of oak / winter moth / great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) (Buse et al., 1999; Evans et al., 2024; Wilkin et al., 2009). Any impacts on herbivore populations will also likely impact the food availability for their predators, which can have knock-on effects on any dependent offspring. For example, changes in herbivore populations can affect the breeding success of predatory tits if these occur within the parent's foraging area, as these bird species rely on caterpillars found on oak trees as a food source for their nestlings (Evans et al., 2024; Verboven et al., 2001).

There have been documented impacts of plant pathogens on a range of insect herbivores, with the direction and strength of these interactions varying across systems. Pathogens of oak have been associated with increased herbivore activity. The oak powdery mildew pathogen *Erysiphe alphitoides* for example, impacts the larvae of the leaf miner moth *Tischeria ekebladella*, which show increased growth rates when feeding on leaves infected with the pathogen (Tack et al., 2012). As outlined in section 1.1, trees represent foundation species, therefore the impacts of plant-pathogen-herbivore interactions can span many trophic levels (Tack & Dicke, 2013). As such, when considering how tree diseases such as AOD can impact entire ecosystems and processes such as breeding in birds, it is essential to examine first how the presence of these bacterial pathogens can impact other trophic levels within this system, specifically invertebrate herbivore abundance. A previous review by (Eberl et al., 2019), demonstrated that fungal plant pathogens can cause changes in insect herbivores, however, the pathogens they examined were localised to leaf tissue so the impacts on folivorous herbivores was more direct. When trees are symptomatic with AOD, the highest concentration of the pathogenic bacteria is found localised to the bleed sites (Denman et al., 2016), and although the bacteria have been identified in other areas of oak trees (Gathercole et al., 2021), it is unknown if they are present in a pathogenic state away from the lesions.

To date, most research into the impact of plant pathogens on insect herbivores has focused on fungal and viral plant diseases. Research into the impact of AOD on the wider ecosystem

has yet to be fully examined, especially in relation to folivorous herbivores which feed on AOD symptomatic trees. The work undertaken here will examine the impact of AOD on herbivory, which will provide a basis for understanding impacts of other trophic levels associated with oak herbivores.

5.3 - Aims and hypothesis

This chapter aims to explore how the presence of Acute Oak Decline (AOD) and its associated pathogenic bacteria can impact the abundance of folivorous caterpillars by examining how herbivory levels vary across oak trees with different AOD statuses. The hypothesis is that the infected trees will have a different level of herbivory to uninfected trees, as has been found in other herbivore systems.

This work will be the first to examine how the impacts of tree diseases can spread into the wider ecosystem and impact other trophic levels. This will have consequences for ecosystem functioning, as we will be able to understand how localised changes in one trophic level, in this case oak health, can impact food chains and species that have associations with oak trees.

5.4 - Methods

5.4.1 - Study area

The field site for this work was Epping Forest, in Essex, on the outskirts of London in southeast England. The field site consisted of 103 habitat plots, which were surveyed for symptoms of AOD and canopy density in 2020 and 2021. Half of the habitat plots contained oak trees displaying symptoms of AOD and half asymptomatic. The central point of each plot was spaced at least 100m from the next, meaning trees sampled in this study were no closer than 50m.

An online random number generator (calculator.net) was used to select six habitat plots, ensuring three of which were symptomatic for AOD and three were asymptomatic following assessments in 2020 and 2021. One oak tree was selected from each of these plots. Oak trees selected for branch removal were also ensured to be symptomatic or asymptomatic for AOD, reflecting the AOD status of the plot. Where trees were symptomatic, they were all at similar stages of development and also at a similar stage of AOD progression.

5.4.2 - Branch Removal

All six trees were visited on the same date in May 2022 to reduce the impact of differences in herbivory rates throughout the spring (Feeny, 1970). Four branches were removed from each tree, one from each of the following compass aspects (North, South, East and West). Branches were removed using a pole pruner, with branches typically being removed being around 6m from the ground.

To prevent desiccation of the leaves before assessment, leaves were removed from branches and photographed within 48 hours of collection. This resulted in only a subsample of leaves being analysed for each branch, which did result in a skew in fewer leaves being assessed from trees with AOD, however the large sample size accounted for this discrepancy. Due to these time constraints, an average of 114 leaves were photographed and assessed for each tree (range 96-165). Approximately 10-15 leaves were photographed from each tree, and this was rotated throughout the trees in order to ensure consistency in how long the leaves had been removed for.

5.4.3 - Herbivory Assessment

Herbivory was quantified by eye, following protocols from the Herbivory Variability Network. Due to the nature of herbivory, it was not possible to use digital estimations to of herbivory as these largely assume symmetrical leaf shape which is not the case with oak leaves. Instead each leaf was visually inspected, and the percentage of herbivory damage was estimated to the nearest 5%. This method is not an exact way to quantify herbivory but has been found to be a sound reliable estimation (Xirocostas *et al*., 2022). As an example, if ~12.5% of a leaf were damaged, then the following step should have been followed to reach that conclusion:

1. Mentally cut the leaf into quarters
2. See that less than a quarter (25%) is damaged
3. Mentally cut the quarter with damage in half, yielding eighths (12.5%)
4. See that the area damaged is equal to an eighth and record 12.5%

The same process can be repeated for example by mentally dividing the leaf into tenths and estimating how many of the ten segments have been damaged and so on.

Figure 1 shows some examples of oak leaves alongside their damage percentages. Herbivory assessment training was carried out using the ZAX Herbivory Trainer (Xirocostas et al., 2022),

and visual assessments of leaves sampled here were only commenced once at least 98% accuracy on the test set was met. The ZAX Herbivory Trainer was then used before each assessment session, with scoring being carried out that day.

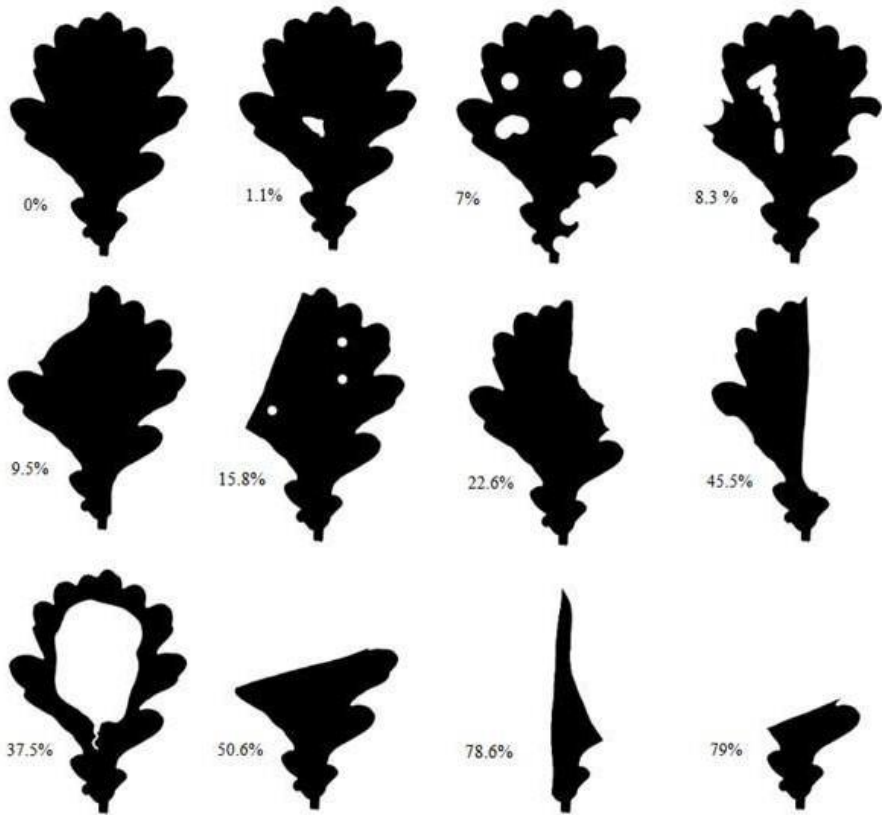


Figure 1. Examples of percentage damage of oak leaves, adapted from protocols from The Herbivory Variability Network (herbvar.org)

Each leaf was assessed twice at different time points at least one month apart. During the second assessment the results from the first assessment were not consulted, ensuring the scores from the first round could not influence the second. An average of the herbivory percentages from the first and second assessment was taken and was used alongside both the first and second assessment in statistical analysis.

5.4.4 - Statistical Analysis

To determine if there were any differences in herbivory rates between trees that were symptomatic for AOD compared to those that were not, a Welch Two sample t-test was used. The data and residuals were normally distributed therefore the use of the parametric t-test was

appropriate. Statistical analysis and data visualisation was carried out using R (v.4.3.1) using the ggplot2 (v.3.4.4) and DHARMa packages (v.0.4.6).

5.5 - Results

A total of 689 leaves were assessed from six trees – 302 from symptomatic trees and 387 from asymptomatic trees (Table 1). The average number of leaves per branch on asymptomatic trees was 129, whereas the average number on symptomatic branches was 100.

Table 1. Number of leaves examined for the herbivory assessments

Tree Sampled	AOD Status	Number of leaves examined
1	Present	96
2	Present	104
3	Present	102
4	Absent	109
5	Absent	113
6	Absent	165

In order to ensure that there was consistency in the scoring of the leaves between the first and second attempt, the difference between the herbivory assessments was calculated and can be seen in the plot in Figure 2. 95% of samples had a difference of less than 20% between the first and second assessment, with 81% of assessments being consistent to within 10%. Herbivory damage on leaves from trees symptomatic with AOD were significantly higher than those from asymptomatic trees ($t(455.35) = -16.1$, $p < 0.05$) (Fig. 3, Table 2).

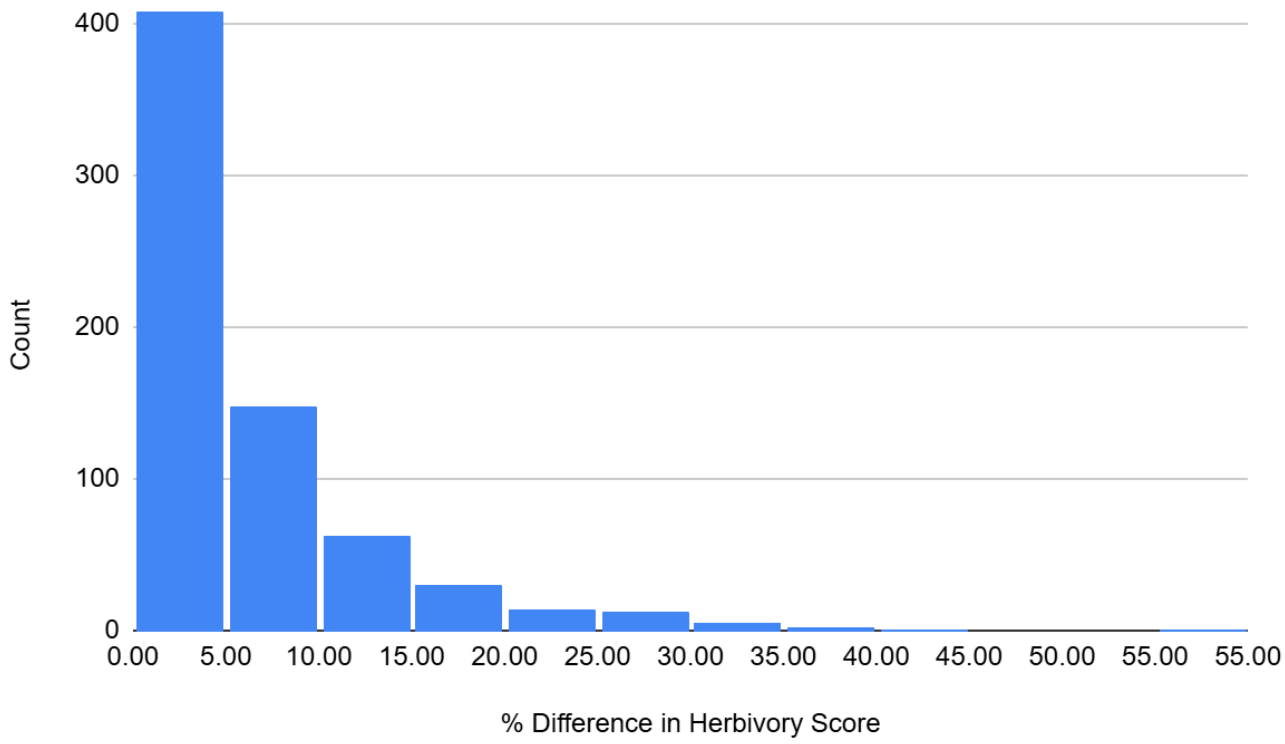


Figure 2. Histogram of differences in herbivory scoring assessments between assessment 1 and assessment 2.

Table 2. Summary statistics for rates of herbivory on leaves from trees with different AOD statuses.

Herbivory (%)	**Tree AOD Status**	
	Symptomatic	**Asymptomatic**
Mean	7	25
Min	0	0
Max	65	80
IQR 1	0	12.5
Median	2	22.5
IQR 3	3	37.5

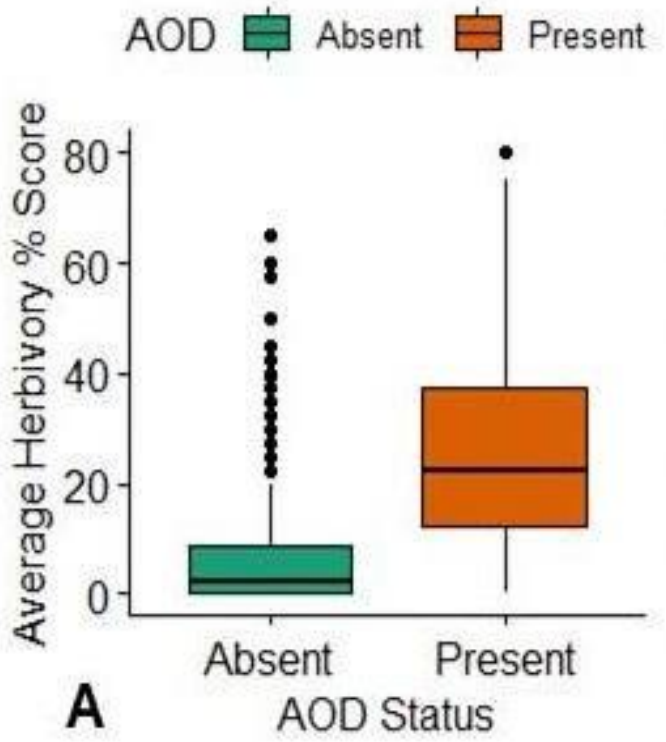


Figure 3. Comparison of herbivory levels of trees symptomatic (orange) and asymptomatic (green) for AOD. Supplementary material 5.1 shows average herbivory scores across the two different scoring replicates.

5.6 - Discussion

The results of this study support the hypothesis that trees that are symptomatic for acute oak decline have higher, in fact much higher rates of herbivory than trees that are asymptomatic. This is the first work that demonstrates an association between the presence of a bacterial tree pathogen and the feeding impact of the tree's herbivores.

Herbivory levels were used here as a proxy for predicting invertebrate herbivore population size, which is a well-used metric (Schowalter, 2006). We can therefore infer from these results that trees symptomatic with AOD likely support greater numbers of invertebrate herbivores. Further studies could use the collection of frass to estimate herbivore abundance, which has been found to be an efficient way of predicting herbivore population size over a season (Zandt, 1994). In this study frass collection was not found to be a successful method as it can be heavily impacted by the environment such as wet weather, and this project lacked the resources for regular checks of frass traps at short enough intervals. To gain further insights into the herbivore community variation on AOD symptomatic

and asymptomatic trees, further work should examine a wider range of invertebrate herbivores through collection and identification over a longer period of time or the study of herbivore eDNA (environmental DNA), which is present in the environment for longer periods of time (Ladin et al., 2021; Macher et al., 2023).

5.6.1 - Tree diseases and herbivores

The results found in this study are consistent with those of other studies that focus on the effects of tree pathogens on herbivory rates. Gypsy moth (*Lymantria dispar*) caterpillars feeding on black poplar (*Populus nigra*) trees show a preference for feeding on foliage infected by the rust fungus *Melampsora larici-populina*, and develop at a faster rate by doing so (Eberl et al., 2020). Other herbivore behaviours can be linked to the presence of tree diseases, for example the aphid *Euceraphis betulae* shows higher settlement rates, individual growth and population growth on fungal (*Marssonina betulae*) infected silver birch *Betula pendula* than non-infected plants (Johnson et al., 2003). In a broad analysis of the interactions between herbivores and pathogens on a range of tree species in China, (Schuldt et al., 2017) found that herbivore damage was significantly related to pathogen damage, although the strength of this interaction depended on the tree species richness of the surrounding area, and the individual plant traits.

Mechanisms leading to differences in herbivory rates within a pathogen / plant / herbivore system appear to be specific to the system in which they occur. In the previous example of the gypsy moth and black poplar, the mechanism behind this preference was attributed to an increased concentration of mannitol within the fungal tissue and the infected leaves. This indirectly resulted in the leaves being more desirable for the caterpillars and resulted in increased weight gain (Eberl et al., 2020). As the current study is the first to show a possible herbivore preference for feeding on trees symptomatic for AOD, the mechanism behind this preference remains to be investigated. Brown et al., (2018) demonstrated that sites with higher levels of AOD were in areas with high levels of dry nitrogen deposition. If intercepted by the tree canopy this could lead to increased nitrogen concentration in the leaves (Guerrieri et al., 2015), which has been shown to be more attractive to insect folivores. As the symptoms of AOD are not closely linked with the leaves (aside from resultant canopy loss), and it is not known if the bacterial pathogens even infect the leaves, further work is needed to examine the structural changes between leaves on trees afflicted with AOD compared to those without the disease, and how these may affect herbivory preferences. Interestingly, oak trees are known to be able to alter their resource investment at different times of the year, focussing on defence mechanisms such as increasing leaf tannin concentration during high levels of herbivory (Feeny, 1970). Investigating if this seasonal change in tannins could be affected by the presence of AOD is a potential starting point. Indeed, understanding if overall leaf palatability

is impacted by the tree's AOD status has also yet to be investigated, which could reveal if trees symptomatic with AOD have more palatable leaves.

A recent review called for the inclusion of microbiomes when assessing interactions between trees and insects with regards to forest health and management (Vacher et al., 2021). Analysing leaf microbiomes alongside chemical and structural differences in leaves from AOD symptomatic and asymptomatic trees, would provide further insights into the mechanisms behind herbivory preferences.

5.6.2 - Species and system specific effects

Reviews examining the extent of plant pathogen effects on other trophic levels beside the host plants, particularly folivorous herbivores, have indicated that these can be as important to consider as interactions and competition between different species of herbivore, however the direction and strength of the interactions vary considerably depending on the specific system (Tack & Dicke, 2013). The presence of plant pathogens within a system has shown both positive, negative, and neutral effects on insect herbivores, be this direct or indirect. Some caterpillars show higher survival and faster development when fed on artificial diet containing plant pathogenic fungus *Botrytis cinerea* (Mondy & Corio-Costet, 2004), whereas the green peach aphids (*Myzus persicae*) on *Zinnia* plants were not able to reproduce when the host plants were infected with the cucumber mosaic virus (Lowe & Strong, 1963), and infection of cucumber (*Cucumis sativus*) with the pathogenic fungus (*Colletotichum lagenarium*) had no effect on spider mites (*Tetranychus urticae*) (Ajlan & Potter, 1991). (Friedli & Bacher, 2001), noted that different developmental stages of the rust fungus (*Puccinia punctiformis*) trigger different responses in herbivores. As such, one should exercise caution when attributing the presence of pathogens to changes in herbivory, as indirect effects through pathogen induced changes in the host plants could have more influence than any direct effects of the pathogen on the herbivores. (Hatcher et al., 1994), found that the same pathogen and herbivore can interact differently depending on which host plant species was in the plant / pathogen / herbivore system. These examples demonstrate the complexities of the impacts of plant pathogens on herbivores and demonstrate the problems in generalising impacts of plant pathogens on herbivores.

5.6.3 - Spatial and temporal effects

Spatial and temporal scales of the effects of plant pathogens on insect herbivores are important to examine. The herbivory assessments in this study were based on tree disease statuses assigned in 2020 and 2021, with leaf collection occurring on a single day in 2022. The temporal pattern of pathogen effects on herbivores can however be subject to differing time scales, with effects varying over several days (Simon & Hilker, 2005) or years

(Lappalainen et al., 1995). This highlights the difficulty in attempting to understand impacts at one snapshot in time, and longer-term monitoring should be considered. It is not known when the trees classified as symptomatic in this study were infected with AOD, and contrastingly it is not known if trees classified as asymptomatic had previously shown symptoms of AOD infection and were in remission, rates of which can be around 40% with this disease (Brown et al., 2016). This is where experimental studies, for example inoculation of healthy tissues, could come in useful.

5.6.4 - Cascading effects

Where symptomatic plants are subjected to higher levels of herbivory, one could assume that plants which may be resistant to diseases such as acute oak decline could potentially suffer from reduced herbivory rates. Over time this could result in selection for pathogen resistance in trees, however we must consider that a disease such as AOD would not necessarily be the only natural enemy for oaks (Wetherbee et al., 2020), and there could be additional pressures influencing selection. Changes in plant species composition at a local level could impact herbivore communities across different plant species.

Contrasting effects of pathogens on different herbivore species within the same system are important to understand. If generalist herbivores are negatively affected by plant pathogens from their preferred plant, they could change to preferentially feed on other plant species which do not have any negative impacts on their reproductive or growth rate. Furthermore if a pathogen preferentially deters a keystone herbivore species over other species, there is the potential for the ecosystem effects of this pathogen to be more wide-reaching, with other herbivore species filling this ecological niche if keystone species show local declines in abundance. This could have cascading effects, increasing herbivory on other plant species and potentially displacing herbivores on those plants, which could have further detrimental effects especially for herbivores which have species specific preferences. Further effects could be felt by predator species such as insectivorous birds which may rely on certain herbivore species as their primary food source.

The impact of diseases such as AOD at a small scale, as was examined in this study, need to be fully understood and then expanded to include ecosystem cascades.

5.6.5 - Impact of herbivory changes on birds

Great tits *(Parus major*) are frequently used as a model when examining trophic cascades, due to this species' dependence on caterpillars from oak trees as a food source during breeding season. Caterpillar abundance in any given year is affected by the health of the oak trees along with many other environmental factors such as temperature (Buse et al., 1999).

This tri-trophic system of oak > caterpillar > great tit, has become almost a ‘poster process’ within ecology, and has been used to demonstrate the fragility of woodland ecosystems (Varley, 1970). Great tits are largely thought to time their broods to coincide with peak caterpillar abundance, due to their heavy reliance on caterpillars as a food source when feeding nestlings, feeding at a rate of 700 visits to the nest per day (Gibb, 1955). Successfully matching the peak food demand with peak caterpillar biomass results in increased offspring success (Ramakers et al., 2020; Thomas et al., 2001; Tremblay et al., 2003; Verboven et al., 2001). Experimental work into the cause of these timings indicated that birds receive their cues from subsequent trophic levels, i.e. the foliage and appearance of the oak tree, which indicates the upcoming peak in caterpillar biomass (Hinks et al., 2015). Some populations of great tits shift their egg laying earlier as a result of warmer temperatures, which has the potential to reduce synchrony with caterpillar populations (Reed et al., 2013). Recent work, however has criticised the use of “start” dates when assessing phenological synchrony and instead looked in favour at “peak” dates in each trophic level - i.e. to what extent is there a discrepancy between the peak prey biomass and the peak predator demand (Ramakers et al., 2020). Certainly, when assessing the impact of phenology on breeding success, using these “peak” dates serves as a better predictor of food availability.

The surrounding environment of a nest box has a large influence on reproductive success, as observational studies have documented that great tits using breeding sites surrounded by lower densities of oak have to work harder, expending more energy to rear their young than those pairs surrounded by higher densities of oak (Hinsley et al., 2008). An increase in the availability and number of caterpillars within a parent’s foraging area could result in higher quality young (Naef-Daenzer et al., 2001) as they are able to be provisioned with a greater amount of food from a closer area, which also means the parents are expending less energy foraging (Hinsley et al., 2008; Oers et al., 2015). In blue tits and great tits, nest site selection can occur where higher quality parents frequently take the more desirable nest sites (Mänd et al., 2005), which can often be categorised by the abundance of oak in the area (Hinsley et al., 2008), and also by potential food availability (Tremblay et al., 2005).

5.6.6 - Future work

The differences in herbivory identified here should be further examined over a longer period and with a larger sample size of trees. Herbivore choice experiments could also be replicated in the laboratory by experimentally inoculating oak saplings and monitoring herbivory rates, which was attempted in this project but was unsuccessful. More controlled experimental analysis could also affect how growth of herbivores varies across trees of different disease statuses, and if any mechanical and structural changes in the plant because of AOD can be seen to impact herbivory through changes in the palatability of the leaves or the ability of the

leaves to be attractive to herbivores.

The trees investigated in this study were also only lightly infected with AOD and still had good canopy cover. As described in section 1.4 of this thesis, trees which are at the more advanced stages of AOD can suffer high levels of canopy thinning. There was not much discrepancy in canopy density of the trees assessed in this study at differing levels of AOD, although they did have slightly lower canopy density than trees asymptomatic for AOD (see Figure 10, section 3.5.1). By re-examining herbivory across trees with a wider range of AOD statuses, the extent of canopy thinning, and crown dieback can be included.

This project also only examined overall herbivory rather than focussing on specific invertebrate species. As demonstrated above, species changes at any level of the system can result in different impacts. By examining both overall herbivore abundance as well as focussing on particular species, we will be able to examine how impacts on specific herbivore species could have impacts in a wider ecological context.

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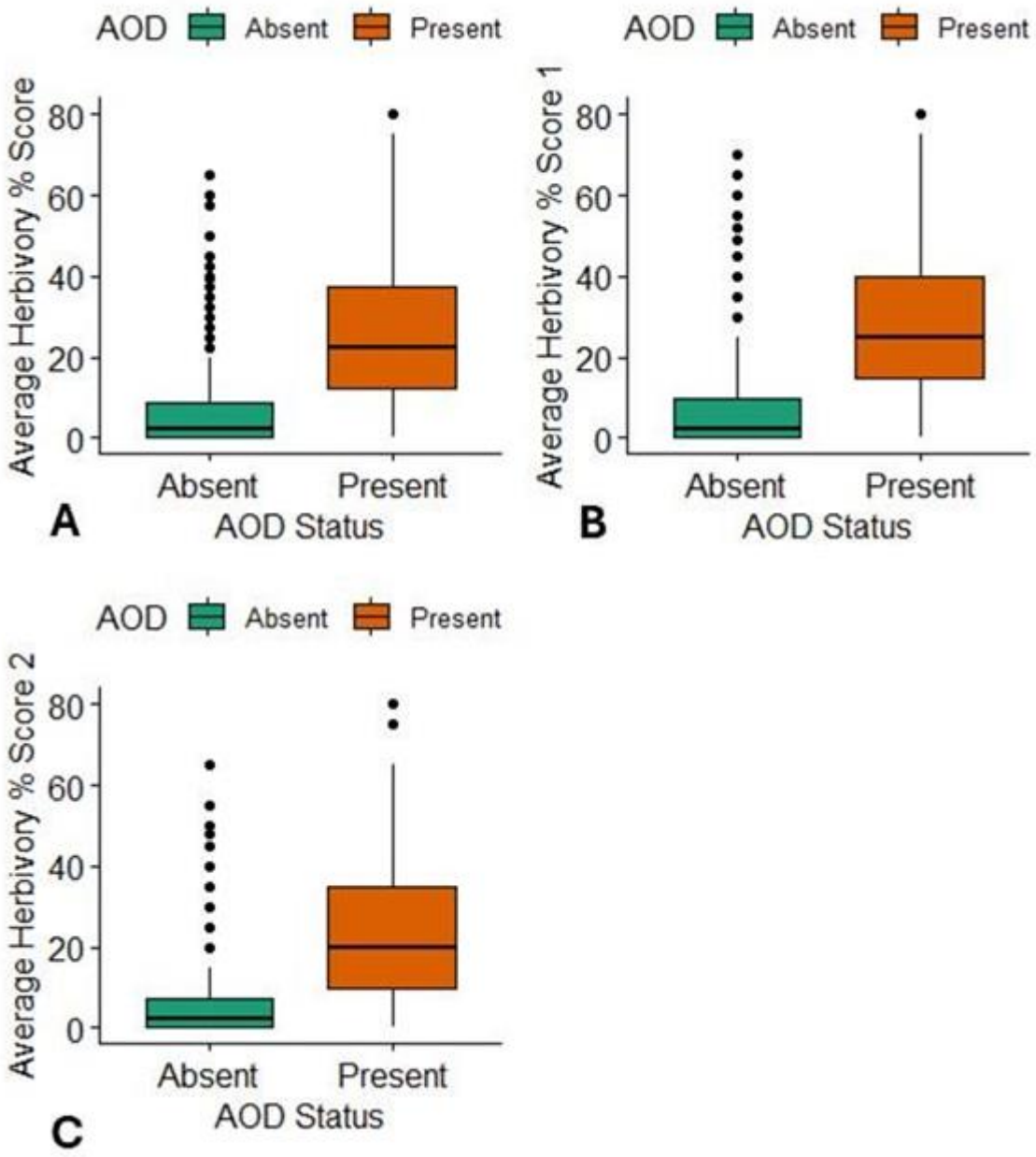
5.8 – Supplementary Material

Supplementary Material 5.1 - Comparison of herbivory levels of trees symptomatic (orange) and asymptomatic (green) for AOD. A) Average herbivory score from first and second assessment. B) Herbivory scores from first assessment. C) Herbivory scores from second assessment.



Chapter 6 -The impact of Acute Oak Decline on breeding success of birds

6.1 - Abstract

Breeding success can be measured by how successful an individual is at breeding, for example by examining the number of fledglings produced each breeding attempt, the health of these fledglings. Breeding success is a measure of fitness and is known to be impacted by a variety of external and internal factors, such as environmental variation. Each species of bird will respond differently to different habitat variables that are specific to their ecological niche. Blue tits and great tits have higher levels of breeding success in habitats with higher quality, however the contribution of any tree diseases has yet to be examined in relation to bird breeding success. In this study, breeding of great tits and blue tits was monitored over a woodland with varying levels of Acute Oak Decline (AOD). nest boxes were monitored for signs of breeding, and morphometrics were taken from any chicks that hatched. It was found that birds were more likely to nest in areas with higher oak condition scores and lower incidences of AOD, however once the nests had been constructed there was no significant difference in any other breeding metrics. This is the first work that has shown a link between the presence of a tree disease and breeding site selection for great tits and blue tits. To fully explore the impact of AOD on bird breeding success, further work is needed to examine how recruitment into the adult population varies across a disease gradient, on a wider range of sites with more extreme levels of AOD.

Keywords; great tit, blue tit, AOD, breeding site selection, tree disease, breeding success

6.2 - Introduction

Globally, bird populations are in decline, with an overall deterioration in conservation status of many species and an increased likelihood of extinction since the 1980s (Lees et al., 2022). This trend is echoed in the UK, with a 15% population decline in wild bird abundance since the 1970s, with the strongest declines being seen with farmland and woodland species (DEFRA, 2023).

A recent review into the state of the world's birds identified many threats to global bird populations, including land-use change, habitat degradation, threats from invasive species and diseases, and climate change (Lees et al., 2022). These pressures can impact birds by

directly affecting the health and behaviour of individuals, as has been documented with increased noise, light and air pollution (Dutta, 2017). Threats can also lead to population declines through increased mortality, or an overall decrease in reproductive rates that fall below a replacement threshold, indirectly, through the reduction of suitable habitats. Certain genotypes and phenotypes may allow for a variation in the response of individuals to different stressors, which lead to the favourable traits being selected and persisting within a population over generations. An example of this in great tits is plasticity in the timing of reproduction, which is a heritable trait found to be selected for in response to changing climates (Nussey et al., 2005). This is an example of relative fitness within a population, where different genotypes and phenotypes convey different evolutionary advantages in specific contexts. Variations in relative fitness within a population push for the selection of advantageous phenotypes which will offer the individual a better chance of survival and reproduction (Alif et al., 2022), leading to these characteristics being retained within a population (Reiss, 2013).

Absolute fitness relates to an individual's fitness within its lifetime and is measured by its reproduction rate. At its simplest form, individuals of a species with higher absolute fitness will produce more offspring within their lifetime, and any advantageous traits will therefore be inherited to the next generation (Orr, 2009). Absolute fitness can be affected by many factors, both intrinsic and extrinsic (Sæther & Engen, 2015), with some examples of stressors outlined in Figure 1. These factors can be grouped into the broad categories of ecological traits, life history, physiology, behaviour and genetic traits (Patankar et al., 2021), many of which have complex linkages and can affect one another. Ecological traits encompass a range of extrinsic factors such as changes in weather and climate. These can impact the absolute fitness of birds, either directly by pressuring chicks in nests which aren't able to thermoregulate, or indirectly by influencing other factors such as food availability (Sauve et al., 2021). Other pressures, such as increased parasite and pathogen loads, have also been associated with lower fledgling survival in passerine birds (Asghar et al., 2011; Lachish et al., 2011). Some environmental changes or variables that can increase the fitness of one species may conversely decrease the fitness of another, especially when predation and competition are considered. The impacts of competition are variable, and, as with most environmental pressures, they are context specific (Gregory, 2009). Some bird species benefit from the presence of interspecific competitors in their breeding territory, showing increased brood size and healthier fledglings (Forsman et al., 2002), whereas others have worse fledgling body condition and increased displacement from nesting sites (Powell et al., 2021). Food availability, particularly during the breeding season, can impact parental provisioning rate, and can impact fledgling body condition and also the proportion of chick survival from the nest (Martin, 1987). These variations in fitness response to stressors highlight the need to consider each system separately and highlight the need to have a sound understanding of

which environmental factors can influence the fitness of species.

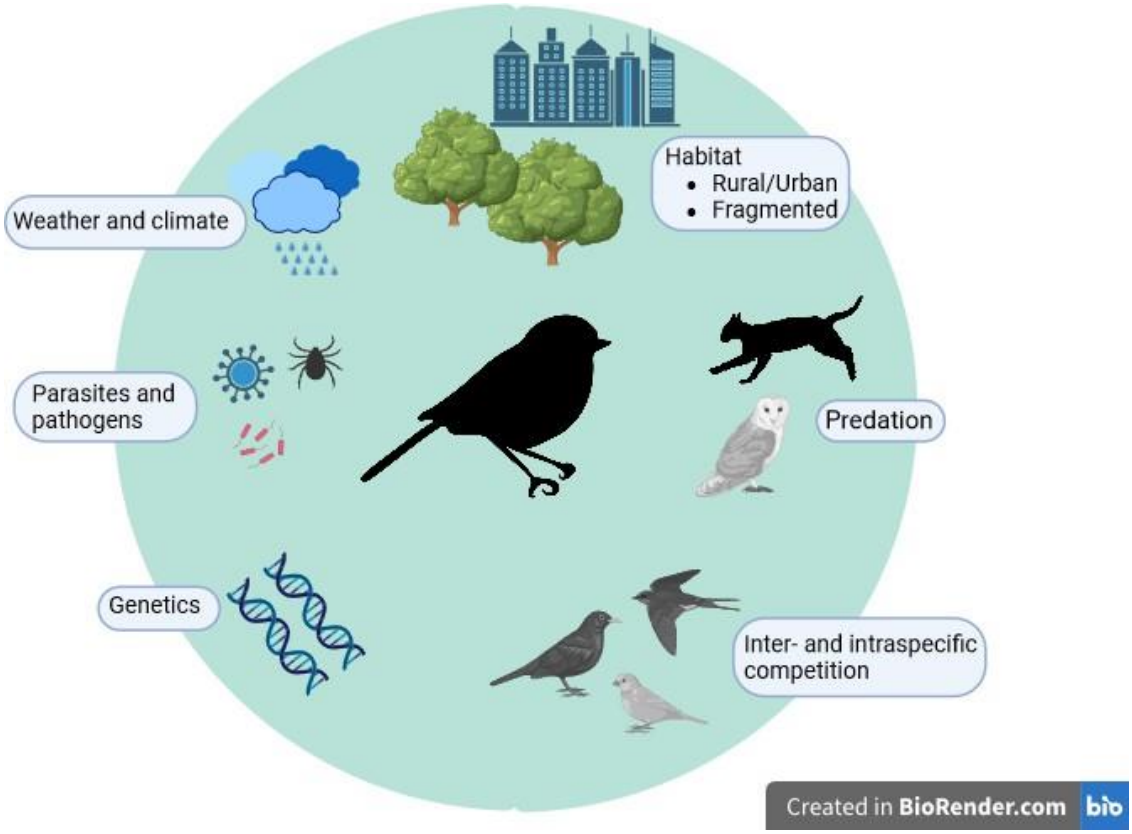


Figure 1. A selection of factors which can impact and be impacted upon by the fitness of individuals, such as the small woodland songbird at the centre.

Among birds, it has long been established that parental quality and fitness can have a direct impact on nestling success, measured by chick size and survival. Lower-quality parents with reduced relative fitness often produce smaller and lower-quality eggs (Krist, 2011), and can provide insufficient incubation, which have knock-on consequences for hatching success and nestling condition (Parker, 2002). Some fitness costs can be amplified by external factors; for example, birds with lower parasite loads have been found to produce healthier offspring, heavier clutches and invest more in nestling provisioning than individuals with higher parasite burdens. This results in birds that have lower fitness levels, attributed to their parasite load, producing lower quality offspring (Schoepf et al., 2022).

It is therefore crucial to understand the extent to which extrinsic pressures can impact on important behaviours, such as breeding and feeding. A thorough knowledge of environmental pressures and their fitness implications allows us to understand why species favour certain

habitats over others and will help to evaluate how environmental changes can affect individuals and species. As complex as the impacts on fitness can be, however, this work focuses on habitat quality, with a particular emphasis on tree and woodland health, and the impact this has on reproductive success of woodland birds.

6.2.1 - Habitat quality and birds' reproductive success

The association between habitat selection and reproductive success has been considered in research and reviews for many decades (Hildén, 1965; Piper, 2011), for example, by comparing the likelihood of birds selecting certain habitats, especially for the purposes of breeding, and the resulting fitness consequences from this (Chalfoun & Schmidt, 2012; Fisher & Davis, 2010; Jones, 2001; Kristan et al., 2007). Nest site characteristics can be crucial to offspring health and survival but are variable depending on the species. One example is forest edges (McCollin, 1998), where the proximity to habitat edges, and smaller habitat patches, are associated with lower nestling success in blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*), and a reduction of food provisioning in great tits (Bueno-Enciso et al., 2016). For other species, such as owls (*Strigiformes)*, however, increased forest edges provide easier access to food (Ries et al., 2004). Breeding in more desirable nesting locations, based on the aspects that are most important for the niche of the species, could result in higher levels of breeding success. In this example, one could assume that owls breeding at forest edges would have increased reproductive success, whereas great tits and blue tits breeding in the same habitat would have lower success.

Measures of parental breeding success can contribute to our understanding of an individual's reproductive fitness, which can be measured annually or over an individual's lifetime. Lifetime reproductive success of individuals can be extended to be a measure of population fitness**.** Breeding success is one way to measure absolute fitness of breeding parents, and can be quantified by:

1. Nest success - measured by the number of eggs produced in each clutch, and subsequently how many chicks hatch and fledge (Mayfield, 1961).
2. Nestling quality - for example, measured by body mass, wing/feather length, growth rate, skeletal size (Krist, 2011).
3. Reproductive success (productivity) - measured by the number of fledglings that survive the breeding season and become independent (known as recruitment) (Streby et al., 2014). This can be measured annually and also across an individual's lifetime and can account for multiple breeding attempts within a year.

In many studies, the term ‘breeding success’ is commonly used to refer to nest success, with reproductive success being less frequently reported (Thompson et al., 2001) due to the additional effort in monitoring the proportion of nestlings recruited into the adult population.

Breeding success in birds is variable across habitat quality, as documented in a range of species occupying different niches with different ecologies (Gunnarsson et al., 2005; Jones et al., 2014). As such, measures of habitat quality are specific to each species and are highly variable (Camacho et al., 2015; Johnson, 2007). For birds such as blue tits and great tits in the *Paridae* family, which typically breed in forests and nest in tree cavities (Mönkkönen & Orell, 1997), habitat quality is tightly linked to the woodland structure and composition that they inhabit. Better quality habitats, which consist of more contiguous and heterogeneous woodlands, have been found to produce better quality young blue tits and great tits (Hinsley et al., 2008; Lambrechts et al., 2004). Where the birds breed is not random, and there is a wealth of evidence demonstrating that birds actively choose nest sites with favourable conditions for that species (Chalfoun & Martin, 2007; Chalfoun & Schmidt, 2012; Hollander et al., 2011). Nesting sites surrounded by a greater density of understory vegetation and fewer forest patches are two such variables that are selected for in some forest-dwelling passerines (Reiley & Benson, 2019), whereas birds that inhabit grasslands have different criteria for habitat selection, such as the proportion of bare ground and grass within a habitat (Fisher & Davis, 2010).

The factors affecting the breeding success of blue tits and great tits have been studied extensively throughout Europe, with the first long-term breeding study of great tits starting in the Netherlands in 1912 (Kluijver, 1951). This study was the catalyst for the implementation of long-term breeding studies of great tits and blue tits across Europe and the UK, with a notable UK system being the Wytham Tit project established in 1947 (Lack, 1947). These studies allow social behaviours to be examined (Beck et al., 2023), as well as how breeding varies across spatial gradients and in response to changing climates and food availability (Shutt et al., 2019). The enormous amounts of data collected through these projects means we have a sound understanding of the intricacies of great tit and blue tit breeding, with great tits being the most studied wild birds in the world (Song et al., 2020).

Blue tits and great tits are abundant in the UK and take readily to nest boxes, enabling their breeding to be easily studied (Ceia et al., 2023; Serrano et al., 2017). These species are commonly found in a range of UK habitats, across woodland, parkland and urban environments (Cramp & Perrins, 1993). Blue tits and great tits represent ideal model species

to examine ecosystem effects on breeding as they are widespread across a range of habitat types, numerous and make useful indicator species. Studies comparing the breeding success of these birds across a range of habitat types has found they are generally more successful in forests as opposed to urban areas, with higher numbers of hatchlings and fledglings, and overall higher reproductive success, which has been attributed to lower inter and intraspecific competition in forests, lower predation rates and higher levels of food availability compared to urban areas (Hedblom & Söderström, 2012; Wawrzyniak et al., 2020). Looking within forests as preferred habitats, factors such as woodland area have been found to be important to breeding success of both blue tits and great tits, with a decline in breeding success with smaller woodland areas (Hinsley et al., 1999). Similarly, tree species composition is important, with the higher levels of breeding success being found in areas with higher proportions of mature, deciduous trees (Blondel et al., 1991; Hinsley et al., 2008), in particular oak (Dekeukeleire et al., 2019).

Death of mature trees is important in maintaining structural complexity within woodlands, and deadwood serves as an important habitat to many species (Thorn et al., 2020). Issues can occur when deterioration and dieback of trees happens at an above natural rate, often spurred on by environmental changes and novel diseases as mentioned in section 1.2 of this thesis. These additional pressures on trees can have knock-on effects on the whole ecosystem (Broome et al., 2021) with, for example, the increased death of common ash trees (*Fraxinus excelsior*) due to infection the fungal pathogen *Hymenoscyphus fraxineus*, causing ash dieback, which reduces woodland connectivity and increasing gaps in the habitat (Plenderleith et al., 2022).

Studies have demonstrated increased breeding success for blue tits and great tits in high density oak-dominated areas (Amininasab et al., 2016; Wilkin et al., 2007), but no studies have yet examined oak tree condition in relation to bird breeding success or the wider ecosystem. Tree diseases, such as Acute Oak Decline (AOD), have the potential to severely reduce stands of oak trees, as discussed throughout this thesis, and also to reduce the health and quality of the trees. As found in Chapter 5 of this thesis, trees symptomatic for AOD had higher rates of invertebrate herbivory, an important food source for provisioning great tit and blue tits, so this may have further significant implications for the birds' breeding success. Overall, if habitat quality affects not only a bird's decision to breed in a certain area, but also has the potential to affect the quality of the offspring produced, then all aspects and variables associated with that habitat have the potential to impact breeding success and fitness. This study is the first to investigate how the influence of tree disease on habitat quality impacts the

breeding success of blue tits and great tits. The findings of this work will significantly add to the wider understanding of the ecosystem effects of Acute Oak Decline (AOD), providing a wider view of the implications of this tree disease away from impacts on the individual affected trees.

6.3 - Aims and hypotheses

Blue tits and great tits were used as model species in this research to investigate how far the effects of acute oak decline can spread outside of the individual affected trees. Work in Chapter 5 of this thesis demonstrated a link between trees which were symptomatic for AOD and folivorous herbivory rates. This chapter extends this ecosystem view of AOD by examining how breeding success of birds can be affected by the presence of this tree disease.

This chapter aims to examine the following;

1. The impact of AOD and oak tree quality on the likelihood that blue tits and great tits will occupy nesting sites, proceed to lay eggs, and produce offspring that successfully fledge. Blue tits and great tits show preferences for nesting in areas with high levels of canopy cover and especially in close proximity to oak trees. As AOD leads to canopy thinning and eventually oak death, it is predicted that birds will show a preference for nesting sites in areas with lower levels of AOD and higher levels of oak tree quality.

2. The impact of AOD on the breeding success of blue tits and great tits, measured by chick weight and mass. Due to the impact AOD can have on oak condition, and from the differing herbivory rates across AOD symptomatic and asymptomatic trees found in Chapter 5, it is predicted that there will be differences in bird breeding success across habitats with differing AOD statuses.

6.4 - Methods

6.4.1 - Habitat analyses

A detailed description of the field site used in this study can be found in section 3.4.1.1 of this thesis. In summary, a network of 103 nest boxes in Epping Forest, Essex were erected across 180 hectares of forest. The surrounding 50 m radius of the nest box was designated as a plot, and each oak tree within that area was examined for the presence of AOD symptoms

alongside canopy density scores. The AOD symptoms being surveyed for were stem bleeds (both active and inactive bleeds), and emergence holes of the *Agrilus biguttatus* beetle, the process of which is detailed in section 3.4.1.2 of this thesis. In Chapter 3, the AOD status of each plot was scored from 1-3, with a score of 1 indicating no AOD symptoms found, a score of 2 indicating moderate infection with AOD, and 3 representing advanced AOD. Figure 9 in Chapter 3 shows the severity of AOD in each plot across the study site.

The scoring system used in this chapter produced a continuous score of overall oak condition within each habitat plot. Each individual oak tree was given a score for the scale of the AOD symptoms (a score of 0-9) and for the individual tree canopy density (a score of 0-15). These were then averaged across the plot to give an average habitat health and oak condition score. The percentage of symptomatic oak within each plot was then determined and scored from 1-5. This was added to the health score, giving an overall habitat health score for each plot from 1-29. Table 1 indicates the scoring criteria for individual trees and for the whole habitat plot in this chapter.

Table 1. Scoring system used for habitat assessments and oak condition scores.

Condition Metric	**Scoring criteria**	**Score**
Agrilus emergence holes (individual trees)	**Number of emergence holes**	
	>10	1
	1-10	2
	0	3
Inactive Stem Bleeds (old bleeds, not actively expelling exudate) (individual trees)	**Number of bleeds**	
	>10	1
	1-10	2
	0	3
	Number of bleeds	

Condition Metric	Scoring criteria	Score
Active Stem Bleeds (bleeds actively expelling exudate) (individual trees)	>10	1
	1-10	2
	0	3
Crown Density (individual trees) Assessed three times per tree - the upper, middle, and lower canopy, giving a score of up to 15 for the whole tree	**Proportion of live Crown per third**	
	0% (dead)	0
	1-20%	1
	21-40%	2
	41-60%	3
	61-80%	4
	81-100%	5
Symptomatic Tree Score (of plot)	**Percentage of symptomatic trees**	
	30-40	1
	20-30	2
	10-20	3
	0-9	4
	0	5

6.4.2 - Bird breeding monitoring

Breeding success of blue tits and great tits was monitored in Epping Forest, Essex. As detailed in Chapter 3 of this thesis, 103 nest boxes were installed in 2019 (Chapter 3, Fig. 4). Nest

boxes had a 32 mm entrance hole which excluded birds larger than great tits from nesting there. For reference, typical breeding metrics of blue tits and great tits are outlined in Table 2.

Table 2. Typical breeding metrics of great tits and blue tits, data compiled from Cramp & Perrins (1993).

Breeding Metric	Great Tits	Blue Tits
Laying date	April	Early April - mid May
Average Clutch Size	8-12	10-12
Incubation Time (days)	12-15	13-16
Fledgling Period (days since egg hatched)	16-22	16-22
Nesting success (proportion of eggs laid that produced fledglings)	95%	90-95%
Number of broods	Up to 2	1 (second broods very uncommon)

Nest boxes were monitored each spring from 2020-2023, with weekly checks commencing from 1st April. Full data was not able to be collected in 2020 due to lockdown restrictions from the Covid-19 pandemic. As such only occupancy data of the nest boxes is available for that year, which were obtained in late May.

A ladder was used to access nest boxes which were mounted approximately 3m high on oak trees. The nest box lid was carefully lifted to determine the presence of adults. If adults were present, then checks were commenced once the parent had left the nest. Boxes were checked for stages of nest construction (Table 3) and the presence of eggs. Birds in the *Paridae* family typically lay one egg per day (Perrins & McCleery, 1989), therefore if eggs are detected during the laying process, the date of the first egg production can be calculated. Eggs were recorded according to the scoring system in Table 4.

Table 3. Stages of nest construction. Descriptions in italics are those advised by the British Trust for Ornithology (BTO), followed by additional descriptors

Nest Code	Description
N0	*Nest not yet built* An empty box
N1	*Nest quarter built* Some bits of new moss/grass, but floor of box still visible
N2	*Nest half built* Floor covered with layer of moss/grass
N3	*Nest three-quarters built* Thick deep layer of moss/grass. Maybe the beginnings of a nest cup forming
N4	*Nest completed and unlined* Well-defined cup, but unlined
NL	*Nest completed and lined* Cup is lined with fur/feathers

Table 4. Stages of egg development

Egg Code	Description
CV	Eggs covered by nest material (egg-laying not complete)
UN	Eggs uncovered
CO	Eggs cold (indicates incubation not yet commenced or not been incubated recently)
WA	Eggs warm (indicates recent incubation)
FN	Female on the nest, incubating eggs

Nest box monitoring continued weekly until any eggs had hatched. One hatched, the number of chicks present in the nest were counted and aged. It is possible to calculate the age of the chicks up to 8 days of age, with the day of the first egg hatching counted as 'Day 0'. After day 8 of development the developmental stages become less distinct day to day, therefore the chicks are unreliable to age. The developmental stages of chicks can be seen in Figure 2, and Figure 3 shows how to determine wing feather emergence. Where several developmental stages were detected in the nest, the most advanced stage was recorded. Chick development typically takes between 18-21 days from hatching to fledging.

Figure 2. Stages of chick development from hatching to fledging alongside the BTO recording codes

On day 11 post hatching, several morphometrics were taken from the chicks. To do this, chicks were gently removed from the nest and placed into a cotton drawstring bag. Another cotton bag was used to plug the entrance hole of the nest box to prevent adults returning to the nest

and assuming it had been predated, which would increase the risk of nest abandonment. All processing was carried out within a few metres of the nest box tree.

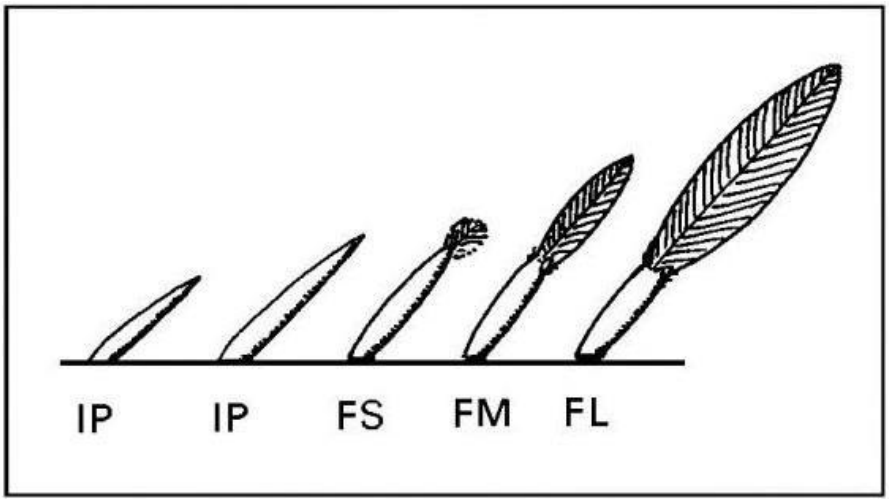


Figure 3. Stages of wing feather growth with corresponding codes as advised by the BTO. IP - In pin, FS - feathers short, FM - feathers medium, FL - feathers long. Image reproduced with permission from the British Trust for Ornithology.

Each chick was fitted with a unique identifier in the form of a metal ring on their right leg. Each metal ring displays a unique ring number which was reported to the BTO. The following information and morphometrics were taken from each nest box, alongside samples for microbiological and molecular analysis (outlined in Chapters 3 and 4).

- Total number of chicks present
- Individual chick weight (g)
- Length of feather emerged from the third wing quill (mm)

All nest boxes were checked until chicks had fledged, which was usually mid-June. Nest boxes were monitored until the last chicks had fledged. Any second broods were noted but were not sampled, as second broods tend to have smaller clutch sizes, higher nestling mortality, and poorer body condition with smaller chicks with a lower probability of fledgling survival (de Lope et al., 1998; Dubiec & Cichoñ, 2001) therefore they would not be useful for comparisons.

In summary, the eight variables below were recorded for each nest box, which have been adapted from Hinsley et al., (1999, 2008) and are established as indicators of breeding success in nest box studies.

- Date of first egg
- Size of the clutch
- First hatch date
- Number of unhatched eggs
- Number of offspring at 11 days
- Mean chick weight at 11 days (g)
- Length of feather emerged from the third wing quill at 11 days (mm)
- Number of young fledged

6.4.3 - Statistical analyses

Data were analysed and visualised in R (v.4.3.1) using the stats (v.3.5.0) and ggplot2 (v.0.6.0) packages. Breeding metrics were analysed between sites that had differing levels of AOD, and then against a continuous health scale as described in section 6.4.1. Generalised linear models with binomial errors were used to assess the variables below based on presence/absence data. These data were not separated by species.

- Nest construction
- Eggs laid
- Eggs hatched

The following data were analysed separately for blue tits and great tits. Logistic regression was carried out using generalised linear models (R package “stats” v.3.6.2) in R were used to look for relationships between habitat condition score and the following variables:

- Day of 1st egg (relative to April 1st)
- Clutch size
- Brood size
- Number of fledglings
- Number of deceased chicks
- Chick feather length (mm)
- Chick weight (g)

An example of the model structure is

```
logit_model <- glm(Nest_Made ~ Overall_Health_Score + Year, family = binomial,
data = OccupancyR)
```

Year and nestbox number were included as fixed effects, so as not to include variation across years or within the same nestbox. Nestbox number was included when assessing averages such as chick weight.

6.5 - Results

A total of 2623 oak trees were surveyed over 103 habitat plots. Section 3.5.1 of this thesis has a detailed breakdown of areas of the locations of the plots within the field site which had differing levels of AOD and shows the breakdown of categorical AOD statuses. Figure 4 shows the distribution of habitat health scores across the plots surveyed, with the mean health score being 21.35 (range 14.68 - 26.8). Due to time constraints each tree and habitat plot was only surveyed once.

The number of nest boxes used and surveyed each year was variable, and a breakdown of the use of each nest box over the four field seasons can be found in Supplementary material 6.1.

Figure 4. Histogram showing the range of habitat health scores across the habitat plots surveyed.

6.5.1 - Occupancy

The condition of oak trees surrounding nest boxes was a significant factor in predicting whether a nest box was used and a nest constructed (β = 0.157 ± 0.666 SE, $p \leq 0.05$). The likelihood of a nest box containing a nest increased with increasing health score (Fig. 5), a relationship which was consistent across years, although the strength of this relationship did vary according to the year.

Figure 6 shows how nest boxes that contained nests typically had higher average habitat health scores than nest boxes that did not, which was consistent across years. In most years, nests were present in nest boxes of most habitat health scores; however, nests were generally "absent" at lower habitat health scores, with much more inter annual variation.

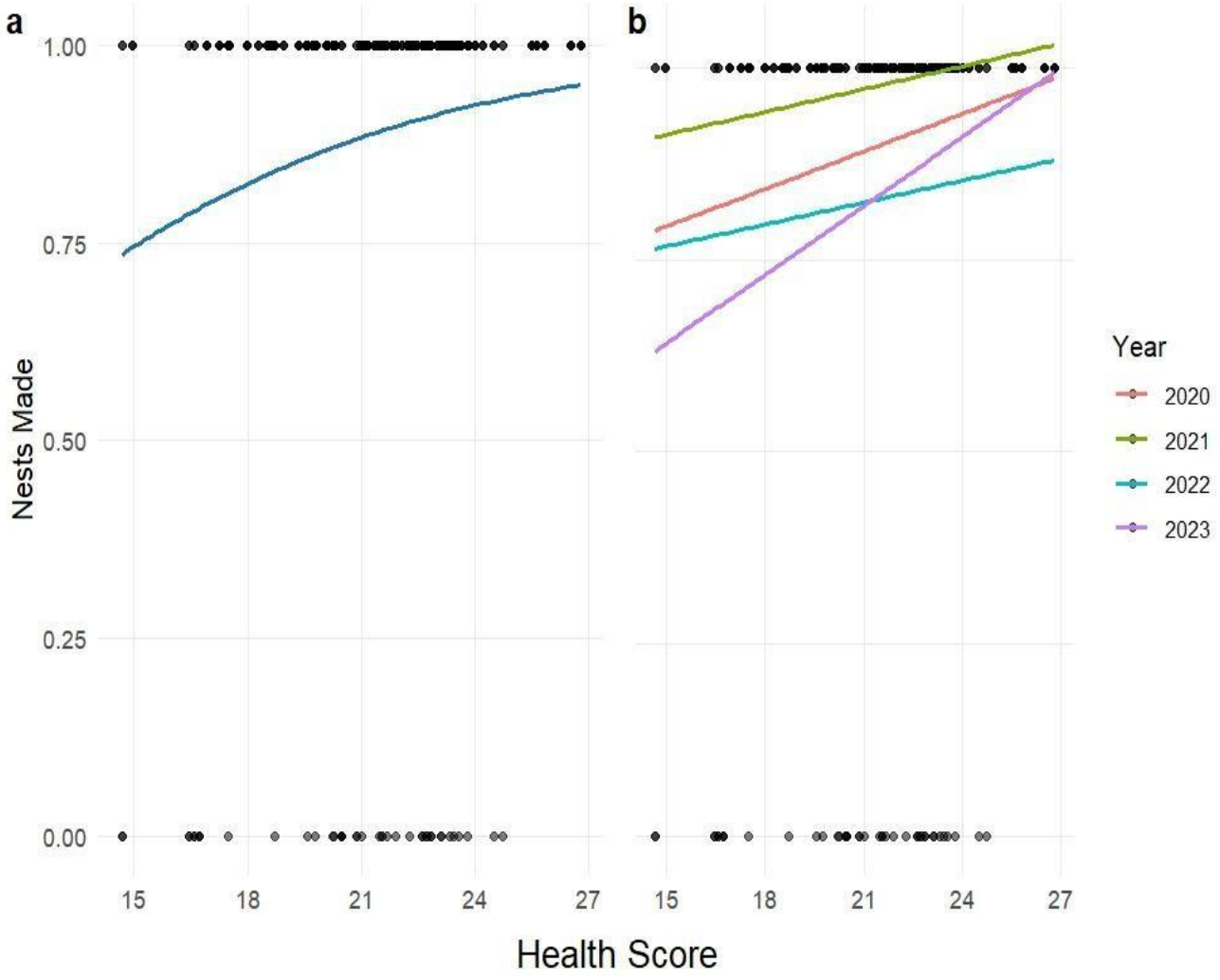


Figure 5. Proportion of nests made across habitats with differing health scores a) average trend over all years of the study (2020 - 2023), b) individual year trends. Both graphs show a positive relationship between health score and the probability of the nestbox being used to construct a nest.

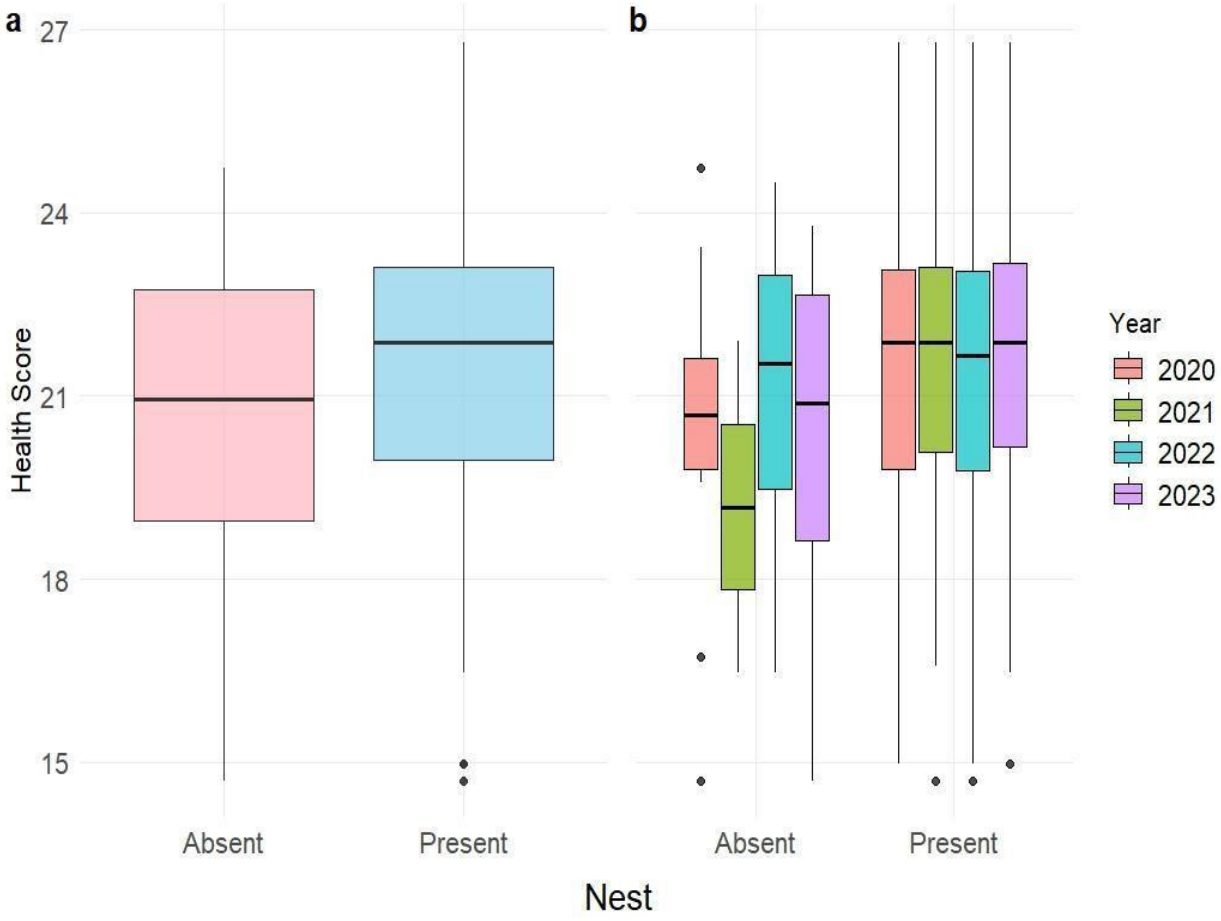


Figure 6. Boxplots demonstrating the spread of habitat health scores of plots that did or did not have nests constructed a) over all years, b) separated by year.

6.5.2 - Date of first egg

Table 5 shows the distribution of days when the first egg was laid, relative to April 1st, across habitats affected with AOD and those without, for both blue tits and great tits. As an example, day 17 would be April 17th. When analysing these data against a categorical basis of AOD (i.e. is AOD present or absent within the habitat or the nestbox), there wasn't a clear trend the between mean and median egg laying dates and AOD status.

When the AOD data were analysed on a continuous scale, there was a slight negative relationship between the day of the 1st egg and health score of the habitat (Fig. 7), with earlier egg laying dates in habitats with higher health scores. This trend however, was not significant for either bird species (blue tits, $\beta = -0.0162 \pm 0.023$ SE, $p = 0.479$) (great tits, $\beta = -0.040 \pm 0.047$ SE, $p = 0.392$). When analysed across years (Fig 7B), we can see the negative trend being reflected in all years except for blue tits in 2022, which shows a very weak positive relationship between habitat health score and date of first egg.

Table 5. Median and mean days of first egg, relative to April 1st +/- standard error

		All			Absent			Present		
		2021	2022	2023	2021	2022	2023	2021	2022	2023
Median Day (relative to April 1st)	All	17	16	20	17	16	19	16	16	20
	Blue Tit	17	20	22	17	22	24	15	17	20
	Great Tit	19	15	16	18	15	15	20	15	20
Mean Day +/- se (relative to April 1st)	All	19 +/- 1.15	16 +/- 0.84	20 +/- 0.86	19 +/- 1.62	16 +/- 1.21	20 +/- 1.15	18 +/- 1.51	17 +/- 1.17	21 +/- 1.29
	Blue Tit	18 +/- 1.23	17 +/- 1.24	22 +/- 1.10	18 +/- 1.70	15 +/- 1.87	19 +/- 1.51	18 +/- 1.83	18 +/- 1.79	22 +/- 1.86
	Great Tit	21 +/- 2.93	15 +/- 1.04	16 +/- 0.95	21 +/- 4.18	14 +/- 1.36	15 +/- 1.15	20 +/- 2.43	16 +/- 1.66	18 +/- 1.46

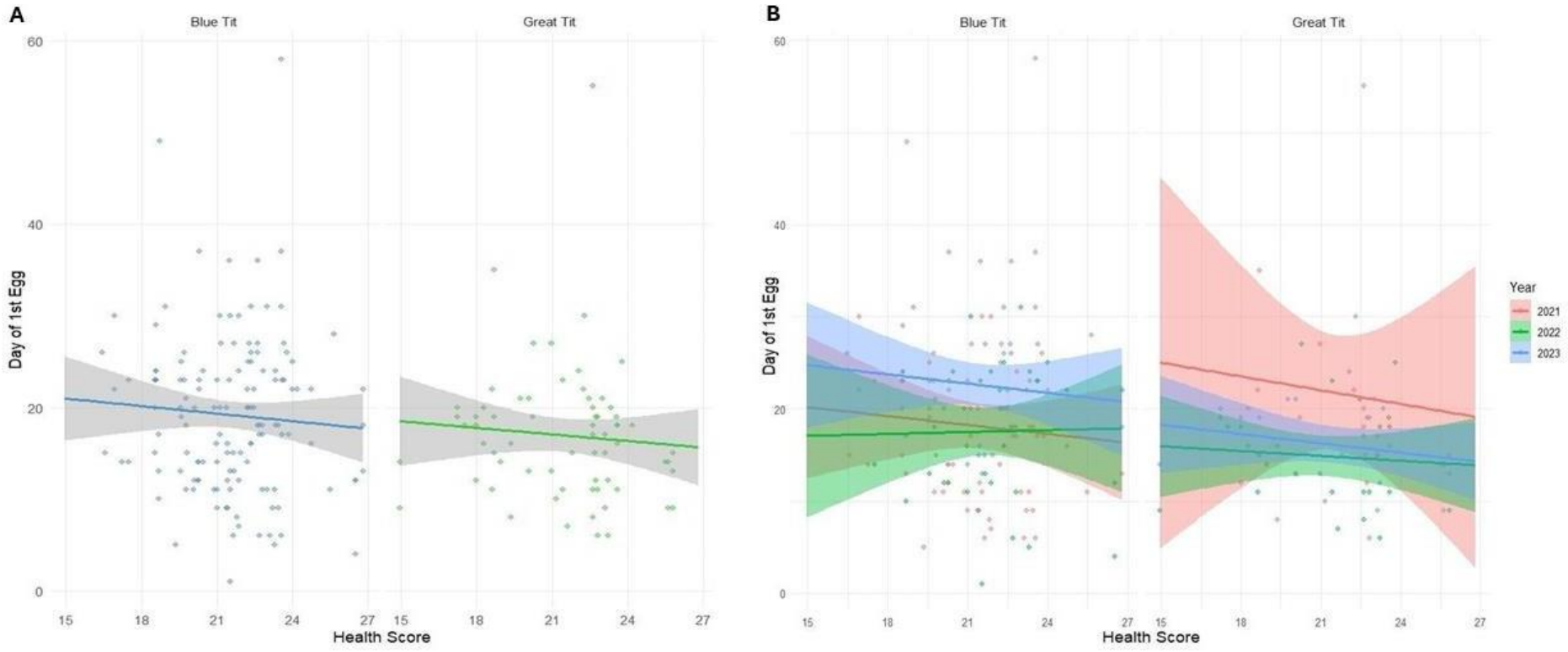


Figure 7. Relationship between egg laying day (relative to April 1st) and habitat health score for blue tits and great tits a) average trend across all years of the study (2020 - 2023), b) individual year trends.

Section 6.5.1 showed that nestboxes in habitats with higher health scores were more likely to be selected as nesting sites than those which had lower habitat health scores, however the likelihood of eggs being laid in nests was not affected by the habitat health score of the plot (β = 0.009 ± 0.075 SE, p>0.05) (Fig. 8) and 9). The probability of eggs being laid was fairly consistent across all health scores when averaged over the four years of the study (Fig. 8A), and when each year was analysed separately there was variability in the direction and the strength of this relationship (Fig. 8B). There was little distinction in the health score of habitats where eggs were laid and also not laid (following nest construction), both across all years of the study (Fig. 9A) and also when compared across years (Fig. 9B).

No significant relationship was found between the likelihood of eggs hatching, clutch size, brood size or number of fledglings, or number of dead chicks across the differing habitat health scores (see Supplementary Material 6.2 – 6.6).

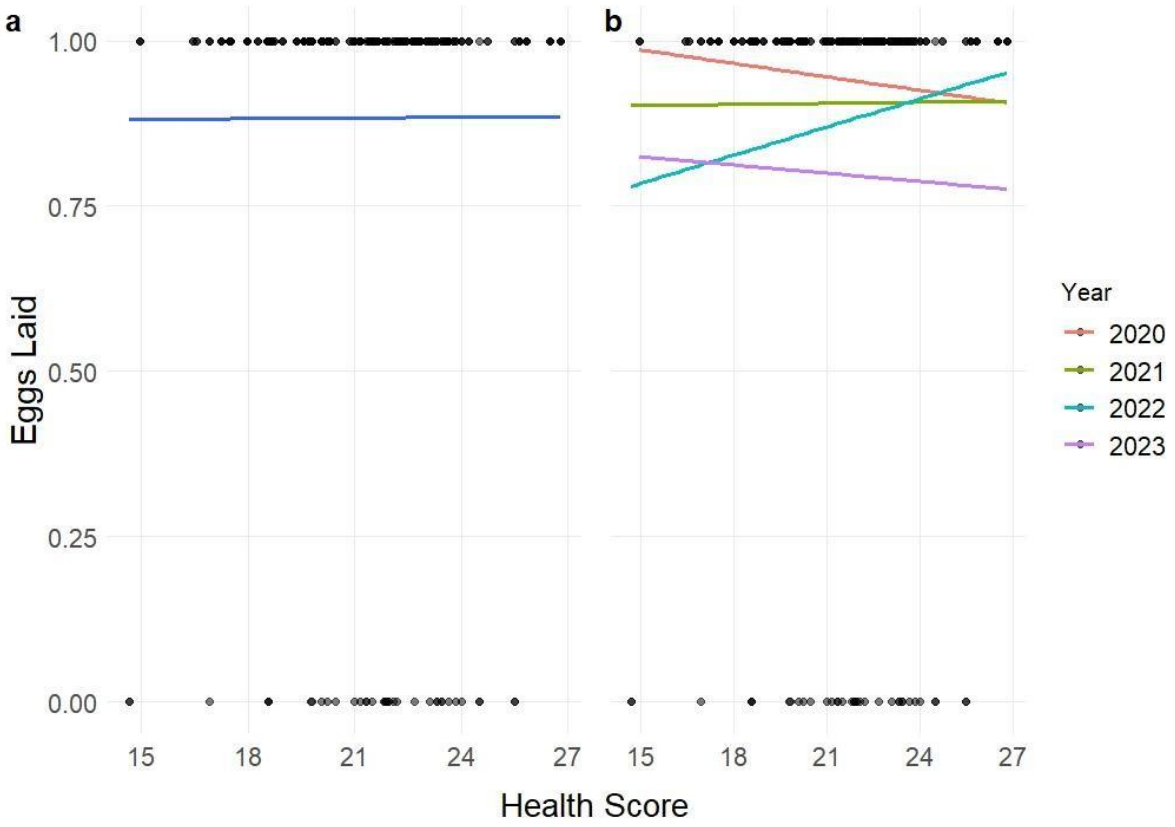


Figure 8. Proportion of nests in which eggs were laid across habitats with differing health scores a) average trend across all years of the study (2020 - 2023), with no relationship between health score and the probability of eggs being laid. B) individual year trends., however this was variable across years.

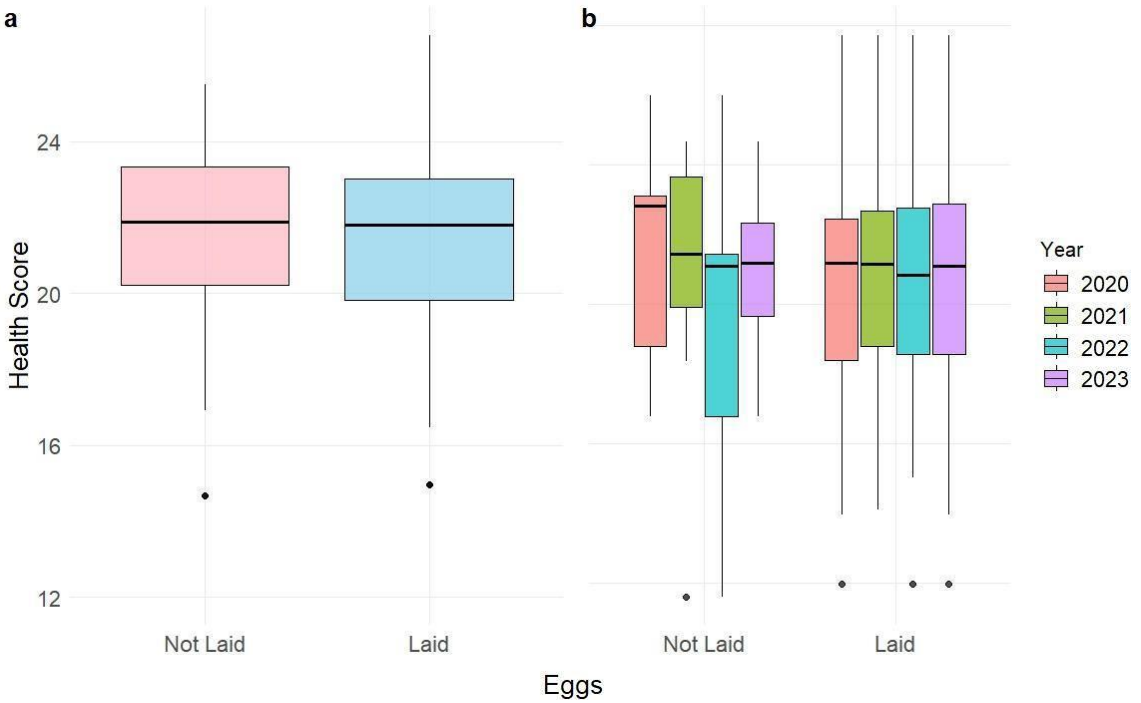


Figure 9. Boxplots demonstrating the spread of habitat health scores of nest boxes that had eggs laid in constructed nests versus nests that were not used for eggs a) across all years, b) separated by years.

6.5.3- Morphometrics

Morphometrics of 1,122 nestlings from 140 broods were collected over three years (Table 6). There was a decrease in the number of blue tit broods after the first surveying year (2021), despite this being the second year the nest boxes were in place. A full breakdown of the use of each nestbox from 2020-2023 can be seen in Supplementary Material 6.1.

Table 6. Number of chicks surveyed for weight and feather length by species and year

Year	Number of Chicks Surveyed		Number of Broods	
	Blue Tit	**Great Tit**	**Blue Tit**	**Great Tit**
2021	381	98	44	14
2022	200	133	23	18
2023	185	125	25	16

Feather length and weight were found to be not significantly affected by the health of the habitat for either species ($p>0.05$) (Supplementary Material 6.6). Figure 10 shows a very slight positive relationship between feather length and habitat health score for both blue tits and

great tits, with some variability across years, however this was not significant (p = 0.426 (Blue tits), p = 0.218 (Great tits).

Figure 11 shows a slight positive but not significant relationship between weight and overall habitat health score for great tits (p > 0.05) When analysed over the different years, we can see there is little difference in the strength or direction of the relationship between weight and health score for blue tits. Great tits however show slightly more variability, with 2023 showing a stronger positive relationship between health score and chick weight than the other years which showed weak negative relationships.

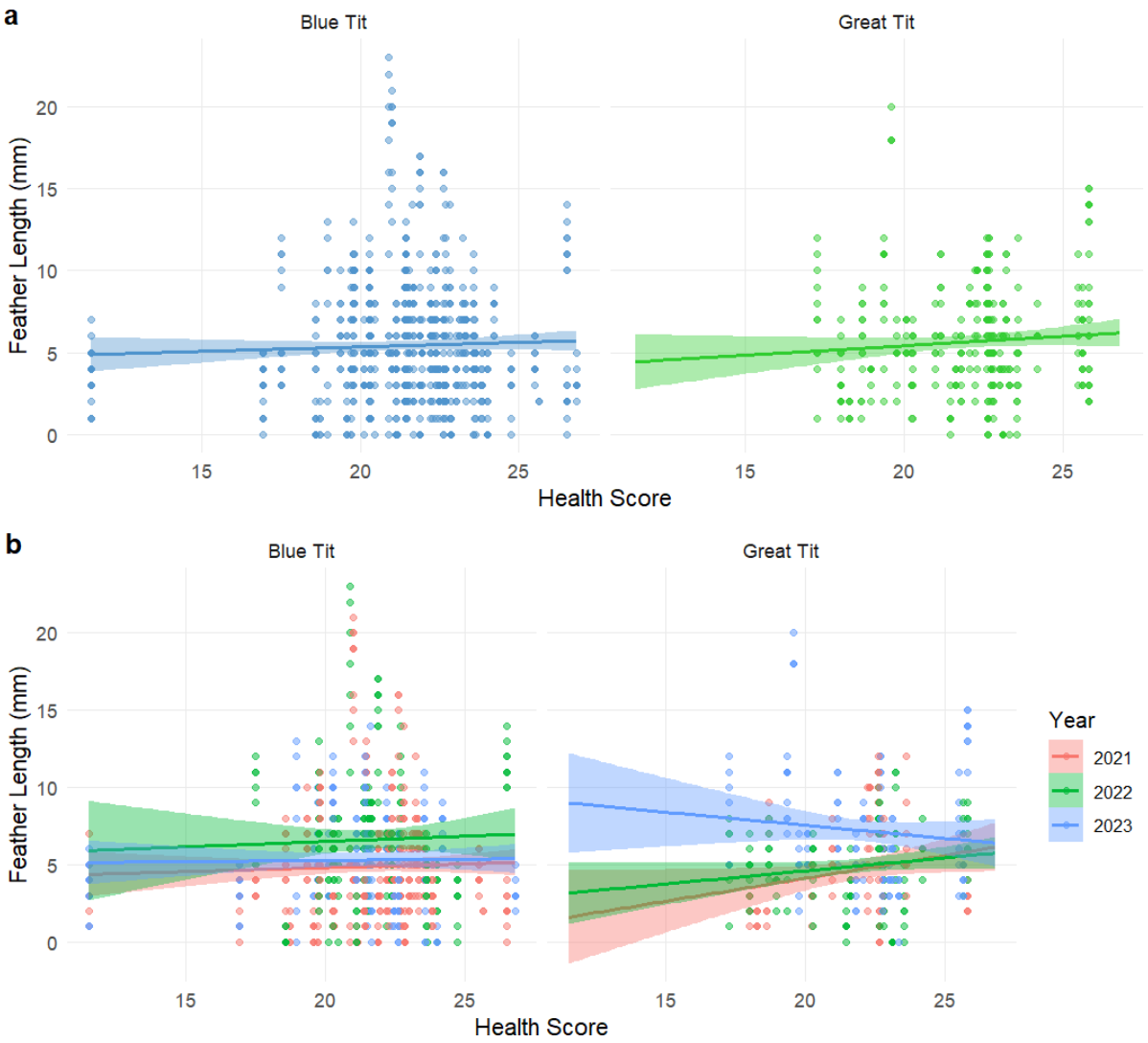


Figure 10. Relationship between habitat health score and chick feather length (mm) a) across all years, b) separated by years

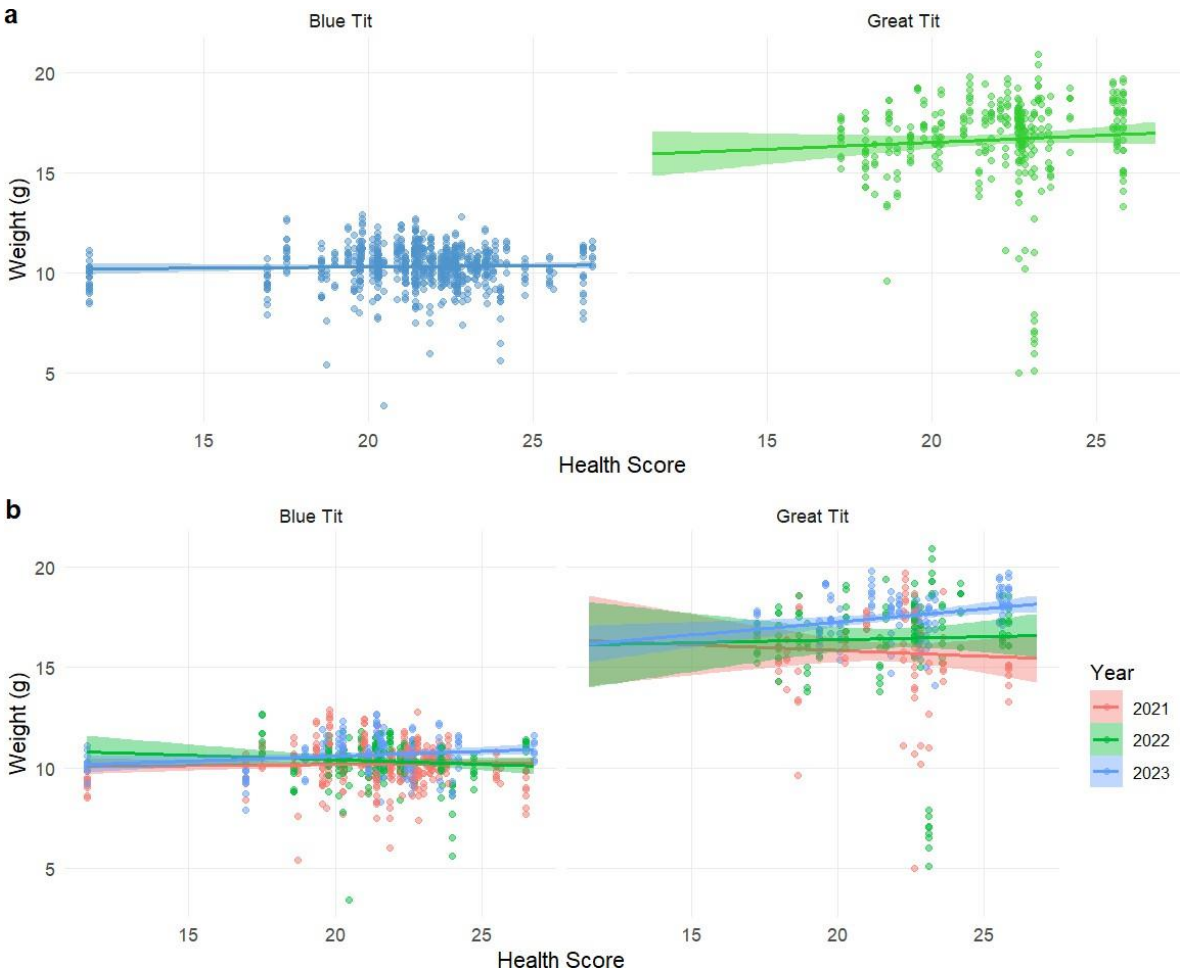


Figure 11. Relationship between habitat health score and chick weight (g) a) across all years, b) separated by years

6.6- Discussion

The results in this chapter indicate that the habitat health had a significant effect on where blue tits and great tits apparently chose to breed. No other breeding metric assessed was significantly affected by habitat health, however the direction and strength of these relationships is discussed here.

6.6.1 - Occupancy

The results from this chapter indicate that blue tits and great tits are more likely to occupy nest boxes and construct nests inside them in habitats that have a lower incidence of AOD. There was no significant effect detected on subsequent breeding metrics, namely the proportion of nests that had eggs or chicks, and the clutch size, brood size, number of fledglings, or the

number of chicks that died. It is interesting to note that out of all the “nest success” variables analysed, nest box occupancy is the one variable that can be used to infer an intent to breed. Documenting nest box occupancy allows us to infer decisions made by the birds as where they should breed. As previous work has shown, birds will preferentially choose breeding sites based on characteristics that will increase their reproductive success, and occupancy rate has been identified as a reliable indicator of breeding site quality for blue tits (Amininasab et al., 2016). All other variables examined in this study have the ability to be affected by external factors which present following breeding, such as predation, changes in food availability etc. By measuring occupancy, we can determine how valuable a habitat is to the individuals choosing to nest there.

As the birds in this study demonstrated a preference for nesting in sites with lower levels of AOD, it would be useful to examine which aspects of AOD sites would be unfavourable to these species. In this study, the severity of AOD within a bird’s breeding habitat was incorporated into a habitat health score that included canopy density. Blue tit and great tit nest site selection have been reported to show preferences for continuous forested sites which have few patches and gaps in canopy cover, have high densities of trees (Redhead et al., 2013) and further from habitat edges (Maícas et al., 2012). Acute oak decline is a tree disease that results in canopy thinning of oak trees, and can also quickly cause death to these trees only a few years following infection (Denman & Webber, 2009). It therefore follows that habitats with higher levels of AOD would have more patchy canopies and a higher proportion of dead or dying oaks, which are less favourable to blue tits and great tits. As a result, it is not too surprising that sites with a lower incidence of AOD and higher habitat health scores would be favourable as nesting locations for these species.

If these findings are replicated across other woodlands suffering with AOD, this tree disease has the potential to lead to shifts in the breeding range of these species. Shifts in the breeding range of bird species has already been documented as a result of climate change (Hitch & Leberg, 2007), and blue tits and great tits have been documented as expanding their breeding range to include more northerly sites during the past century in several countries (Rytkönen & Orell, 2001; Schölin & Källander, 2012; BTO, 2024). If these species are forced to breed in less suitable and unfavourable habitats due to being displaced out of high quality woodland, this could have knock on effects on the population, as these species are known to produce smaller clutches of lower quality in less favourable habitats (Lambrechts et al., 2004). By repeating the work in this chapter across woodlands with greater severity of AOD, we could

examine how later stages of breeding such as egg laying could be affected, as only nestbox occupancy was found to be impacted at the site used in this study.

We must be cautious when extending the potential implications of AOD on blue tit and great tit breeding to other bird species. Some species are known to thrive in habitats with higher proportions of dead wood, for example woodpeckers (Jackson & Jackson, 2004). Therefore, these species may actively choose to nest in areas with higher proportions of dead and decaying wood, for example areas infected with tree diseases such as AOD. The impact of habitat variability on fitness is likely more of an issue for habitat specialists rather than generalists that can be relatively plastic in their use of their environment and any changes to it (Wimp et al., 2019).

Nest construction and occupation of nest boxes is a good indicator of an intent to breed. Anything that interrupts the breeding process, such as failure to lay eggs, failure of eggs to hatch, or chick death is more likely to be impacted by external factors, such as death of parents, particularly the nesting female, than to be a decision by the parents to cease the breeding process. Therefore it is unlikely that birds would invest time and effort into constructing a nest if they had no intention to lay eggs or complete the whole breeding process, which is supported by parental investment theory (Verboven & Tinbergen, 2002).

The results from this study indicate there was little difference in nest abandonment across habitats with varying habitat health scores and differing measures of AOD, and habitat health of the habitat only impacted where the birds chose to breed in a way that was consistent with the literature and known nesting preferences of these bird species. Previous work analysing post-nest construction breeding metrics have found both effects and no effect of habitat variability on hatching and fledging success, emphasising the importance of species and habitat specificity in breeding success (Atiénzar et al., 2010; Garrido-Bautista et al., 2023).

Indeed, without direct monitoring of nest boxes via cameras, the causes of nest failure can never be fully understood (Ribeiro-Silva et al., 2018). Often, chick deaths are attributed to factors such as predation as there is no other clear explanation as to why the checks have died, however other factors can be at play, for example parasitism (Møller et al., 1990).

6.6.2 - Date of first egg

Although not statistically significant, there was a slight negative relationship between habitat health score and the date of the first egg being laid, with birds using nest boxes in habitats with higher health scores laying eggs slightly earlier than in habitats with lower health scores. This trend was seen both for blue tits and great tits and was consistent across years, with the exception of blue tits in 2022. The mechanisms behind egg laying date for blue tits and great tits are thought to be largely controlled by environmental variations. Earlier egg laying dates have been documented in habitats with larger proportions of mature oak trees (Amininasab et al., 2016; Van Noordwijk et al., 1995), with habitats such as smaller woodlands (Hinsley et al., 1999) and urban parklands having later egg laying dates (Wawrzyniak et al., 2015).

Previous work has found that the woodland with increased oak densities have increased abundance of caterpillars on trees, and that woodland composition can shape the availability of caterpillars as food for nestlings (Macphie et al., 2020). This caterpillar abundance and food availability for nestlings typically declines throughout the breeding season. Nestlings produced from eggs which are laid later in the season, in particular past the peak of food availability, are typically of poorer physiological quality (Kaliński et al., 2019). It is therefore not surprising that the results in this study showed earlier egg laying in preferential breeding sites, which had higher health scores. The results in Chapter 5 of this thesis indicate a higher proportion of herbivory on AOD symptomatic trees, however there is still much to unravel in those results, for example if the increased herbivory is due to a decrease in predation pressure due to these habitats being less favourable for nesting birds.

Great tits and blue tit are both known to time the laying of their eggs with leaf phenology and bud burst (Nilsson & Källander, 2006), therefore the small association between impact of oak habitat health on the timing of reproduction on these species is interesting. The first egg-laying date for both of these species is known to have advanced over the past few decades, which has been attributed to a changing and warming climate, however the timing of these species' first eggs has stayed in synchrony with the population size of their caterpillar food supply (Matthysen et al., 2011). This demonstrates the ability for behaviours such as egg laying to be adaptable and plastic in response to environmental changes over several generations.

6.6.3 - Morphometrics

Weight and feather length in this study did not appear to be significantly impacted by habitat health score or the severity of AOD within the habitat. Mass and wing length have been found

to be associated with post-fledging survival, with larger individuals being less at risk from predation than their smaller conspecifics (Naef-Daenzer & Grüebler, 2016). By collecting data on weight and feather length of nestlings, we can also make inferences about the level of food provisioning by the parents. This in itself can indicate habitat quality, food availability etc. (Redhead et al., 2013). Juvenile survival is often positively correlated with fledgling mass in *Paridae* species, therefore taking these measures in the nest allows for a good indication if the birds will survive to be recruited into the population as adults (Naef-Daenzer et al., 2001). In the system studied here, it is likely that the severity of AOD was not strong enough to have a significant impact on the provisioning of food for the nestlings, or that there are other environmental variables which play a stronger role in determining this. The study system used in this research was designed to limit the influence of extra environmental variables that could impact breeding success. Nest box style was consistent throughout the study, and when boxes needed replacing they were done so like for like, as differing styles have different occupancy rates (Browne, 2006). The nest boxes were also suitably spaced to ensure no overlapping territories of breeding parents, however they were erected in the same woodland environment and the habitat structure was very similar across all habitat plots.

Figures 9b and 10b demonstrate inter-annual variation in the weight and feather length of great tits compared to habitat health score, thereby looking at variables that may have changed during those years, such as rainfall and temperature, which are known to impact fledgling mass and survival (Bodey et al., 2021; Grzędzicka, 2019; Marques-Santos & Dingemanse, 2020; Sauve et al., 2021) would provide a clearer picture to why this variation occurs. The interannual variation in weight and feather length seen in great tits was not as distinct in blue tits which made up a larger proportion of the sample size, and the trends were fairly consistent for this species across years.

Some studies that have compared morphometrics of blue tits across environmental gradients and distinct environmental patches have found no differences in the mass of nestlings across differing forest types (Garrido-Bautista et al., 2023), whereas other studies have found that forest patch size is important, with better body condition of blue tit nestlings and larger great tit nestlings in larger forest patches (Bueno-Enciso et al., 2016). Clearly the outcomes of environmental influence on nestling condition are variable and context specific, and can be attributed to small scale environmental variables rather than characterisations of the wider habitat. A nest's proximity to oak trees is a variable that has been indicated as having an influence in fledgling mass, with those nearer to oak trees receiving higher food provisioning rates and thereby having greater mass (Wilkin et al., 2009).

It is important to note, however, that trophic systems involving oaks have the potential to be disrupted by the presence of AOD. As seen in Chapter 5 of this thesis, trees that were symptomatic with AOD showed higher levels of herbivory, however the mechanism for this being due to reduced predation pressure by birds potentially avoiding nesting in these areas, or because insects are more attracted to diseased trees is unclear. Although the presence of AOD within a breeding site was not a factor affecting fledgling growth or mass in this study, it would be interesting to examine a range of trees all at different stages of disease and see how AOD intensity has the potential to affect the weight and size of nestlings.

6.6.4 - Recommendations for future work

The research in this chapter provides a good overview into the breeding behaviour of blue tits and great tits, however there are further breeding metrics that could be explored for a deeper understanding of how AOD and habitat health could impact breeding in this field site. Other research programmes have deployed the use of cameras within and outside nest boxes that can analyse feeding rates (how frequent provisioning of the young is) (Grüebler et al., 2018; Pagani-Núñez et al., 2017; Surmacki & Podkowa, 2022). The use of cameras and additional surveillance would also allow us to understand why certain nests fail mid-way through the breeding cycle, for example predation of the chicks. Going one step further, radio-tracking of adults would also allow for energy expenditure during nestling provisioning to be analysed (Naef-Daenzer, 1994; Telve et al., 2020), for example do parents in certain habitats have to travel further to forage for their nestlings. By also radio-tracking young prior to fledging, post-fledging survival and dispersal in these areas can also be analysed (Naef-Daenzer et al., 2001), as the current data do not reflect any behaviours once the birds have left the nest box. Diet is also an important factor in determining the health and quality of offspring (Oers et al., 2015; Wilkin et al., 2009). Diet analysis of nestling faecal samples that were used for microbiome analysis in Chapter 4 could also be used to determine if there are differences in the invertebrates being provisioned to nestlings across different habitats. Frass analysis, as discussed in Chapter 5, is a further way to more accurately determine the herbivore abundance on specific trees and could also be used for identification of prey species being provisioned to the nestlings. These could answer further questions into the nutritional content of the food being provided to the young, and if this affected nestling survival or quality. All of these methodologies discussed do require increased time, effort and funds than were available for this project, but should be utilised in future to examine additional environmental impacts on breeding success and nestling quality.

There are several slight but nonsignificant trends that have been documented in the results of this chapter. As this is the first work to incorporate a tree disease as a potential variable affecting breeding and success of *Paridae* species, it would be interesting to see if the directions of these relationships were stronger and indeed more significant, in systems which had much more prevalent or advanced stages of tree diseases. In this study the average percentage of symptomatic trees in plots infected with AOD was 6.8%. If the AOD infection in this site became more advanced, or if this study were repeated at another site with a higher level of AOD infection, it is possible that the subsequent breeding metrics analysed here, such as chick weight, feather length etc. could have been affected more strongly. By using long-term datasets from other studies in habitats without AOD, it should be possible to compare breeding success at geographically distinct sites.

When looking at the set-up of the site in this project, it is possible that the nest box sites were not distinct enough from each other, and the sites were quite interspersed. Figure 9 in Chapter 2 shows the spread of habitat plots used in this study, and their AOD status. The habitat plots to the North of the field site were quite mixed in terms of AOD status, with neighbouring plots often having differing AOD statuses. It is possible therefore that any impacts of AOD may have been diluted by the varying disease statuses, and in fact it could be that some plots appeared not to suffer from AOD, but indeed simply were not showing any external AOD symptoms. Rather than examining differences within a woodland or habitat, future work should examine impacts of tree diseases at distinct sites, having diseased and disease-free sites in distinct geographical locations.

The results from this study, as with many ecological studies, do not well represent all of the potential ecological factors at play, one notable influence being density dependence and competition. Research has shown that competition during breeding season is variable depending on tree species composition, with higher levels of competition in large oak-dominated habitats. A flaw of the experimental design in this study is that there is no way of determining how many “natural” competitors are around. The field site in this study was set up to limit competition between birds breeding in the experimental nest boxes, however there may be variability in the numbers of competitors nesting in natural cavities.

The results in this chapter are indicative of a 4-year snapshot in time. Longer studies which span several decades are able to account for environmental impacts on breeding, for example temperature and weather fluctuations (Gladalski et al., 2015; Marques-Santos & Dingemanse, 2020). As discussed earlier in this chapter there are different measures of breeding success -

nest success and reproductive success, where reproductive success represents the number of offspring recruited into the population as adults. By measuring reproductive success over a breeding season, we would understand much more about how habitats impacted by tree diseases such as acute oak decline would affect fledglings. In fact a recent assessment of using different measures of breeding success has found that measuring fitness variables at recruitment stage rather than nest stage is the best predictor for longer term fitness (Alif et al., 2022). Habitat is a factor known to influence post-fledging survival (Naef-Daenzer & Grüebler, 2016; Remeš & Matysioková, 2016), so studying these seems like a natural next step following on from this research. This however requires much more resources than were available in this project, however future work could utilise technology such as PIT tags which have been successful in monitoring recruitment of *Paridae* species over a breeding season (Crates et al., 2015).

As well as examining nestling mass and feather length at fixed time points, it can also be useful to study these over the course of their development in the nest. Studies have examined growth rates of *Paridae* species across different environments and have found no difference in growth rate (Marini et al., 2017).

The results from these studies did demonstrate between year variation, which has been found across a number of studies into the breeding of blue tits (Gladalski et al., 2015; Maícas et al., 2012) and great tits (Broggi et al., 2022; Saulnier et al., 2023; Slagsvold, 1976), and shows the impact of environmental stochasticity and inter-annual variations on breeding.

6.7 - References

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6.8– Supplementary material

Supplementary material 6.1 – Individual nestbox use and outcome over the duration of the study

	YEAR			
NESTBOX NUMBER	**2020**	**2021**	**2022**	**2023**
1	No Nest	Nest Made	Nest Made	No Nest
2	Chicks - successful	Chicks - successful	Nest Made	Chicks - successful
3	Missing	Missing	Missing	Missing
4	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
5	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
6	No Nest	Damaged	No Nest	No Nest
7	No Nest	Chicks - successful	Chicks - successful	No Nest
8	Chicks - successful	Nest Made	Chicks - successful	Nest Made
9	Missing	Missing	Missing	Missing
10	No Nest	Nest Made	No Nest	No Nest
11	Chicks - successful	Chicks - successful	Chicks - dead	Chicks - successful
12	Missing	Missing	Missing	Missing
13	No Nest	Chicks - successful	No Nest	Chicks - successful
14	Chicks - successful	Damaged	Chicks - successful	Chicks - successful
15	Chicks - successful	Damaged	Eggs - unhatched	Nest Made
16	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
17	Chicks - successful	Chicks - successful	Chicks - successful	No Nest
18	Chicks - successful	Damaged	Chicks - successful	Chicks - successful
19	Chicks - successful	Damaged	Eggs - unhatched	Damaged
20	Chicks - successful	Chicks - successful	Eggs - predated	Damaged
21	Missing	Missing	Missing	Missing
22	Nest Made	Chicks - predated	Chicks - successful	Chicks - successful

NESTBOX NUMBER	YEAR 2020	2021	2022	2023
23	Chicks - successful	Chicks - successful	Chicks - successful	Damaged
24	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
25	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
26	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
27	Chicks - successful	Eggs - unhatched	Chicks - successful	Chicks - dead
28	Chicks - successful	Eggs - unhatched	Chicks - successful	Chicks - successful
29	Chicks - successful	Chicks - successful	Nest Made	Chicks - successful
30	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
31	Chicks - successful	Eggs - unhatched	Chicks - successful	Nest Made
32	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - dead
33	Chicks - successful	Nest made - invaded by bees	Chicks - successful	Chicks - successful
34	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
35	Chicks - successful	Chicks - successful	Eggs - unhatched	Eggs - unhatched
36	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
37	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
38	Chicks - successful	Chicks - successful	Chicks - dead	Nest Made
39	Chicks - successful	Nest Made	Chicks - successful	Chicks - successful
40	Chicks - successful	Damaged	Nest Made	Chicks - successful
41	Chicks - successful	Chicks - successful	Damaged	Chicks - successful
42	Nest Made	Chicks - successful	No Nest	Nest Made
43	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
44	Eggs - unhatched	Chicks - successful	Eggs - unhatched/predated	Chicks - successful
45	Chicks - successful	Chicks - successful	Damaged	Nest Made
46	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
47	Chicks - successful	Chicks - successful	Chicks - successful	Eggs - unhatched

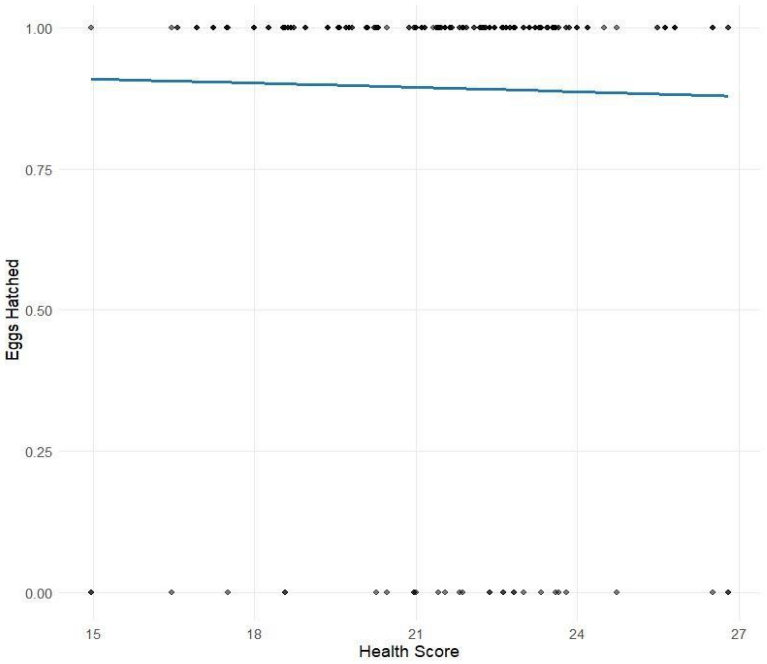
NESTBOX NUMBER	YEAR 2020	2021	2022	2023
48	Chicks - successful	Nest made - invaded by bees	Chicks - successful	Eggs - unhatched
49	Chicks - successful	Missing	Missing	Missing
50	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
51	Chicks - successful	Chicks - successful	Damaged	No Nest
52	Chicks - successful	No Nest	No Nest	Eggs - unhatched
53	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - dead
54	Chicks - successful	Chicks - successful	Nest Made	Nest Made
55	Chicks - successful	Chicks - successful	Eggs - unhatched	No Nest
56	Chicks - successful	Chicks - successful	Chicks - successful	Nest made (tree fell mid season)
57	Chicks - successful	Chicks - successful	No Nest	Nest Made
58	Chicks - successful	Chicks - successful	Nest Made	Nest Made
59	Chicks - successful	Chicks - successful	Chicks - successful	Eggs - unhatched
60	Chicks - successful	Missing	Missing	Missing
61	Chicks - successful	Chicks - successful	Damaged	Missing
62	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
63	Chicks - successful	Chicks - successful	No Nest	Chicks - successful
64	No Nest	Nest Made	Chicks - successful	Chicks - successful
65	Chicks - successful	Chicks - successful	No Nest	Nest Made
66	Chicks - successful	Chicks - successful	Nest Made	Chicks - successful
67	Chicks - successful	Nest Made	Damaged	Nest Made
68	Chicks - successful	Chicks - successful	Eggs - unhatched	Nest Made
69	Chicks - successful	Chicks - successful	No Nest	Nest Made
70	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
71	Chicks - successful	Chicks - successful	Damaged	Nest Made

NESTBOX NUMBER	YEAR 2020	2021	2022	2023
72	Chicks - successful	Chicks - successful	Damaged	Nest Made
73	Nest Made	Chicks - successful	No Nest	Nest Made
74	Chicks - successful	No Nest	Damaged	Nest Made
75	Chicks - successful	Chicks - successful	No Nest	Chicks - successful
76	Chicks - successful	Nest Made	Chicks - successful	Chicks - successful
77	Chicks - successful	Chicks - successful	Eggs - unhatched	Chicks - successful
78	Chicks - successful	Eggs - nest used by bees	Eggs - predated	Chicks - successful
79	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
80	Chicks - successful	Chicks - successful	Eggs - unhatched	Chicks - successful
81	No Nest	Chicks - successful	Not checked - safety	Not checked - safety
82	No Nest	Damaged	Chicks - successful	Eggs - unhatched
83	Chicks - successful	Chicks - successful	Eggs - unhatched	Nest Made - predated
84	Chicks - successful	Chicks - successful	No Nest	No Nest
85	Chicks - successful	Chicks - successful	No Nest	Damaged
86	Chicks - successful	Nest Made	No Nest	Nest Made
87	Chicks - successful	Eggs - unhatched	Chicks - successful	Chicks - successful
88	Chicks - successful	Eggs - unhatched	Nest Made	Chicks - successful
89	Chicks - successful	Chicks - successful	Chicks - successful	Nest Made
90	Chicks - successful	Chicks - successful	Eggs - unhatched	Chicks - successful
91	Nest Made	Chicks - successful	Nest Made	Chicks - successful
92	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - dead
93	Chicks - successful	Chicks - predated	No Nest	No Nest
94	Chicks - successful	Chicks - successful	Chicks - successful	No Nest
95	Nest Made	Eggs - unhatched	Nest Made	Eggs - unhatched
96	No Nest	Damaged	Chicks - successful	Chicks - successful

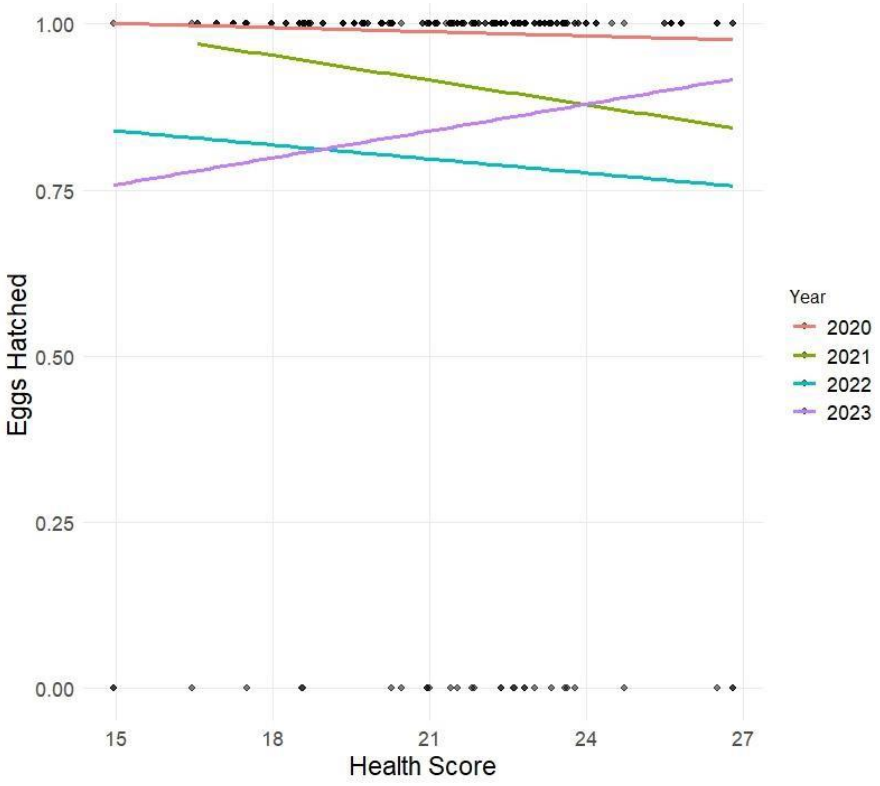
	YEAR			
NESTBOX NUMBER	**2020**	**2021**	**2022**	**2023**
97	Chicks - successful	Chicks - successful	Chicks - successful	Eggs - unhatched
98	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
99	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
100	No Nest	Eggs - unhatched	Chicks - successful	Nest Made
101	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
102	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful
103	Chicks - successful	Chicks - successful	Chicks - successful	Chicks - successful

Supplementary Material 6.2 - Proportion of nests in which eggs hatched across habitats with differing health scores a) average trend across all years of the study (2020 - 2023), b) individual year trends.

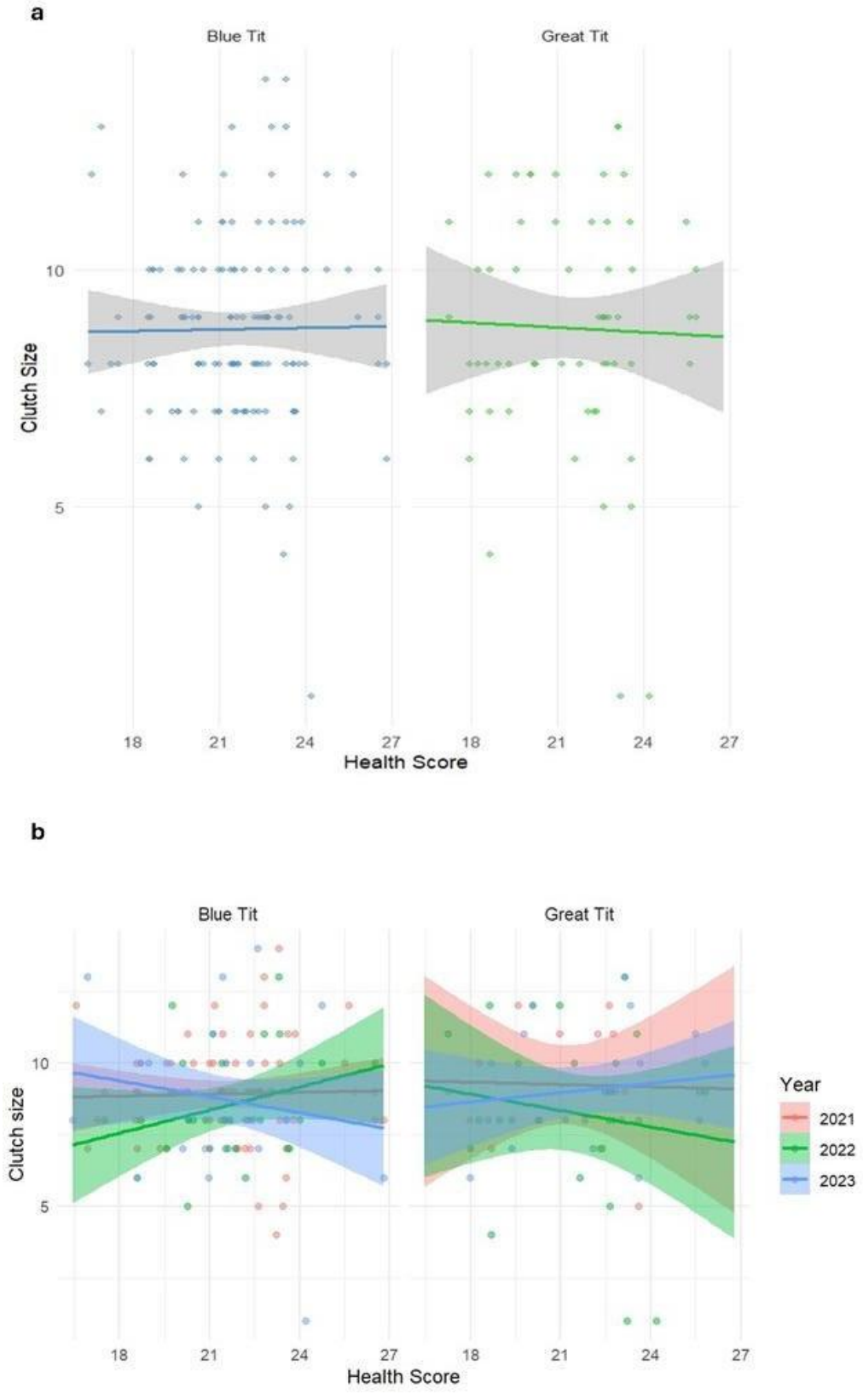
a



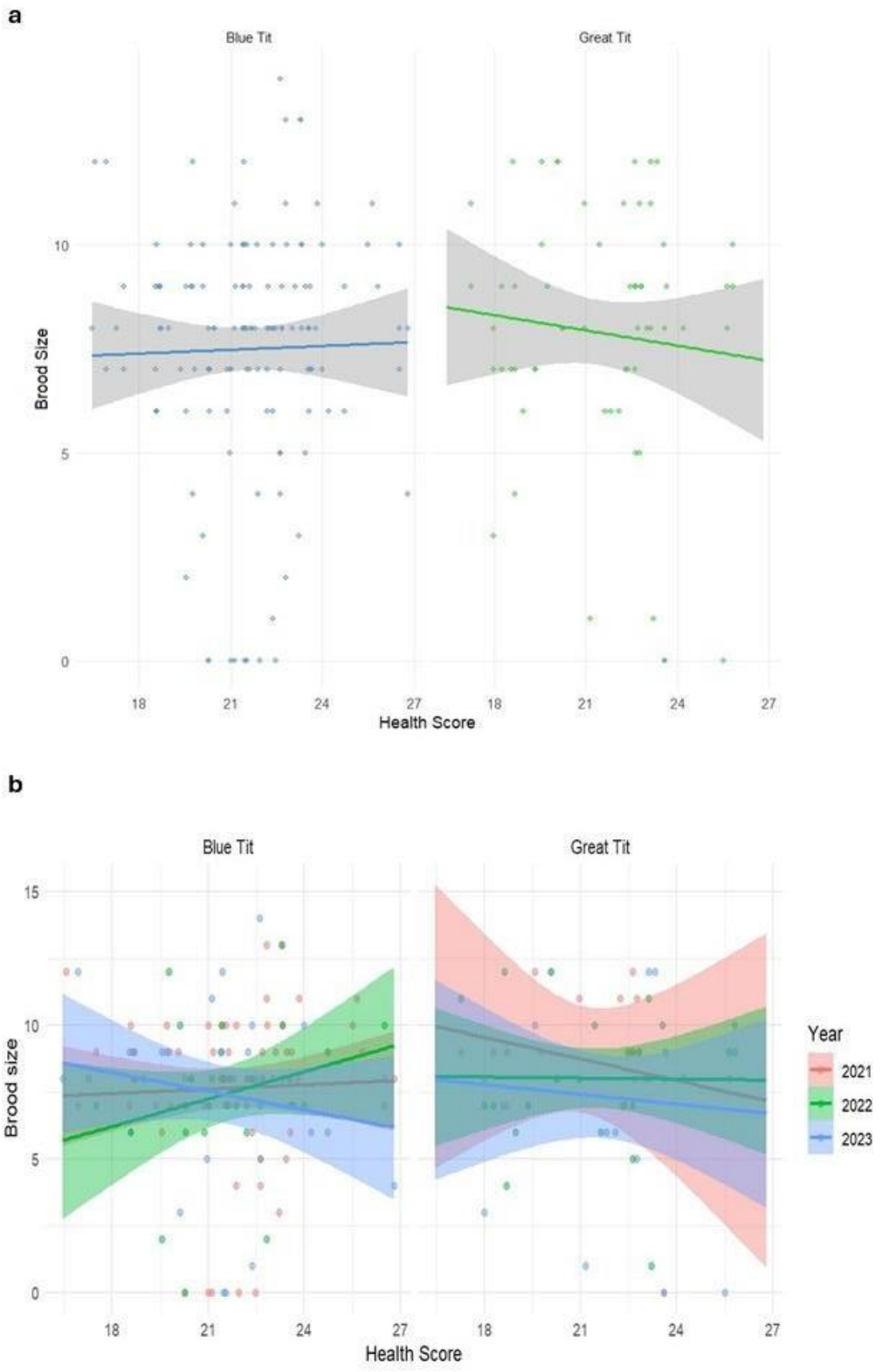
b



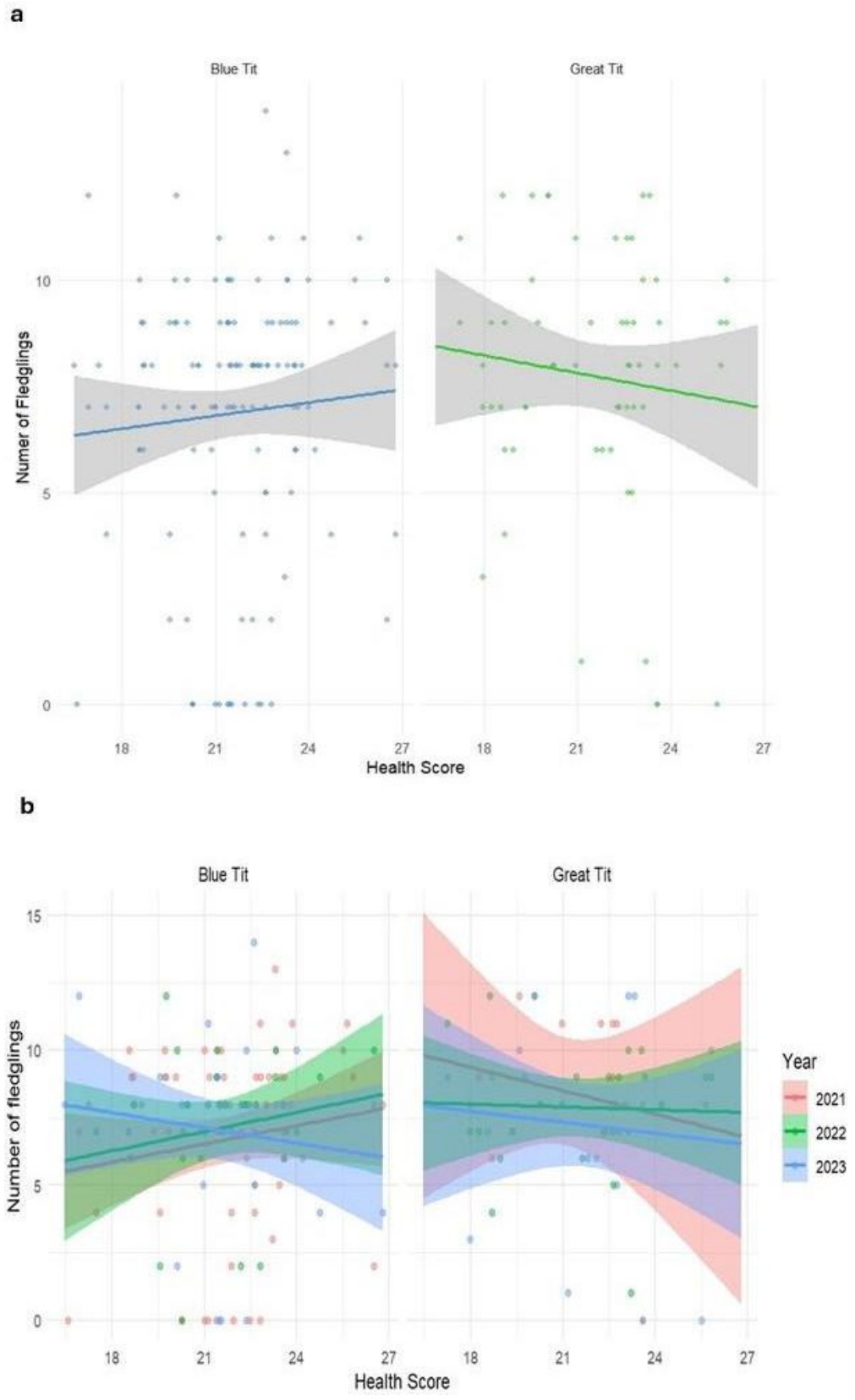
Supplementary Material 6.3 - Relationship between habitat health score and clutch size a) across all years, b) separated by years.



Supplementary Material 6.4 - Relationship between habitat health score and brood size a) across all years, b) separated by years.



Supplementary Material 6.5 - Relationship between habitat health score and number of fledglings a) across all years, b) separated by years.



Supplementary material 6.6 – Output of the general linear models, tested against habitat health score. Estimate indicated estimated coefficient, and Std. Error indicates the standard error of this coefficient. Test Statistic – 't' indicates a t-distribution (used in Gaussian family models) and 'z' indicates a z-distribution (used in binomial family models). Bold p-values indicates significance <0.05.

Variable Tested	Species	Estimate	Std. Error	Test statistic	p-value
Day of 1st Egg	Blue Tit	-0.01621	0.02285	-0.709 (t)	0.479
	Great Tit	-0.04041	0.04690	-0.862 (t)	0.392
Nest Made		0.1573	0.0658	2.390 (z)	**0.0168**
Eggs Laid		0.009206	0.075008	0.123 (z)	0.9023
Eggs Hatched		-0.02259	0.08486	-0.266 (z)	0.79007
Clutch Size	Blue Tit	0.009829	0.081941	0.120 (t)	0.905
	Great Tit	-0.03607	0.14387	-0.251 (t)	0.80296
Brood Size	Blue Tit	0.02904	0.11916	0.244 (t)	0.80786
	Great Tit	-0.09803	0.17468	-0.561 (t)	0.57689
Number of Fledglings	Blue Tit	0.1040	0.1297	0.802 (t)	0.424
	Great Tit	-0.1166	0.1725	-0.676 (t)	0.50211
Chick Feather Length	Blue Tit	0.04086	0.05159	0.792 (t)	0.428576
	Great Tit	0.07955	0.07549	1.054 (t)	0.293
Chick Weight	Blue Tit	0.01691	0.01373	1.232 (t)	0.218
	Great Tit	0.04445	0.05073	0.876 (t)	0.3814

Chapter 7 – Overall Discussion

This thesis has explored five main themes: the knowledge of plant pathogen vectors; the occurrence of Acute Oak Decline (AOD) pathogens associated with birds; how AOD can impact avian gut microbiomes; how herbivorous insects respond to AOD; and how AOD can impact bird breeding success. By using an interdisciplinary approach to examine the interactions between AOD and woodland birds, evidence and technology from different branches of biology was assimilated to enable a well-rounded investigation. This chapter provides an overview of the results and highlight remaining knowledge gaps and how research could be expanded from this thesis.

7.1 - Thesis overview

In Chapter 2, “A scoping literature review examining the vectors of plant pathogens", the current literature on vectors of plant pathogens was interrogated, categorising over 700 papers relating to plant pathogen vectors into their individual pathosystems. This review identified clear gaps in the literature relating to non-arthropod vectors of plant pathogens, with a striking lack of knowledge regarding birds as vectors. My review demonstrated a substantial bias in the literature towards research involving the pathogens of crops, which highlighted the need for a wider understanding of all plant pathogen vectors, especially as the potential impact of pathogens is increasing with the rapidly changing climate.

Chapter 2 showed that the following research themes were uncommon when analysing plant pathosystems and their vectors: 1) birds as plant pathogen vectors, 2) bacterial plant pathogens, and 3) tree pathogens in particular. Following this, the research focused on one particular example in Chapter 3, “The role of woodland birds as vectors of bacteria associated with Acute Oak Decline”. That chapter outlined the suitability of birds as potential vectors of AOD pathogens and investigated this experimentally. A variety of samples from blue tit and great tit nestlings and adults were taken in an attempt to recover the three species of AOD associated bacteria - *Brenneria goodwinii, Gibbsiella quercinecans,* and *Rahnella victoriana*, using culture-based microbiological methods. This is the first work to attempt to recover the bacteria associated with AOD on organisms outside of oak trees. Despite culturing and sequencing hundreds of bacterial samples from buccal, faecal and body samples, only three bacterial cultures were positively matched to the AOD bacteria, and these were all *Rahnella victoriana.* As *B. goodwinii* and *G. quercinecans* are the main driving pathogens behind AOD, it is therefore not possible to conclude that birds act as vectors of AOD pathogens based on the recovery of the few cultures of *R. victoriana*, however this result is the first to show an association between AOD bacteria and birds. The inability to recover *B. goodwinii* and *G. quercinecans* could have been a factor of experimental design however,

so more in-depth molecular methods were subsequently adopted in Chapter 4. The inability to recover AOD bacteria from environmental samples is not uncommon, as the bacteria are slow growing and therefore often outcompeted by other environmental bacteria when cultured from mixed samples in the lab (A. Ordonez, personal communication, 2022). The work in this chapter was also the first to attempt to recover plant pathogens using buccal sampling of birds, therefore experimental techniques may need to be refined before further work is carried out.

Chapter 4, “The impact of Acute Oak Decline on avian gut microbiomes”, focussed on attempting to detect within faecal samples any AOD bacteria that had not been recovered using culture- based methods in Chapter 3. Whole community microbial composition was analysed across areas with differing levels of AOD to assess whether the presence of this tree disease impacted bird’s gut microbiomes. By using Illumina Next Generation Sequencing, bacteria in the samples were identified to the lowest taxonomic classification possible, which most commonly was genus level. The taxonomic composition of the samples was compared across a range of sites with a differing frequency of AOD, along with alpha and beta diversity, to determine what impact AOD had on microbial composition. There were small variances in the taxonomic composition of bacteria within the samples; however, the differences in alpha and beta diversity could not be attributed to the presence or severity of AOD. This work is pioneering in exploring the association between avian gut microbiomes and the presence of tree diseases. These techniques could be applied to a range of other warm blooded woodland species which associate with oak, such as squirrels, to assess their suitability as carriers of AOD associated bacteria and determine how AOD may impact their microbiomes. It is possible that the sites used in this study were not distinct enough to impact avian microbiomes, as these have been found to be shaped by habitat variability such as urbanisation, pollution and captivity (Alba et al., 2023; Murray et al., 2020; Ruuskanen et al., 2020).

In addition to assessing birds as potential vectors of AOD pathogens, an overarching theme of this thesis has been examining the wider ecological impacts of AOD on the surrounding habitats, with a particular focus on birds. To examine the effects of any changes in trees on birds that use them for foraging, it is important to examine the intermediate trophic level, which is the folivorous caterpillars that act as important prey items for blue tits and great tits during the breeding season. The oak > caterpillar > great tit and blue tit trophic system is well studied, especially in relation to environmental changes such as climate change (Visser et al., 2006). This classic tri-trophic system can be impacted by environmental variables such as tree species composition (Shutt et al., 2019), but this is the first work to examine this system with

a particular focus on a tree disease. In Chapter 5, “The impact of Acute Oak Decline on oak insect herbivory damage”, insect herbivory rates between trees that were symptomatic for AOD were compared against those which are asymptomatic. Herbivory levels were significantly higher in trees that were symptomatic for AOD, however the reasoning for this is not yet understood. An increase in herbivory could be due to symptomatic trees being more attractive to folivorous insects, as has been found in studies on other herbivore and plant systems (Eberl et al., 2020; Johnson et al., 2003). Symptomatic trees also could have weakened defences which would usually allow them to protect against herbivory, or there could be a combination of these two factors on symptomatic trees. If either or both are true for AOD symptomatic trees, the mechanisms behind this would need to be fully understood. Alternatively, an increase in invertebrate herbivory rates could be due to decreases in predation pressure from birds, which echoes the call for a wider understanding of ecosystem impacts of AOD, which are explored in more depth in Chapter 6.

The results from Chapter 5, indicating higher levels of herbivory on AOD symptomatic trees, also align with the findings from Chapter 6, “The impact of Acute Oak Decline on breeding success of birds”. Chapter 6 involved collecting and analysing four years of nest box breeding data for great tits and blue tits across areas with differing levels of AOD in the surrounding trees. The results showed that birds were more likely to select breeding sites in areas with lower levels of AOD, however once nest construction had commenced AOD severity did not have a significant impact on any other metric of bird breeding success. This result serves as a possible explanation for the increased herbivory seen on AOD symptomatic trees in Chapter 5. If birds actively avoid areas with higher levels of AOD when they choose nesting sites, there will be less predation pressure on folivorous caterpillars, allowing their numbers to remain high throughout the breeding season and leading to increased herbivory of oak trees.

7.2 - Limitations of the thesis and existing knowledge gaps

As with any thesis and research project, there were significant constraints with time and resources available as part of this doctoral programme. As such, there were limitations to the extent of the work that could be carried out and the opportunities to expand on the work, which will be touched on below.

Chapters 3 and 4 focussed on identifying the AOD associated bacteria in samples taken from birds; however, there was very little evidence that birds acted as vectors of these bacteria. One of the limitations of this thesis is that only two species of bird were investigated - the blue

tit and the great tit. These species both represent ubiquitous well studied species, making them useful to examine in novel work such as this, however it is possible they aren't as intimately associated with oak trees as some other species. When examining if birds can act as vectors for AOD associated bacteria, it would make sense to examine bird species which have the closest association with oak. In the work presented in Chapter 3, mist netting was carried out to attempt to capture a wider range of oak associated birds, such as woodpeckers, treecreepers and nuthatches, which have more of a direct relationship with the bark of oak trees. These species have direct contact with the bark of the tree through moving around the tree and feeding. Nuthatches have been anecdotally observed feeding around active bleeds of AOD symptomatic trees (R. Jackson, personal communication, 2020). However, despite attempting to capture birds of these species for sampling, this was not successful. These species have estimated population sizes between 200,000 - 260,000 in the UK and are not as numerous as blue tits and great tits, which have estimated population sizes of 2-3 million (BTO, 2024). Aside from making blue tits and great tits more efficient to capture, their large population size also allowed a larger sample size to be used. Further work should employ a larger team of researchers that could allow for direct targeting of species of interest, possibly through identification of nesting and roosting sites rather than a somewhat passive capturing technique such as mist netting used here. This represents a notable limitation of this thesis, and further studies should be carried out using a wider range of bird species.

The samples analysed using Next Generation Sequencing in Chapter 4 were all faecal samples. Additional funding had been applied for to cover this analysis (detailed on page 4 under "Grants"). However, this funding was limited to only around 96 samples. Following advice from NEOF, where the analysis was taking place, faecal samples were prioritised as these were the most likely sample type to recover a good amount of bacterial DNA from. To fully explore more potential vectoring routes, analysis of all samples taken in this study (buccal, foot and body) should be carried out to detect the presence of the AOD associated bacteria. It is possible that transfer of the bacteria from the tree to the bird during direct contact, would be a more likely vectoring route, therefore detection of AOD bacteria could be more likely with external body samples.

Knowledge around AOD is rapidly expanding, in fact when my PhD commenced there was arguably only basic knowledge around the causes and severity of this tree disease. As my research and thesis progressed, so did that of more established and well-funded groups researching the bacteria associated with AOD. The AOD scoring system used here (first presented in 3.4.1.2) was developed in line with existing disease scoring systems used by the

biodiversity team at Epping Forest, adapted slightly to ensure relevant data were collected for this thesis, but so that the information would still be useful to the forest managers on the ground. In 2021, Finch et al. published oak decline severity indices, developed to assign severity of both AOD and Chronic Oak Decline (COD). These indices go above and beyond the assessments carried out for the analysis carried out in this thesis, as they include for example the length of the stem bleeds and the presence of pathogenic fruiting bodies. Reviewing the habitat assessments carried out here in light of Finch's work would allow for more in depth comparisons across different sites, allowing for a wider analysis of the impact of AOD on woodland birds by incorporating a range of breeding sites and long term datasets.

Recent unpublished data has also found more of a direct link between *Agrilus biguttatus* and AOD symptomatic trees than previously thought. As introduced in section 1.4 of this thesis, AOD symptomatic trees are often associated with larval galleries and emergence holes of *A. biguttatus,* however the link between the beetle and the disease was not fully understood. Current research has found *A. biguttatus* adults are attracted to diseased trees due to VOCs emitted by the AOD associated bacteria (pers comm S. Denman, June 2024). This work is still developing, however it is known that adult *Agrilus* beetles feed in oak canopies (Reed et al., 2018). When we look at the limitations of Chapter 5 of this thesis, one of the main issues concerned the fact that we did not know which folivorous insects were responsible for the increased herbivory levels on symptomatic oak trees. It is possible that if adult *Agrilus* beetles are attracted to symptomatic AOD trees, that they could be responsible for these increased herbivory levels. *Agrilus* species do represent important food sources for some bird species such as woodpeckers (Brown et al., 2015), but not so much for the blue tits and great tits studied in this thesis. As such it would be important to discern which folivores are responsible for the increased herbivory of AOD symptomatic trees, which can be done through branch beating, and collection of insect DNA from the tree canopies (Weber et al., 2024). These results would allow us to make stronger hypotheses about the impact of AOD on subsequent trophic levels within an ecosystem.

Further work is needed to determine the source of the bacteria associated with AOD. There is a growing consensus around AOD researchers that the bacteria are present within oak trees naturally in the form of endophytes, (personal communication, R. Jackson 2024) and recent research appears to support this idea (Maddock et al., 2023), particularly regarding the primary AOD pathogen *Brenneria goodwinii*. *B. goodwinii* has been documented as not surviving well in rainwater and soil, whereas *Gibbsiella quercinecans* is able to survive and be recovered (Pettifor et al., 2020). DNA from these bacteria has also been found in the canopies of both

symptomatic and asymptomatic oak trees (Gathercole et al., 2021), indicating the bacteria are not just localised to symptomatic lesions on diseased oaks.

Chapter 2 highlighted knowledge gaps in relation to birds acting as vectors of plant pathogens, with very little research having been done into this potential vectoring system. It is possible that work has been carried out into birds as vectors of plant, and in particular tree pathogens, but has produced negative results which aren't as often reported on (Mlinarić et al., 2017).

The absence of work on what impact plant pathogens might have on avian microbiomes is somewhat surprising. Birds are well known vectors of human and animal pathogens (Benskin et al., 2009), which can have deleterious effects for wild bird populations (Hansen et al., 2015). It would be interesting to see if plant pathogens have a similar effect, as any negative impacts of plant pathogens on birds could increase interest and funding into the spread of plant and tree diseases, examining wider ecosystem impacts. As knowledge of birds as vectors of plant pathogens and with research into wild bird microbiomes steadily increasing, this could become more prevalent in coming years. A sound understanding of the avian gut microbiome is imperative to understand how environmental variations can impact bacterial species composition, and what impacts these can have on the host birds.

Sequencing carried out as part of microbiome analysis in Chapter 4 identified over 8,000 different bacterial taxa present in avian faecal samples, with over 10 million individual bacterial reads detected. Due to the direction of this thesis, the data were only examined to identify AOD associated bacteria and their relatives, however such a large dataset could be used to investigate other questions and attempt to identify other human and plant pathogens of interest. The microbiome dataset produced in this thesis could also be examined to determine if there is a core microbiome shift associated with the presence of AOD. A core microbiome is defined as ASVs being present in >50% of samples (Grond et al., 2018), and microbiomes are known to fluctuate in response to environmental changes (Kolodny & Schulenburg, 2020). By taking repeated faecal samples from individuals over time, any shifts in microbiome attributed to environmental changes such as increased associations with tree diseases, which may give additional information about the ability of birds to adapt their microbiomes according to environmental variability (Kogut, 2019).

7.3 - Concluding remarks

The overarching aim of this thesis was to investigate the wider ecological impacts of acute oak decline, with a particular focus on birds. This aim has been achieved through the

monitoring of bird breeding in Epping Forest, a large woodland site with variable levels of AOD, alongside measuring insect herbivory levels on oak with differing severities of AOD. The results from this work have provided a small insight into the potential tree diseases have on their wider ecosystem, giving scope to extend this work to a wider range of bird species and across sites with more drastic variations in AOD levels. A further aim of this work was to examine what role, if any, birds had as vectors of the bacteria associated with AOD, and what impact the presence of this tree disease had on their gut microbial composition. There were no clear links between AOD and birds as vectors, or indeed a contribution towards the birds' gut microbial community, however this work provided the foundation for further bird species and sample types to be studied. As threats from novel and existing tree diseases increase, it is important that we view their presence and effects as not being localised to the individual trees, but to take stock of the wider ecosystem impacts of diseased and declining trees.

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