Development and Deployment of Rapid Molecular Diagnostic Tools to Assess Biocontrol Strategies of the Highly Invasive Oak Processionary Moth (*Thaumetopoea processionea*) for Integrated Pest Management

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Abstract

Oak Processionary Moth (*Thaumetopoea processionea* - OPM) is a serious forestry pest and risk to public health in the UK. The unsustainable nature and spiralling cost of chemical pesticides in managing OPM has driven the need for sustainable, integrated pest management including the use of novel biocontrol methods. The challenges posed by the health risks of OPM make many morphological methods impractical. Molecular methods offer a solution to this by reducing contact between humans and OPM material. Here the use of molecular methods for the discovery and surveillance of biocontrol prospects, such as Carcelia iliaca, in OPM is implemented. A field applicable molecular approach to diagnosing parasitism in OPM was successfully developed and validated with diagnostic sensitivity (91%) and specificity (75%) that is considered suitable by industry standards. This was then applied to OPM samples from the Greater London area over a two-year study to reveal parasitism rates of *C. iliaca.* Parasitism rates were found to be variable with no spatial pattern that could be linked to environmental covariates and the data set being too short in duration to infer reliable temporal trends. To investigate the possibility of pathogen control of OPM, tagged nested metabarcoding was used to characterise the bacterial and fungal communities associated with diseased and healthy OPM samples. Overall, species richness and diversity were higher in diseased samples which was either caused by opportunistic coloniser species in diseased samples or a reduced species richness and diversity in healthy samples driven by a bacterial symbiont. Non-metric multidimensional scaling analysis found little overall community difference although the presence of *Wolbachia*, *Beauveria*, and *Fusarium*, do drive some differences. These results demonstrate how novel molecular methods can be adapted for use in informing the management of an invasive pest species. The successes and lessons from this work will be applicable to the next generation of forestry pests that are predicted to arrive in the UK.

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1 General Introduction

Introduction and Rationale

In England the oak processionary moth (*Thaumetopoea processionea* – OPM) (Lepidoptera: Notodontidae) (Linnaeus, 1758) is an invasive species considered to be a serious forestry pest because of its propensity to defoliate oak species (Tomlinson, Potter and Bayliss, 2015) and potential public health risk due to the urticating hirs on late instar larvae. (Rahlenbeck and Utikal, 2015). Control of OPM in England has been ongoing since its initial discovery on a housing estate in 2006. However, the approach changed from eradication to control of the spread when it became apparent that eradication had failed and OPM was discovered to be breeding in the country (Townsend, 2013). OPM control currently takes the form of pesticide application and physical removal of nests. However, the financial cost of both approaches, and the ecological costs of widespread pesticide application, has led to an increased call for alternative control strategies. Among these strategies are increasing the prevalence of natural enemies including parasitoids. OPM are parasitised by ~40 species across its entire range (Sobczyk and Others, 2014) with some parasitoids reaching ~80% parasitism in certain areas (Sands *et al.*, 2015). However, in the English population OPM is only parasitized by 3 larval parasitoids, *Carcelia iliaca* (Diptera: Tachinidae) (Ratzeburg, 1840)*, Compsilura concinnata* (Diptera: Tachinidae) (Meigen, 1824)*,* and *Pales processionea* (Diptera: Tachinidae) (Ratzeburg, 1840) (Kitson *et al.*, 2019; Raper, 2023). In Richmond, London, parasitism rates in OPM larvae have been observed for both *C. iliaca* (~50%) and *C. concinnata (*~0.4%) (Kitson, J., Evans, D., Straw, N., 2019). Due to the high levels of parasitism observed with *C. iliaca* this species has been identified as a suitable bio-control agent. However, there are still gaps in our understanding of *C. iliaca* ecology, and local *C. iliaca* and OPM interaction dynamics which prevent biocontrol from being included in decision making frameworks. Due to the health and safety issues presented by the urticating hairs of larvae, including dermatitis and agitation of respiratory disorders, which become particularly prominent when working with OPM in enclosed environments, the OPM control program has identified a need for a rapid, field-friendly, molecular method of detecting *C. iliaca*. Sequencing-based methods could be employed if the parasitoid cohort were unknown. However, sequencing can be expensive, and, in this instance, a single parasitoid (*C. iliaca*) is being targeted. Therefore, in this thesis loop-mediated isothermal amplification will be used as this is a faster and cheaper molecular diagnostic approach compared with similar molecular methods. The diagnostic assay will be

used to sample OPM populations for *C. iliaca* which will allow for cheaper and safer sampling of *C. iliaca* in OPM across London. This data, along with local environmental conditions, will be used as inputs for predictive models to determine the distribution of *C. iliaca* and provide understanding on the ecological correlates that explain the presence of *C. iliaca.* Understanding this information will allow land managers to make more informed management decisions and tailor management strategies to increase *C. iliaca* abundance. This would increase the availability of *C. iliaca* in areas where parasitism may previously have been less well established such as the OPM expansion front.

Aims and Objectives

The aim of this thesis is to develop a parasitism diagnostic test to determine the UK distribution of *C. iliaca* to inform the management strategy of OPM. To achieve this, the following objectives and sub-objectives have been identified:

Objective One: *Develop a rapid, cheap, DNA-based field assay to confirm the presence of C. iliaca in OPM larvae.*

- Develop a field friendly DNA extraction method to generate input for the diagnostic assay.
- Use loop mediated isothermal amplification using the Cytochrome Oxidase subunit I (COI) locus to develop a diagnostic assay to detect *C. iliaca.*
- Validate the *C. iliaca* assay according to EPPO standards ("PM 7/98 (4) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity," 2019).
- Analyse the diagnostic performance of the LAMP assay using industry standard metrics ("PM 7/98 (4) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity," 2019).

Objective Two: *Apply the assay developed in Chapter 3 to real world samples collected in field seasons 2021 and 2022 to assess the rate of C. iliaca parasitism for those years across London, UK.*

- Collect *T. processionea* samples from different sites across London, UK and diagnose parasitism with the assay developed in Chapter 3.

- Determine parasitism rates for different sites across London, UK.
- Model the variables that may predict presence of *C. iliaca* to explore any significant relationships.

Objective Three: *Use DNA metabarcoding to better understand the fungal and bacterial communities associated with healthy and diseased OPM larvae to determine whether there are novel entomopathogens that could be used for OPM management.*

The specific sub-objectives for this chapter are:

- Optimise a PCR workflow to amplify bacterial DNA extracted from diseased OPM caterpillars.
- Analyse the bacterial communities associated with diseased OPM caterpillars.
- Compare the bacterial communities with an existing data of fungal communities associated with diseased OPM caterpillars.

Thesis Structure

To address the objectives detailed above this thesis will form five additional chapters:

I. Chapter 1 provides an overview and rationale to the project whilst also giving a breakdown of the thesis structure and the overall aims.
II. Chapter 2 introduces the ecology and life history of OPM, the core concepts and challenges of integrated pest management and the potential of molecular techniques to revolutionise this.
III. Chapter 3 “Development of a LAMP Protocol to Identify *Carcelia iliaca* from Oak Processionary moth (*Thaumetopoea processioneae*) Larval Tissue”. Details the development and validation of a molecular diagnostic assay to diagnose parasitism in OPM larvae.
IV. Chapter 4 “Application of a Novel Molecular Diagnostic Method to Detect a Larval Parasitoid in Oak Processionary (*Thaumetopoea processioneae*)” Shows the results of applying a diagnostic assay to real world samples to better understand *C. iliaca* distribution.
V. Chapter 5 “Using tagged metabarcoding to explore the relationship between the bacterial and fungal microbiomes of diseased and healthy Oak Processionary moth

(*Thaumetopoea processionea*) uses metabarcoding to investigate the relationship between bacterial and fungal microbiomes of healthy and diseased OPM to identify novel entomopathogens.

VI. Chapter 6 synthesises the main findings and challenges from this work whilst also providing an overview of how this work fits into the wider literature and future directions that are evident from this work.

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Chapter 2 Literature Review

Introduction

The oak processionary moth (*Thaumetopoea processionea* - OPM) (Linnaeus, 1758) is considered a serious pest across many European countries because of the threat it poses to the health of oak trees and human health (Godefroid *et al*, 2020). Current trends show OPM expanding into areas of northern Europe which is facilitated by a mixture of climate change, land use change, and globalisation (Figure 2.1) (Townsend, 2013). Countries, such as Germany the Netherlands, Belgium, and the UK are seeking to contain the spread of OPM to reduce the pressure on Oak tree populations (Tomlinson, Potter and Bayliss, 2015; Csoka *et al.*, 2018; Marzano *et al.*, 2020; Buist *et al.*, 2021). OPM larvae attack the crown of their host trees and can quickly reach "plague-like" proportions (Forestry Research, 2020) causing defoliation of Oak trees (Figure 2.2). Whilst the direct impact of this on tree health is still uncertain, it is argued that defoliation by OPM alone cannot kill the host plant (Poulsom, 2015) although the defoliation can exacerbate the effects of other Oak threats such as, drought, disease, and other Oak defoliators (Groenen and Meurisse, 2012; Poulsom, 2015).

In the UK the most common form of pest control for OPM is the application of *Bacillus thuringiensis* var. *kurstaki* which is a specific strain of *Bt* that is most commonly used as a pesticide to control Lepidopteran larvae (Deltamethrin is licensed for use but due to concerns around toxicity to other forms of life it is discouraged and not commonly used), and nest removal. However, these control strategies are failing due to high cost, inconsistent quality of application, and a push for more sustainable practises (Townsend, 2013; Tomlinson, Potter and Bayliss, 2015; de Boer and Harvey, 2020; Buist *et al.*, 2021). However, more sustainable practises such as biocontrol and cultural practises and can be limited due to a lack of knowledge of species biology and interactions with other species and its environment. This chapter will review our current understanding of OPM life history, ecology and distribution, before assessing current pest management techniques. It will then consider the potential of recent molecular approaches to aid in integrated pest management programs.

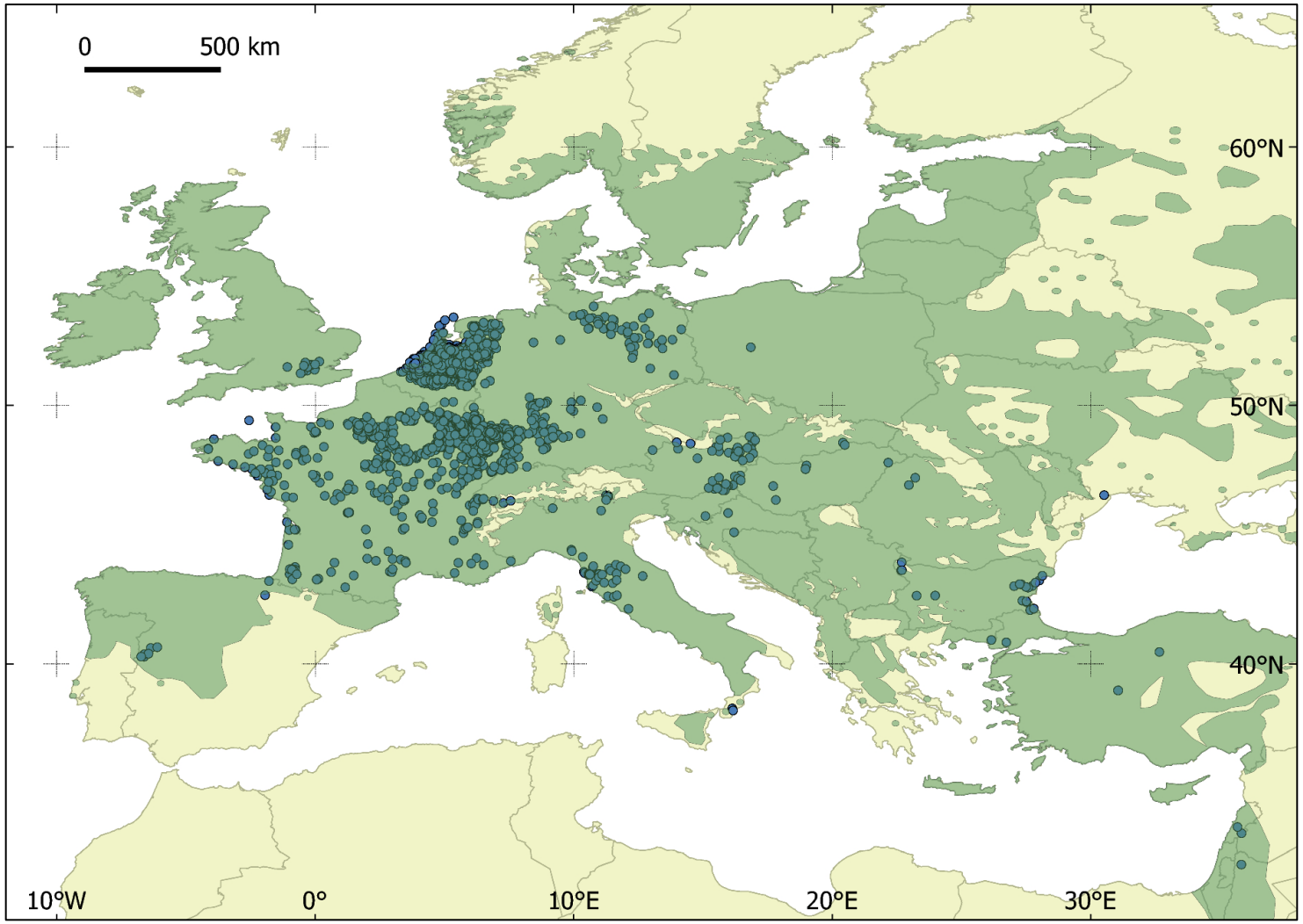


Figure 2.1: Figure from Godefroid et al (2020). Occurrence of OPM across Europe represented by green points with host availability shown by shaded green area.

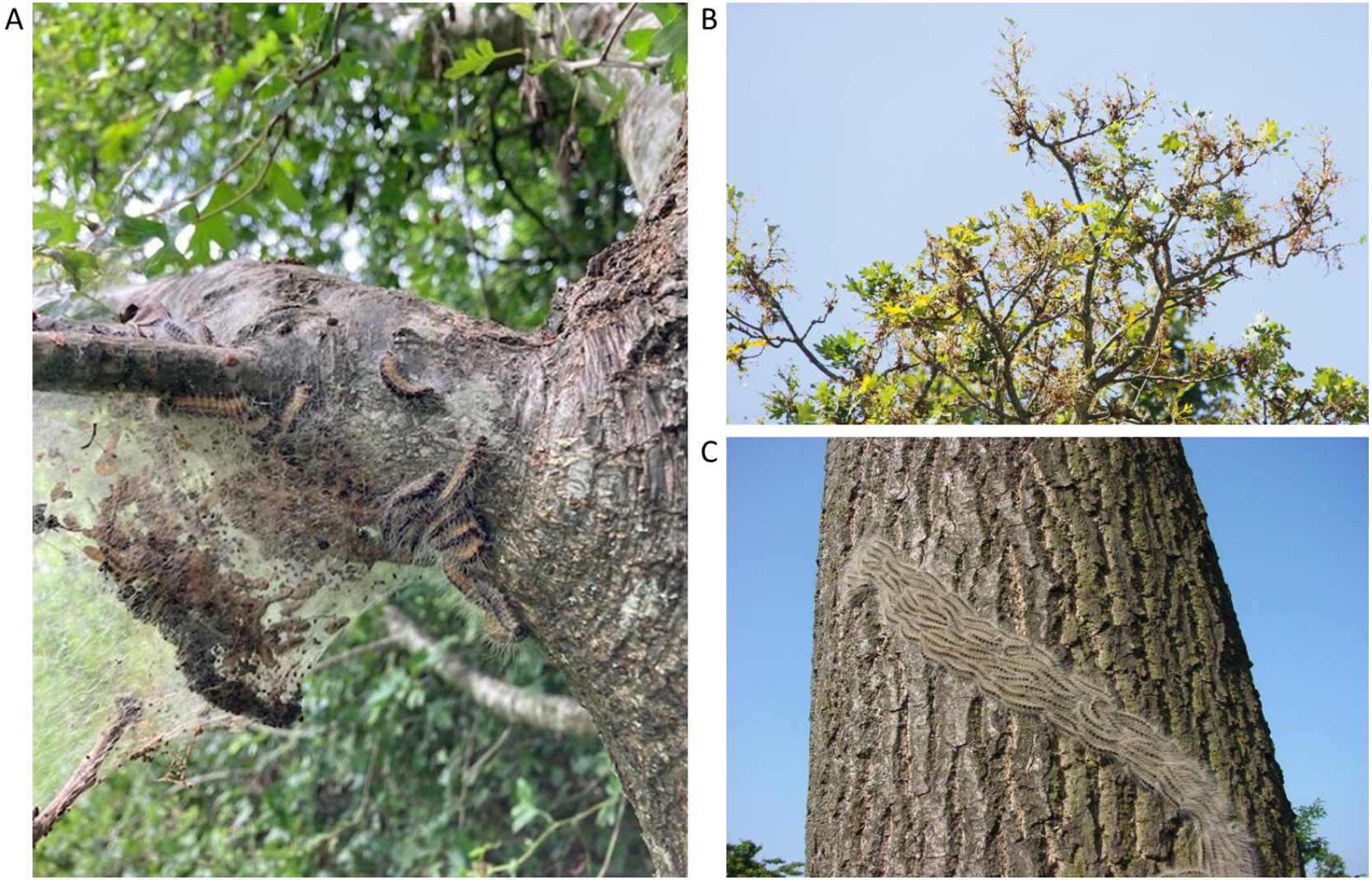


Figure 2.2: A) Nest of OPM larvae (Taken by author). B) Defoliation of pedunculate oak (*Quercus robur*) caused by OPM larvae (Forest Research, 2022a). C) Procession of OPM larvae (Forest Research, 2022a).

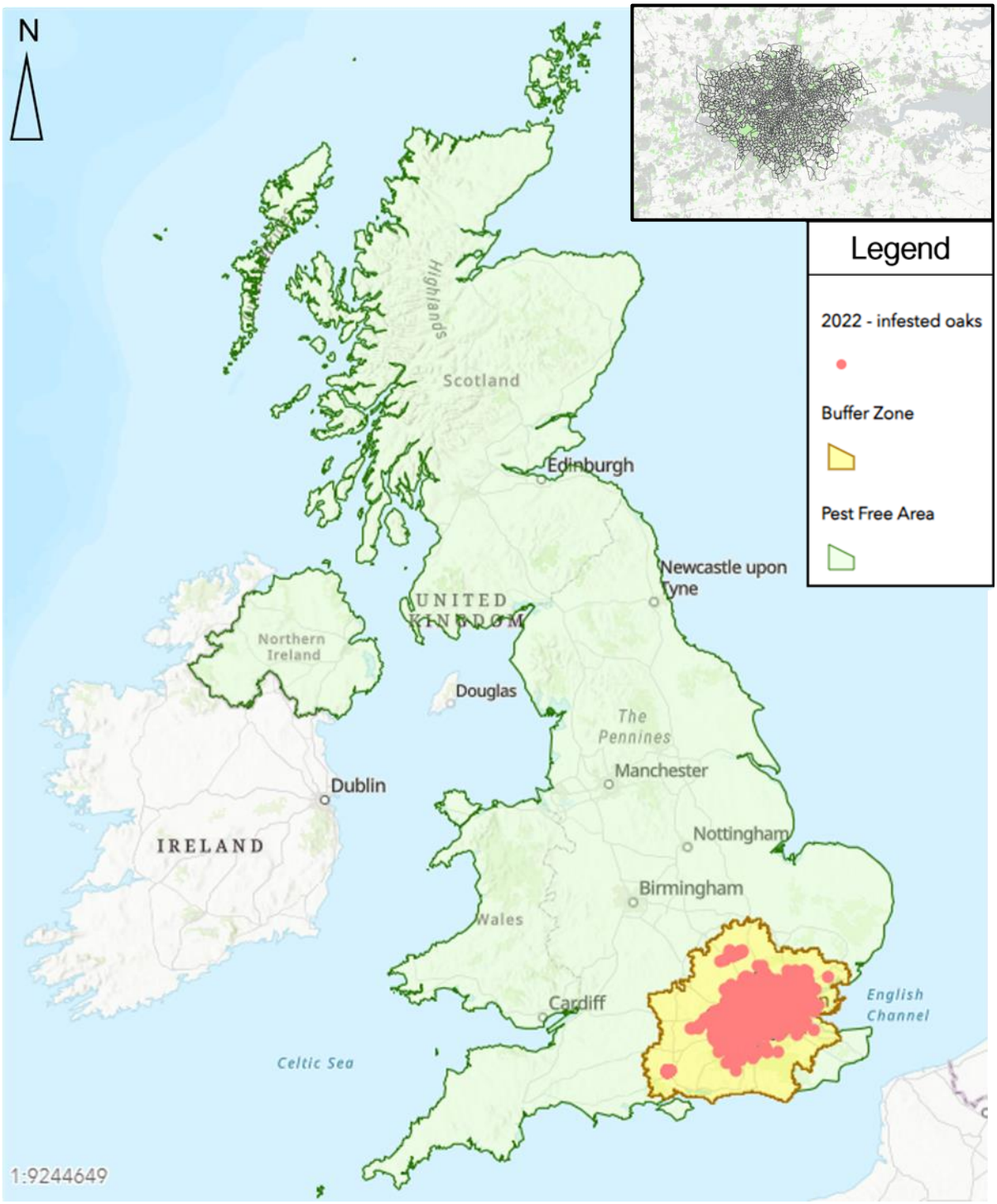
Distribution and Host Range

The oak processionary moth is native to the central and southern regions of Europe (Rahlenbeck and Utikal, 2015), where they primarily inhabit the edges of broadleaf oak woodland (Battisti *et al*, 2014). OPM are univoltine and almost exclusively use *Quercus* (oak) species as their host plant although larvae have been observed utilising other species, namely beech (*Fagus*), birch (*Betula*), hawthorn (*Crataegus*) and *Robinia sp* (Móricz, 2018) when OPM outbreaks have been severe. There is consensus that only Oak and Beech can lead to the development of adult moths (Battisti *et al*, 2014), which means that the presence of oak trees are considered vital to the success and spread of OPM.

Over the last few decades OPM has been steadily expanding its range with populations established in France, Germany, Belgium, Netherlands, and the UK (Groenen and Meurisse, 2012; Godefroid *et al.*, 2020). However, it is debated as to whether this expansion is OPM colonising new territory or recolonising its previous range (Groenen and Meurisse, 2012). The expansion of OPM has previously been attributed to climate change (Fransen *et al.*, 2006; Townsend, 2007) however, (Groenen and Meurisse, 2012) argue no specific mechanism is given to explain this. Groenen & Meurisse (2012) also present historical distribution records as evidence to suggest that OPM was present in much of its current range pre-1920s. However, during two of the time periods given (1750 – 1910, 1911- 1970) OPM were much less abundant than compared with more recent records, with currently populated areas having either no records or records of a few individuals during these time periods. Groenen & Meurisse (2012) suggest two possible explanations for this; (i) the presence of small cryptic populations or (ii) that closer to the northern range boundary OPM populations may cycle through periods of being established, extinct and re-establishing, the latter occurring largely due to unfavourable climatic conditions such as variable spring rainfall and late frosts. It is most likely that there is a mixture of recolonisation and expansion into new territories occurring which could be tested using a population genomics approach. The records of OPM presented in (Groenen and Meurisse, 2012) suggest that populations were present in northwest Europe, however, Denmark, the UK and Sweden had no records prior to 1971. In the case of the UK an invasion pathway has already been described with either OPM egg plaques or larvae arriving on “instant impact” trees planted on a new-build housing estate near Kew Gardens, Richmond, London (Townsend, 2007). These trees were imported from the

Netherlands although they originated in Italy. No specific invasion pathway has been described for Denmark and Sweden. Therefore, a lack of records prior to 1971 may be due to sampling effort in those areas, however, it is more likely that these records are truly the first records of OPM in those countries.

In the UK OPM have been present since 2006 with suspected breeding occurring in the same year (Townsend, 2013). The initial governmental response was to try and eradicate the species however, this approach failed, and a move was made to contain the species within as small an area as possible (Jarvis, 2016). The remainder of the UK was declared a pest free area, and the focus was made to keep OPM from expanding further (Townsend, 2013; Jarvis, 2016). Despite heavy investment in pesticide application, nest removal, and survey efforts, OPM has continued to expand a rate of around 2 km per year, although this is expected to increase (Townsend, 2013; Cowley, Johnson and Pocock, 2015; Jarvis, 2016; Godefroid *et al.*, 2020). A map of their distribution in the UK as of 2023 can be found in Figure 2.3.



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176 Figure 2.3: Map of OPM distribution based on Forestry Commission (2023) with reference map of
177 Greater London boundary.

Biology

OPM have four life stages: egg, larvae, pupae, and adult. Larvae hatch from eggs synchronously to coincide with Oak bud burst which allows them to feed on high quality foliage (Forestry Research, 2020). Early instar larvae have been recorded favouring buds and freshly emerged leaves whereas later instars will seek out older leaves (Wagenhoff *et al*, 2014). Phenological asynchrony and late spring frosts are thought to negatively affect population persistence at the limits of the species range (Móricz, 2018). Larvae are also gregarious and migrate from a communal nest, which is woven from silk, to feed in a procession which contributes to their name (Forest Research, 2022a). Each nest is based on instar, so several nests occur as larvae develop with nest positioning dependent on climate, tree size, and situation of the tree (Battisti *et al*, 2014). As larvae progress through instars, they develop hairs (or setae) which produce an irritation response upon contact with human skin caused by a protein called Thaumetopoein, with instars 5 and 6 having up to half a million hairs (Mindlin *et al*, 2012; Rahlenbeck and Utikal, 2015). Adult moths emerge at a similar time across their range, usually in August or September depending on local climatic conditions (Wagenhoff *et al*, 2014).

Adult moths have a short lifespan, on average living around 4 days. During this time the moths will disperse and breed, with females laying around 300 eggs in contiguous rows along young tree branches. Males and females will both move away from their emergence sites, however males will disperse much further than females (Groenn and Meurisse, 2012). A flight distance of up to 20 km has been estimated for male moths based on pheromone trapping studies (Forest Research, 2020). However, males have been recorded further than this from nests, where no persistent population has occurred afterwards which suggests their dispersal can be aided by strong winds or facilitated by humans through poor horticultural practises (Groenen and Meurisse, 2012). Female moths are known to be capable of dispersal, however it is uncertain if they are capable of the same flight distances as males (Forest Research, 2020).

OPM have a host of natural enemies (Table 2.1) and are predated by a range of birds, mammals, and other insects (Sobczyk and Others, 2014). Around 40 species have been observed parasitising OPM (Table 2.1) at the egg, larval, and pupal, life stages, across their native range. These species come from four main groups: Diptera, Hymenoptera, Coleoptera, and Heteroptera (Sobczyk and Others, 2014). OPM-parasitoid dynamics are poorly

understood, particularly in its non-native range (Kitson *et al*, 2019). In the UK three species have been confirmed to parasitise OPM, *Carcelia iliaca, Compsilura concinnata* and *Pales processionea. C. iliaca and C. concinnata* were both recorded prior to 2020 with *C. iliaca* responsible for parasitising ~50% of larvae and *C. concinnata* parasitising ~0.4% (Kitson *et al.*, 2019). The third species, *Pales processionea,* was recorded in OPM for the first time in 2020 (*Tachinid Recording Scheme*, 2020), however parasitism rates and its distribution are unknown. All three are tachinids and target OPM larvae. Another known parasitoid of OPM is present in the UK, *Zenillia libatrix*, (CABI, 2020), however, it has yet to be recorded in the OPM population.

Table 2.1**:** List of species that have been observed predating or parasitising OPM and at which life stage. P - predator, Pa – parasitoid, Ecp – Ectoparasitoid, E – egg, L – larvae, Pu – pupae, R – recorded, U – Unknown, Y – Yes, N - No.

Order	Family	Species	Interaction	Life Stage Target	Observer	UK Presence	Parasitoid Presence in UK OPM
Hymenoptera	Encyrtidae	*Ooencyrtus masii*	P	E	Mercet (1921)	N	-
		Ooencyrtus sp.	P	E	Mirchev *et al* (2011)	-	-
	Eupelmidae	*Anastatus bifasciatus*	Pa	E	Goeffroy (1785); Dissescu and Ceianu (1968); Mirchev *et al* (2011)	Y	U
	Trichogrammatidae	*Trichogramma* sp.	Pa	E	Tiberi *et al* (1991)	-	U
	Pteromdaliae	*Pteromalus chrysorrhoeae*	P	E	Dalla Torre (1898)	Y	-
		Pteromalus processionae	P	E	Ratzeburg (1844)	Y	-
	Ichneumonidae	*Pimpla processioneae*	P	L	Ratzeburg (1849); Wakhals (2005)	N	-
		Pimpla ruipes	Pa	L/Pu	-	Y	U
		Lissonota clypeator	P	L	Ravenhorst, (1820); Yu and Horstmann (1997)	Y	-
		Neischnus germari	P	L	Ratzeburg (1849); Sawoniewicz (2003)	Y	-
		Theronia atalantae	P	L	Poda (1761); Fankhänel (1959)	Y	-
	Braconidae	*Meteorus veriscolor*	Pa	L	Wesmael (1835); Fankhänel (1959); Dissescu and Ceianu (1968); Mirchev *et al* (2011)	Y	U
		Aleiodes testaceus	Pa	L	-	Y	U
	Torymidae	*Monodontomerus aereus*	Pa	L	Walker (1834); Noyes (2012)	Y	U

		Monodontomerus minor	Pa	L	Ratzeburg (1848); Noyes (2012)	Y	U
	Formicidae	*Formica polyctena*	P	L	Förster (1850); Dittmer (2010)	Y	-
	Pteromalidae	*Dibrachys mircogastri*	Ecp	Pu	Bouché (1834)	Y	U
		Dibrachys cavus	Pa	L, Pu	Walker (1835); Fankhänel (1959)	Y	U
		Pteromalus puparum	Pa	Pu	Linnaeus (1758)	Y	U
		Roptrocerus mirus	Pa	L	Walker (1835); Herting (1978)	N	U
		Eurytoma appendigaster	Pa	L	Wederus (1795); Fankhänel (1959)	Y	U
		Gelis aerator	Pa	L	Panzer (1804); Fankhänel (1959)	Y	U
		Gelis sp.	Pa	L	Fankhänel (1959)	-	U
	Eurytomidae	*Eurytoma verticillata*	Pa	Pu	Fabricius (1798)	Y	U
Diptera	Tachinidae	*Pales processionea*	Pa	L	Ratzeburg (1844)	Y	Y
		Pales pavida	Pa	L	Meigen (1824); Mirchev *et al* (2011)	Y	U
		Zenilia libatrix	Pa	L	Panzer (1798); Fankhänel (1959); Dissescu and Ceianu (1968); Tschorsnig and Herting (2005); Mirchev *et al* (2011)	Y	U
		Blondelia nigrips	Pa	L	Fallén (1810); Mirchev *et al* (2011)	Y	U
		Phryxe caudata	Pa	L	Rondani (1859); Tschorsnig and Herting (2005)	N	U
		Phryxe semicaudata	Pa	L	Herting (1959); Dissescu and Ceianu (1968)	N	U
		Compsilura concinnata	Pa	L	Meigen (1824); Tschorsing and Herting (2005)	Y	Y

		Phorocera grandis	Pa	L	Rondani (1859); Tschorsnig and Wagenhoff 2012)	N	U
		Carcelia iliaca	Pa	L	Ratzeburg (1840); Maksymov 1978); Tschorsnig and Herting (2005)	Y	Y
		Carcelia laxifrons	Pa	L	Villeneuve (1912); Mirchev *et al* (2011)	Y	U
		Platymyia nemestrina	Pa	L	Meigen (1824); Mirchev *et al* (2011)	N	U
		Platymyia westermanni	Pa	L	Zetterstedt (1844); Mirchev *et al* (2011)	N	U
Coleoptera	Carabidae	*Calosoma sycophanta*	P	L	-	Y	-
		Calosoma inquisitor	P	L	-	Y	-
	Silphidae	*Xylodrepa quadripunctata*	P	L	-	Y	-
Heteroptera	Entatomidae	*Troilus luridus*	P	L	-	Y	-
	Reduviidae	*Rhynocoris iracundus*	P	L	-	N	-
		Rhynocoris annulatus	P	L	-	N	-
Passeriforms	Paridae	*Parus Major*	P	L/Pu	-	Y	-
	Passeridae	*Passer montanus*	P	L/Pu	-	Y	-
	Orioloidae	*Oriolus oriolus*	P	L/Pu	-	Y	-
Cuculiforms	Cuculidae	*Cucuclus canorus*	P	L/Pu	-	Y	-
Bucerotiforms	Upupidae	*Upupa epops*	P	L/Pu	-	Y	-

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Climate Driven Shifts in OPM Distribution

Widespread changes in species distribution are expected due to climate change (Serra-Diaz and Franklin, 2019). Many of these changes are predicted to be negative with species unable to track their preferred habitats because of natural and human barriers. However, some species are expected to benefit from a change in climate, at least in the short term (Poloczanska *et al*, 2008). The expansion of OPM is often loosely linked to climate change although there is no current direct evidence for this.

Evidence for a climate-driven range shift?

Climate driven shifts in distribution has been described in the closely related pine processionary moth (*Thaumetopoea pityocampa*) (PPM) which has demonstrably benefited from a warming climate (Battisti *et al*, 2014; Robinet *et al,* 2013; Battisti *et al*, 2017). PPM, like OPM, is considered a major pest and is the most studied species in the *Thaumetopoea* genus. PPM is widely distributed across the Mediterranean basin and display a different development cycle compared to other members of the genus. Larvae will hatch in autumn and feed in both autumn and spring after over wintering. However, if climatic conditions allow, larvae will also leave nests during winter to feed with this strategy being possible due to the host plant retaining its foliage over winter (Huchon and Démonlin, 1971). PPM range and development have typically been restricted by winter temperatures at the northern and altitudinal range boundary (Huchon and Démonlin, 1971; Battisti *et al*, 2005). However, warming winters over the last few decades have seen an expansion at both range boundaries (Battisti *et al*, 2005; Battisti *et al*, 2006). Warmer winter temperatures mean a reduction in the occurrence of lethal temperatures and an increase in larval performance through longer feeding times (Robinet *et al*, 2007; Roques *et al*, 2014). Furthermore, warmer summer nights have benefitted adult moth mobility, with an increased likelihood of the flight threshold temperature being achieved, which aids dispersal (Battisti *et al*, 2006).

Whilst OPM and PPM are very closely related and share some similar traits many have argued against a comparison between the two species (Groenen and Meurisse, 2012; Roques, 2015; de Boer and Harvey, 2020; Godefroid *et al.*, 2020). In particular, Groenen (2012) suggesting that the differences in lifestyle make a comparison redundant.

A common factor that is observed when discussing climate driven range shifts in the northern hemisphere is that as northern range boundaries expand, southern range boundaries may start to retreat as a species tries to stay within a suitable climatic zone (Thomas, 2010). This has been observed across all major well studied, freshwater, marine and, terrestrial groups, with strong effects occurring with polar and montane species (Parmesan, 2006). However, the drivers that determine a species range are complex with land use often cited as an important driver of range boundaries in Lepidopterans (Fox *et al.*, 2014; Costache, Crişan and Rákosy, 2021). Therefore, it should be unsurprising that Groenen and Meurisse (2012) found that there was an extension of the southern range limit in OPM. This was associated with records of the subspecies *T. processionea pseudoso litaria* and indicates that OPM population dynamics at the southern range boundary may currently be influenced primarily by land use rather than climate change.

How two invasive species have responded to climate change

The pine-tree lappet (*Dendrolimus pini*) (PtL) is considered an invasive species that is present in the Beauly catchment area in the northwest of Scotland (although there is the possibility that it is an overlooked remnant population) (Forest Research, 2021a). PtL are widespread across continental Europe and have been reported as far east as Asia, and as far south as North Africa (Meshkova, 2003). The first official report of the species in Beauly catchment was in 2008 although unofficial reports suggest species presence in 2004 (Forestry Research, 2021b). Over its native range, PtL feed on a range *pinus* species with Scots Pine (*Pinus sylvestris*) being the most commonly consumed species across Europe (Moore and Evans, 2016). Like OPM and PPM, PtL populations occasionally have outbreak years which result in severe defoliation, and in some cases dieback, of the pine species in the area (Siepinska, 1998). The main areas for outbreaks appear to be Poland (Łukowski *et al*, 2020), and Germany (Skrzecz *et al*, 2020). However, outbreaks have also been reported in Norway (Hopkins, 1908), Ukraine (Meshkova, 2003), and Sweden (Björkman *et al*, 2012), preceding extremely hot and dry summers (Moore and Evans, 2016). This behaviour has not yet been reported in Scotland. Although no formal climate matching has been conducted it is thought that the cool and wet oceanic climate of areas like Scotland, Norway, and Sweden suppresses outbreaks of PtL (Moore and Evans, 2016), unlike the warmer and drier continental European climate.

Unlike OPM, PtL appear to have been restricted to its current area despite an abundance of suitable habitat surrounding it. In 2015 another breeding population was discovered at Glenn Strathfarrar although it is uncertain if this is another introduced population, a population established by natural spread from the previous population or whether this is evidence for PtL being an overlooked native species (Moore and Evans, 2016). Nevertheless, it is uncertain why PtL remained restricted to such small areas despite surrounding suitable habitat. There is no confirmed driver for this behaviour, however, there are several possible reasons as to why PtL have not spread including, a natural lag time, reduced fitness due to climate, populations being suppressed by a natural enemy, or a combination of these drivers (Sakai *et al.*, 2001; Sims, Finnoff and Shogren, 2016).

Similar to (Groenen and Meurisse, 2012)dismissing comparisons between OPM and PPM over lifestyle, PtL have a different life cycle to OPM and show more similarities to PPM. Larvae hatch between July and August, then hibernate at the base of trees over-winter and then continue developing in the spring with adults emerging between June and July (Moore and Evans, 2016). However, it is interesting to note the differences in how these two invasive species have responded to their new environments. Particularly with the arguably less successful species establishing in the colder, wetter north of the UK and the other, more successful, species establishing in the warmer, drier south.

How OPM may benefit from climate change

Whilst OPM has not currently shown definitive benefits from climate change there is the possibility this will change in the future. As the climate continues to warm there are concerns around OPMs ability to take advantage of a wider range of its host distribution alongside worries of how warmer temperatures and drier years may impact on outbreaks.

OPM expansion in its introduced range

As mentioned previously OPM is expanding throughout southern England and is predicted to continue to do so. Its spread has in part been facilitated by the environments it has been introduced to such as urban gardens and parks (Cowley *et al*, 2015). This is because OPM favour forest edges, due to the microclimate produced by a mix of canopy cover with access to appropriate growing temperatures. These features are well replicated by the low tree density in many urban parks and tree lined avenues of UK cities. The presence of these

favourable areas has allowed OPM to spread quickly despite control efforts. Godefroid *et al* (2020) used models to predict that most of the UK will be climatically suitable by 2050 under four different warming scenarios. This increased climatic suitability and the presence of its host plant across much of the UK means that OPM are likely to continue to spread north into new territories. This hypothesis is backed up by modelling work conducted by (Wadkin *et al.*, 2022) with OPM shown to be able to spread across the UK at a steady rate based on data from Richmond Park and Bushy Park. This approach may also be able to inform how OPM may react to climate change in spreading beyond its current range boundary into areas further north such as Finland, Sweden, and Norway.

OPM stabilising at its northern range boundary

Despite the controversy over whether OPM is invading or recolonising, it is generally agreed that a warming climate will make areas at its northern range boundary and beyond more climatically suitable by reducing the likelihood of late spring frosts and providing an increased number of days where growth is possible making survival more likely (Godefroid *et al*, 2020; Groenn and Meurisse, 2012). If unsuitable climatic conditions have been the main driver for OPM populations in northwest Europe remaining small or transient, then this may start to change which could have ramifications on the economy and, ecological and social health, of these countries. It is unknown if *Quercus* species in northwest Europe are less well adapted to deal with the additional pressure a permanent population of OPM could bring.

It is also unclear what this means for human OPM interactions in these areas. Several medical studies have been published from countries where OPM has recently invaded ((Townsend, 2013; Marzano *et al.*, 2020; Buist *et al.*, 2021)), whereas there are few medical studies regarding OPM in its native range. This brings to light several questions such as are: a) OPM human health impacts as serious across its native range?; b) communities that have cohabited with OPM for long periods of time more familiar with the risks therefore no investigation is deemed necessary?; c) communities that have cohabited with OPM for long periods immune, or partially immune to the response caused by OPM hairs?; and d) woodlands in north-west Europe more fragmented compared to those across central and southern Europe therefore leading to higher chance of exposure to OPM? While there is little to no literature regarding communities relationships to OPM across its native range, there is evidence to suggest that

forests across north west Europe are more fragmented, which could in turn lead to higher chances of exposure to OPM (de Rigo, D., Estreguil, C., Caudullo, G., and San Miguel, J., 2013).

It should be noted however that under a +1.5ºC warming scenario, precipitation across northern Europe is expected to become more frequent (Donnelly *et al*, 2017). With spring rain known to negatively impact OPM larvae, there may be a shift in the limiting factor for this species from temperature to precipitation (Móricz, 2018). Although, increased precipitation may not reduce the spread of OPM, it may limit the number of outbreaks (Csoka *et al.*, 2018).

Increased frequency of OPM outbreaks

Outbreaks of OPM have been reported across Europe since the early 1900s, with frequency varying in individual countries. OPM does not always behave like a classic outbreak species, with populations remaining stable for long periods of time with no sign of sudden expansion or collapse (Csóka *et al*, 2018). The factors that facilitate OPM outbreaks are poorly understood, however a study by Csóka *et al* (2018) described the effect that weather fluctuations can have on OPM populations. Over a period of 15 years (1998-2012) they monitored OPM populations with light traps and found that the combined effects of both precipitation and temperature explained 54.8%-68.9% of a population's yearly fluctuation. This suggests that warm and dry weather during spring and summer is a strong factor in facilitating an OPM outbreak. It should also be noted that this is also the case in Pine-tree Lappet moth populations in continental Europe (Leśniak, 1976).

Impacts to Human and Animal Health

Aside from the impact to oak health, there are also concerns from countries where OPM has been introduced surrounding the human and veterinary health impacts (Rahlenbeck and Utikal, 2015). These health impacts are well understood and are caused by coming in to contact with the hairs that cover OPM larvae (Figure 2.4). The setae, contain a protein called Thaumetopoein which is responsible for the irritation caused by contact with the hairs (Lamy *et al*, 1986). This irritation can be diagnosed as 'caterpillar dermatitis' (Rosen, 1990), however, this diagnosis is rare with many medical practitioners unlikely to recognise the irritation as such (Konstat-Korzenny *et al*, 2020).

Larvae from the third instar onwards develop setae and later instar larvae (5^{th} and 6^{th}) can be covered with up to half a million hairs (Rahlenbeck and Utikal, 2015). It is presumed most

incidents involving OPM hair irritation are through direct contact with larvae and nests or from hairs on bark (Rahlenbeck and Utikal, 2015; Tan *et al*, 2020). Hairs remain "active" in the environment for several months, and in the right conditions several years, meaning that the risk of irritation is present year-round (The ESFA Journal, 2009). It is also possible for incidents to occur from hairs that have been blown on the wind although it is difficult to quantify the regularity of these cases. The dispersal ability of hairs on the wind has been modelled by Fenk *et* al (2007). The results of this estimate that hairs can travel roughly 2 km from the source colony. However, it should be noted that the model assumed nests would be positioned at the tops of trees which is unlikely as OPM nests are more commonly positioned in more sheltered areas such as the underside of branching points (Forestry Commission, 2022). Therefore, the real dispersal capacity of the hairs may be lower.

Exposure to hairs can occur through several pathways; (i) dermal, (ii) ocular, (iii) inhalation, and (iv) ingestion. Exposed areas, such as the arms and face, are more likely to be affected and the irritation caused by hairs can range in severity. Severe itching is present in almost every case, conjunctivitis is present in between 15%-20% of cases, and irritation of the upper respiratory tract is present in around 10% of cases (Mindlin *et al,* 2012). Most symptoms clear up within 1-2 weeks of initial exposure, however, some cases have been reported to persist for up to a month afterwards (Mindlin *et al*, 2012).

The discovery of OPM in London in 2006 followed an outbreak of dermatitis. Mindlin *et al* (2012), published the results of an outbreak investigation following residents' reports of itchy rashes. Households (n = 32) surrounding the infested tree were surveyed with questionnaires and active case finding conducted with GPs, hospital emergency departments and dermatologists. The authors received a 63% response rate (20/32) and found that 47 out of 69 residents (68%) reported having symptoms in line with caterpillar dermatitis. All cases suffered from a rash although severity of the rash is not detailed. The other two symptoms reported were itchy eyes, of which 14 respondents (20%) suffered, and 2 respondents (3%) reported respiratory problems. Of the affected households 10 sought medical advice and received five different diagnoses. However, none were diagnosed with caterpillar dermatitis. The authors state reports of similar symptoms in affected individuals prior to April 2006 were low, therefore, suggesting that the symptoms were caused by exposure to OPM.

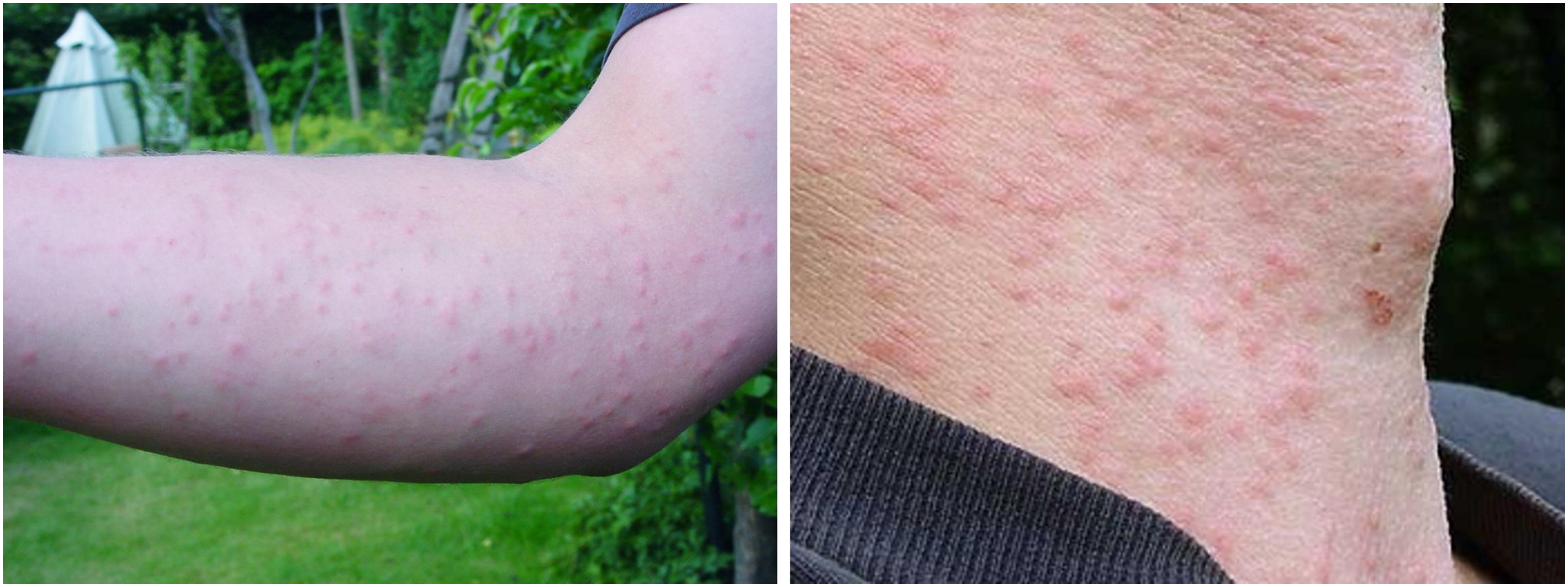

Figure 2.4: Example of rash caused by contact with OPM larvae hairs (Forest Research, 2022b).

Control of OPM and Integrated Pest Management

As previously stated OPM first established in the UK in 2006 with the first evidence of breeding occurring in the same year. In the first years of the establishment the OPM control program aimed to eradicate the population via two main methods pesticide application both aerially, via helicopter, and by ground spray, and to physically remove nests. Later, more holistic forms of management were implemented such as aiding biocontrol via parasitoids and predators. Jarvis (2016) provided a full evaluation of the OPM control program which is summarised in Figure 2.5**.** Modelling work as part of this evaluation suggests that government intervention has slowed OPM expansion compared with zero intervention. However, climate modelling has predicted that more areas of the UK will become suitable for OPM, and the current population will expand into these areas despite current management.

Pesticide application has been successful in slowing the progress of OPM expansion with aerial spraying proving more effective than targeted spraying (Jarvis, 2016). However, an increasingly negative public perception surrounding pesticide application (Remoundou *et al*, 2014), along with cost of application because of a growing population, and the growing concern on the long-term impacts on other Lepidopterans (Rastall *et al.*, 2003; Scriber, 2004) has led the government to seek a more sustainable approach in integrated pest management (IPM). IPM seeks to integrate chemical, biological, physical, and cultural tools to develop management strategies that are economically justifiable whilst reducing damage to human and environmental health (Barzman *et al*, 2015). This practise is rooted in "supervised insect control" which was developed in California as a solution to combat the decline in natural enemies and increase in pesticide resistance seen due to widespread pesticide use (Smith and Smith, 1949). The original technique used field surveys to determine if the parasitoid population of the target pest would prevent an outbreak or if pesticide application were necessary (Smith and Smith, 1949; Stern *et al*, 1959). This has since evolved to include more sophisticated techniques like modelling and crop cultivation techniques (Stenberg, 2017).

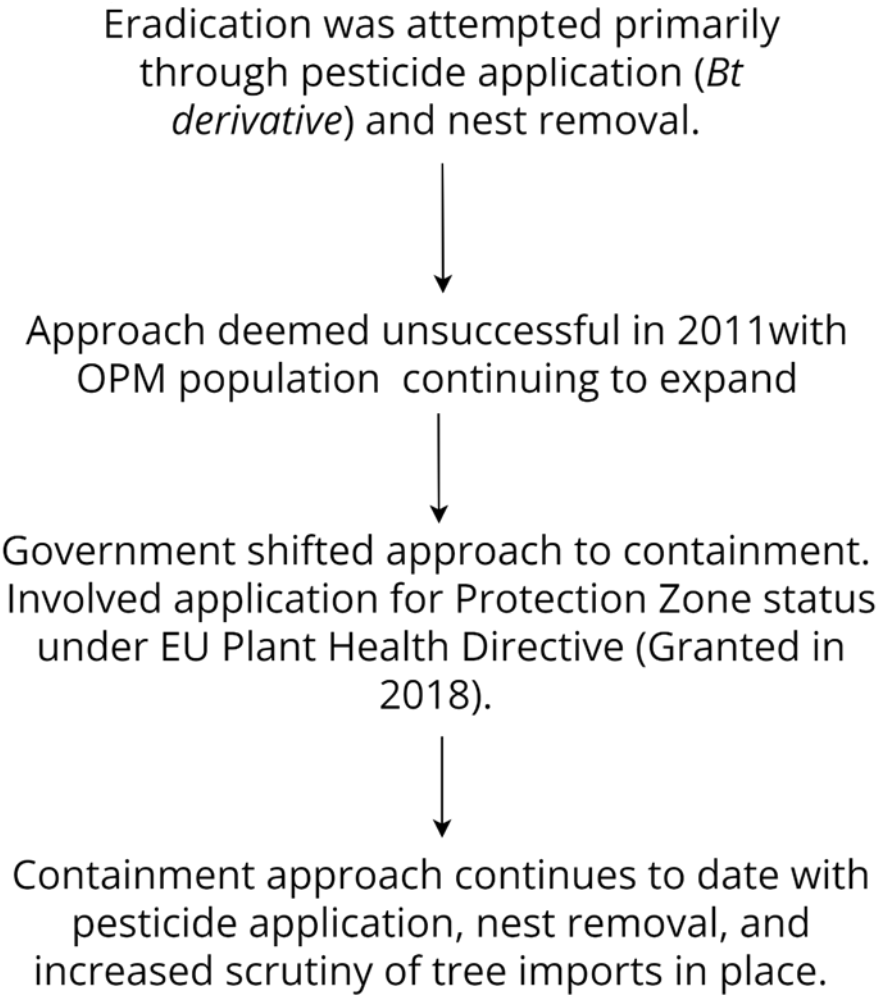

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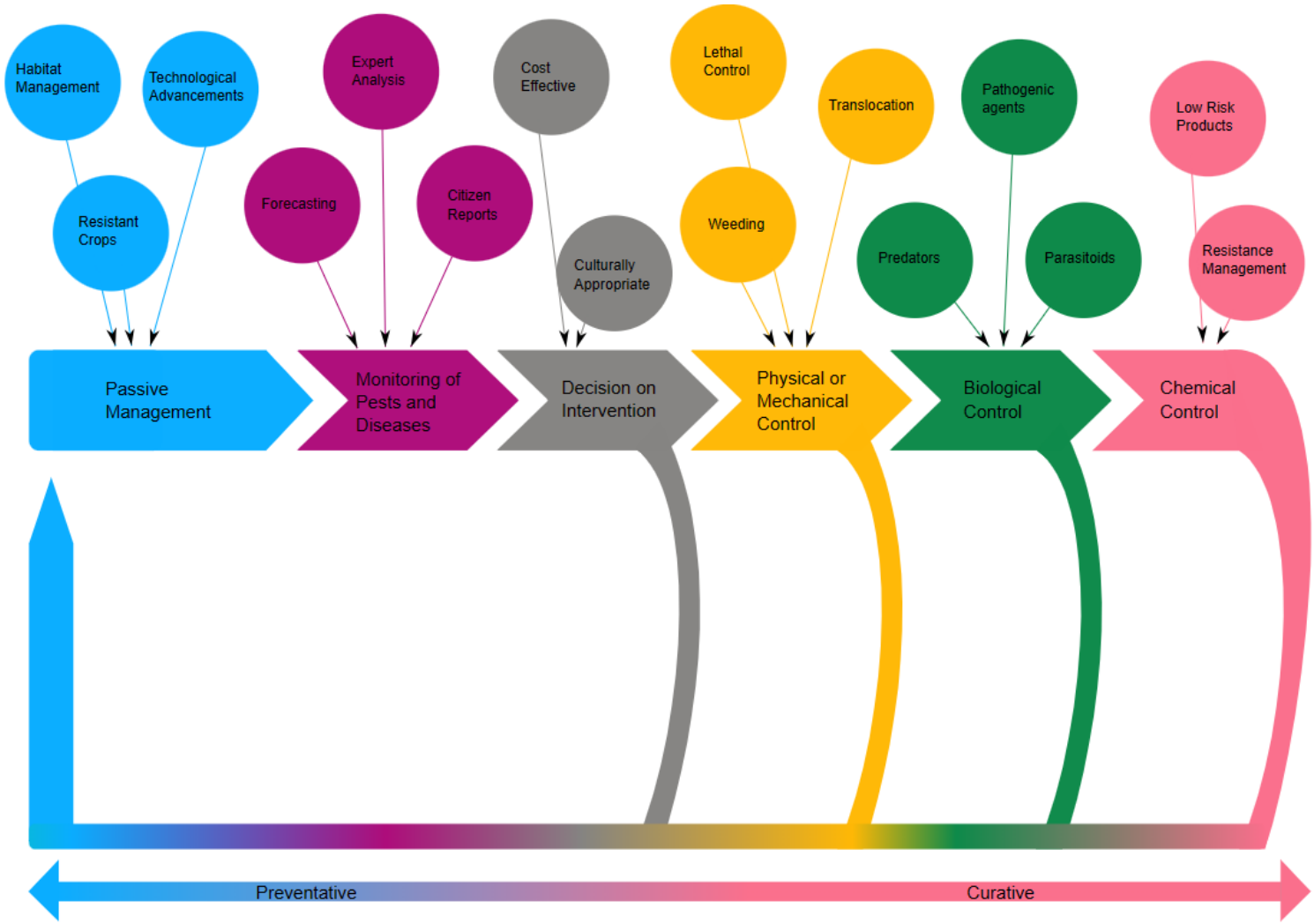
Figure 2.5: Flow chart showing the progress of the UK government OPM response since the initial observations of OPM in London (Based on Townsend, 2007).

IPM has been a focus of the EU under the EU Framework Directive 2009/128/EC which seeks to achieve the sustainable use of pesticides. The directive requires member states to develop an action plan which ensures the implementation of the eight principles of IPM, outlined in (Barzman *et al.*, 2015), by the 1st of January 2014 (Figure 2.6).

1. **Prevention and Suppression** – Populations of harmful organisms should be prevented and/or suppressed with good farming practises e.g., crop rotation, resistant cultivars.
2. **Monitoring** – Use adequate methods and tools to monitor harmful organism populations.
3. **Decisions based on monitoring and thresholds** – Data gained from monitoring should be used to make decisions regarding plant protection measures.
4. **Non-chemical methods** – Sustainable biological and physical methods should be preferred over chemical methods.
5. **Pesticide selection –** When pesticides need to be used, they must be as specific to the target as possible with minimal side effects.
6. **Reduced pesticide use –** When pesticides need to be used, they must be kept to the necessary level.
7. **Anti-resistance strategies –** Where the risk of resistance against a plant protection measure is known available anti-resistance strategies should be employed.
8. **Evaluation –** Applied interventions should regularly be checked for success or failure.

While IPM is currently viewed as the gold standard in pest management it does have limitations particularly when discussing biological control programs for invasive species. These limitations are often similar across all alternative techniques, and they include, inadequate research, regulatory issues, limited resources, and poor uptake by end users. The following section will expand further on these limitations from a biological control program perspective.



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456 Figure 2.6: Eight principles of IPM presented as a potential workflow for site managers (Barzman *et al.*, 2015).

Inadequate research

A poor understanding of a species life history is a common problem across the field of ecology for biocontrol programs, which can make the difference between governing bodies approving or rejecting a program due to the risk involved. For example, the introduction of the Cane toad (*Rhinella marina*) to Australia in 1935, is classically acknowledged as a failure and the impacts of this introduction are still not fully understood (Radford *et al*, 2020).

Inadequate research is in part fuelled by a lack of funding which has been attributed to high profile failures making funding bodies unwilling to risk investment in this area (Barratt *et al.*, 2018). Although many countries are now seeking more sustainable solutions to pest management which has led to an increase in research funding (Barzman *et al.*, 2015; Barratt *et al.*, 2018). However, when funding does become available it is important that researchers follow best practise to ensure successful control programs to increase trust amongst authoritative bodies (Blossey and Skinner, 2000). One method of achieving this is through post-release studies which are vital in providing insight into how certain programs function and how different methods can be applied across a range of scenarios. Whilst there appears to be an increase in the volume of post-release studies being published, Schaffner *et al* (2020) argue they are still largely underutilised.

Regulatory issues

Due to previous failures, classic biological control has faced increased scrutiny from regulators over the past 20 years (van Lenteren *et al.*, 2006; Moran and and Hoffmann, 2015; Hulot and Hiller, 2021). Most countries require extensive risk assessments which involve weighing up potential environmental risks with the benefits of using biocontrol agents (Sheppard *et al.*, 2003; Barratt, 2011). It should be noted these issues tend not to be present for conservation biocontrol where native species are typically encouraged through habitat management practises.

Societies that are risk averse tend to have more restrictive regulations and require more thorough risk assessments for biocontrol programs (Sheppard *et al.*, 2003). This approach lends itself to regulators blocking more applications than less risk averse societies. However, this can result in the rejection of programs that have been well researched and show few risks (Barratt *et al*, 2018). Restrictive regulation has been cited as the reason why many researchers

have abandoned the field and a reduction in the number of active researchers has been reported in many countries (Moran and and Hoffmann, 2015). Despite this, there are countries that have managed to mitigate the impact of regulation whilst still maintaining a high standard of risk assessment. New Zealand for example has achieved this by introducing a maximum statutory period for which applications receive a response (100 days at time of writing) (Barratt *et al*, 2018).

Land Manager buy-in

Globally land managers struggle with low incomes or limited resources, so they are more likely to heavily depend on chemical pesticides for all forms of pest control because the outcomes are easy to predict (Parsa *et al.*, 2014; Jayasooriya and Aheeyar, 2016). Changing from chemical pesticides to a biological control agent, which are less well understood, represents a risk that many cannot financially afford or are disinclined to take because of factors such as age and education (Cullen *et al*, 2008). There is also the issue that biocontrol requires more specialized knowledge (monitoring pests, knowing when to release biological control agents, knowing what volume to release), this knowledge requires an initial time and financial investment which also can be off-putting to land managers (Parsa *et al.*, 2014).

Without land manager buy-in, biological control can rarely be successful as land managers are often the people required to implement elements of these programs. Lack of commitment from land managers will often see them fall back on previous tried and tested techniques. However, it is possible to increase the successful uptake of biocontrol. Education and awareness is often cited as a key driver in improving uptake of biological control methods (Rezaei *et al.*, 2019). Education programs have proved to be successful in improving the uptake of biological control techniques particularly with farmers. This has been seen in Indonesia where following a five-year education program, that took farmers into the field to observe the effects of natural enemies on pest species, a 60% decrease in pesticide use was observed (Ooi, 1996). This was followed by a 13% increase in overall rice production showing that a shift from complete reliance on chemical pesticide use, to incorporating biological control, can still be profitable.

Limitations of Integrated Pest Management in the OPM scenario

The most obvious limitation with regards to the OPM scenario is the lack of research surrounding not only the pest but also the parasitoids with which the pest would be managed. This is particularly true of the four recognised OPM parasitoids present in the UK. The current distribution of all four parasitoids is currently unknown and the parasitism rates are only known for *C. iliaca* and *C. concinnata* (Kitson *et al.*, 2019). Without access to this information, it is difficult to integrate parasitism into decision making frameworks and makes it more likely that land managers would need to rely on pesticide application (Miller, Polaszek and Evans, 2021). Therefore, to ensure the successful application of IPM an evaluation of the current distribution of the parasitoids would be needed as well as continued monitoring of parasitoid populations to ensure that management decisions are based on up-to-date information. A monitoring scheme like this would ideally be part of any IPM strategy (Barzman *et al,* 2015).

Molecular Technology to aid IPM

As previously stated, the primary issue with integrating biocontrol into the OPM management strategy is a lack of information regarding *C. iliaca*. Without an understanding of its distribution, it would be difficult to make decisions regarding pesticide application and land management. Therefore, the creation of a diagnostic test to detect OPM parasitoids is a priority in this scenario. This would allow for an understanding of how parasitoids are distributed in the environment which would give clues to their ecological requirements. This information would provide inputs for distribution models which would give predictions as to where these parasitoids could be present on a wider scale. Health and safety issues associated with handling and processing OPM larvae in an enclosed environment would make dissection surveys for parasitoids time consuming and costly due to the need for specialist facilities and equipment. Kitson et al (2019) use tagged metabarcoding to investigate parasitoid assemblages in OPM larvae however, this approach requires highly specialised lab equipment and technical training. Using an approach such as rapid molecular diagnostics would allow for an increased survey effort as this would only require tissue samples which could be gathered from larvae in the field, and less technical equipment. This removes the need for ventilated lab space and also highly specialised lab equipment.

Molecular diagnostics have been used widely for the detection and management of a range of pest groups (Gariepy *et al.*, 2007; Singh, Arora and Gosal, 2015). Primarily this involves the

use of PCR (polymerase chain reaction) usually in the form of qPCR (quantitative PCR sometimes referred to as real-time PCR). PCR, as it is currently used, was developed by Kary Mullis in 1985 (Kaunitz, 2015), although the first description of the concepts underlying PCR are described by Kleppe in (Bessman *et al.*, 1956).

Since its development PCR has seen many variations and enhancements. However, two major improvements have been the use of thermocycling machines which replaced water baths and the addition of fluorescent dyes which make it possible to detect the amplification of PCR products in real time, removing the need for gel electrophoresis to visualise PCR products (Chiang *et al.*, 1996; Gibson, Heid and Williams, 1996; Heid *et al.*, 1996). Both enhancements have allowed PCR to become the foundation for the vast majority of molecular diagnostics work (Powledge, 2004; Valones *et al.*, 2009; Basu, 2015). It is considered the "gold standard" and is often used to validate other forms of molecular diagnostics tests (Mackay, 2004). PCR is well validated with assays having high sensitivity and specificity, is capable of high throughput workflows and, can provide useful quantitative measurements when implemented in real-time PCR (qPCR) (Schmittgen, Lee and Jiang, 2008; Zhu *et al.*, 2020). However, because PCR can be a time-consuming process that is limited to being conducted in labs with expensive thermocyclers, it can reduce the suitability of a diagnostic test when the test is required to provide a simple presence or absence result. To reduce costs and increase accessibility, it is possible to look at other methods of amplification such as isothermal nucleic acid amplification techniques.

There are several isothermal nucleic acid amplification techniques currently used in the literature including, Loop-mediated isothermal amplification (LAMP), Recombinase polymerase amplification (RPA), Nucleic acid sequence-based amplification (NASBA), and Helicase dependent amplification (HDA). Each technique is optimised for different uses and a summary of strengths and weaknesses is available in Table 2.2.

However, with regards to IPM, any isothermal nucleic acid amplification techniques would need to retain similar diagnostic performance to PCR, be cost effective, have a rapid time to positive, and be fully or partly field accessible.

574 Table 2.2: Isothermal nucleic acid amplification technique strengths and weaknesses.

Technique	Developer	Method	Strength	Weakness
Loop-mediated isothermal amplification	Notomi *et al* (2000)	Uses 4-6 primers and an enzyme with high strand displacement activity to produce auto-cycling DNA synthesis.	• Portable • Short reaction time (15 – 90 minutes) • Cheap • Maintains high sensitivity and specificity • Requires only small amounts of template • Low reaction temperature (60 °C - 65 °C) sustainable with low powered instruments	• Highly specific primers increase likelihood of dimerization • Only useful for targeting a small number of sequences • Single temperature amplification can result in lack of temperature gating • SNP induced false-negative
Recombinase polymerase amplification	Piepenburg *et al* (2006)	Uses recombinase to form a complex with the primers. This initiates amplification of the target without thermal denaturation.	• Portable • Low reaction temperature (37°C – 42 °C) sustainable with low powered instruments • Short reaction time (<30 minutes)	• Low reaction temperature can cause higher rates of non-specific amplification artefacts compared to other isothermal nucleic acid amplification technique • Lower sensitivity compared to LAMP
Nucleic acid sequence-based amplification	Compton (1991)	Hijacks transcription using modified primers to incorporate T7 RNA polymerase promoter into double stranded DNA. This promoter is then able to transcribe the RNA product.	• Useful for detecting viable cells in pathogen assays • Highly sensitive • Short reaction time (~90 minutes)	• Reliance on two-step protocol removes suitability for out of lab use • Unsuitable for DNA amplification
Helicase dependent amplification	Vincent *et al* (2004)	Uses a helicase to separate target DNA which allows primer binding and extension using DNA polymerase.	• Short reaction time (30-90 minutes) • Reaction can be run at a single temperature	• Restricted to lab • Mainly used for short sequence amplicons (70-90bp)

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LAMP

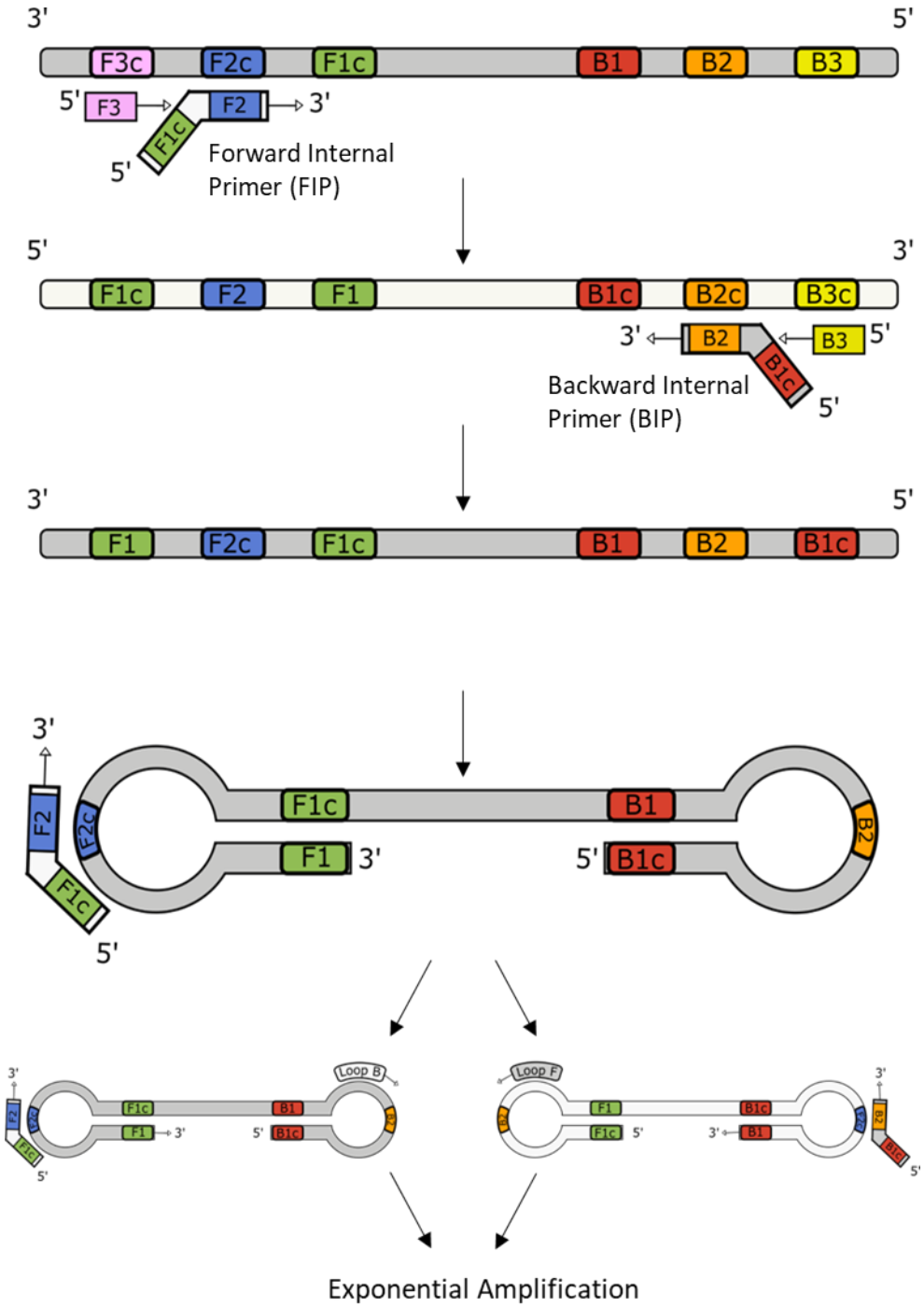
Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification technique developed by Notomi (2000). LAMP relies on specific primers and DNA polymerase with a high strand displacement activity performing continuous DNA synthesis at fixed temperatures. The technique was further optimised by Nagamine *et al* (2002) with the addition of two loop primers which increased sensitivity and further decreased the reaction time. A further modification was developed by Gandelman *et al* (2011) which involves the use of one or more "stem" primers again to improve assay performance and allow for more flexibility during primer design. Despite the benefits, stem primers do not appear to be as common in the literature as loop primers.

LAMP is well validated for both lab and field use (Notomi *et al*, 2015). It is valued for its rapidity and cheap running costs while still maintaining sensitivity and specificity comparable to other mainstream amplification methods such as PCR (Zhang *et al*, 2014). The initial applications of LAMP were in remote testing such as in Thailand where it was used to detect malaria (Poon *et al*, 2006) and for detecting MRSA in hospitals (Chen *et al*, 2017). LAMP appears to be the most popular choice of isothermal nucleic acid amplification technique in the published literature (Mori and Notomi, 2020) with a web of science search conducted by Becherer *et al* (2020) showing LAMP to be the most frequently used method (60.7%).

Mechanism

LAMP uses three pairs of primers (internal, external and loop), and a DNA polymerase with high strand displacement activity, to generate an amplification product that is capable of auto cycling DNA synthesis. Reactions do not require any prior target denaturation and are conducted at one temperature (most commonly 65°C however, assays have used commonly used temperatures between 60°C and 70°C).

Amplification is initiated by the inner primer (FIP/BIP) displacing the double stranded target DNA to bind to its complimentary 2c region. Strand displacing DNA polymerase then extends the primer and completely separates the target sequence. This first amplification product is again displaced by the



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605 Figure 2.7: Mechanism of the LAMP amplification process starting with the displacement by the
606 FIP/BIP and F3/B3 primers, followed by the product capable of forming a self-hybridising loop, then
607 the product with multiple priming sites, and finally the attachment of the Loop primers to increase
608 reaction speed which is then followed by exponential amplification.

outer primer annealing to its complimentary 3c region and the DNA polymerase. As this displacement occurs the end of the original amplification product forms a self-hybridising loop, due to the inclusion of a reverse complimentary region in the inner primer, that is open at the 3' end. This process occurs again resulting in a structure with a loop at either end, often referred to as a LAMP dumbbell structure. It is this dumbbell structure that forms the basis for the exponential amplification that LAMP is known for as it contains multiple sites for DNA synthesis (3' ends of the loop and annealing sites for the inner and loop primers). As amplification occurs from these sites the amplification products grow into long concatemers that again have more sites that can initiate DNA synthesis. This exponential amplification results in large quantities of target DNA and amplification by-products that can be used for detection. Figure 2.7 shows the LAMP reaction from initiation by the FIP/BIP primers to exponential amplification.

Despite its widespread use, LAMP assays do have limitations. LAMP has been shown to be less versatile than PCR (Ranjan Sahoo, *et al* 2016), however, its primary purpose is as a diagnostic technique which still renders it suitable for scenarios that require diagnoses rather than exploration. Proper primer design can be a constraint with poorly designed primers resulting in primer-primer interaction, false-positives, and false-negatives (Torres *et al*, 2011). There are guides available which provide best practise for primer design which should aid researchers in avoiding the pitfalls of poor primer design. Single nucleotide polymorphisms (SNPs) can also undermine LAMP assays. If a SNP is present at a primer binding site this can cause a false-negative result if the primer fails to anneal (Blaser *et al,* 2018). However, because LAMP assays tend to show similar sensitivity to PCR a successfully designed assay should not show abnormal rates of false-negatives. Similar to the previous issue, well designed primers can prevent false-negatives being a constraint.

There are also reports that false-positive amplification events are more common than are reported in the literature (Priye *et al*, 2017). However, whilst specific studies give individual false-positive rates no published literature is available that describes whether there is an underreporting issue present.

Detection of LAMP products

isothermal nucleic acid amplification techniques require a detection method to process the results of any amplification. Some detection methods can be broadly applied across a range of amplification techniques including for LAMP (Schweitzer and Kingsmore, 2001; Tanner, Zhang and Evans, 2015). However, due to differing levels of amplification product some isothermal nucleic acid amplification techniques require detection methods which are more sensitive than some of the more broadly used methods. LAMP is a highly efficient amplification method, with extremely high levels of amplification product being produced in each run (Becherer *et al.*, 2020). These high levels of amplification allow for relatively insensitive detection methods to be used. This is evidence by the formation of a magnesium pyrophosphate precipitate (a by-product of DNA polymerisation) that causes the solution to become sufficiently turbid to be visible to the human eye (Mori *et al*, 2001). It is also possible to visually detect LAMP products through either fluorescence or colorimetry which either produce a change in fluorescence or colour due to the presence of LAMP products. It should be noted that many of the fluorescent dyes require exposure to UV radiation to be seen by the naked eye therefore a UV light is required for these assays.

Because LAMP is primarily used as a diagnostic tool and is only required to produce a presence or absence result it is generally not as important to have quantitative detection methods as is the case for other amplification techniques. However, with certain LAMP applications it is often useful, and sometimes necessary, to have more information. For example, when LAMP is used to detect crop pathogens information regarding inoculum levels can influence decision making on crop management or the storage of crops (Guo *et al*, 2010). Quantitative detection of LAMP products is usually achieved through the measurement of fluorescence in real-time using specialist instruments (Genie: Optigene, Quantstudio: Thermofischer Scientific) or the measurement of colour changes.

It is possible to use melt curve analysis and gel electrophoresis as part of any post reaction analysis. Melt curve analysis involves measuring the fluorescence given off by denaturing double stranded DNA. The temperature dependant fluorescing and can be used to distinguish between species in a multiplex assay and to detect SNPs (Ayukawa *et al*, 2017).

Gel electrophoresis is a technique that uses an electrical current to push DNA through an agar gel with smaller fragments pushed further through the gel than larger fragments. The technique is primarily used to analyse PCR amplicons although, it is also possible to analyse LAMP products in a similar manner. However, opening LAMP products in a laboratory risks contamination due to the amount of DNA produced in the reaction. Therefore, it is recommended any post-reaction analysis occur either outside of a laboratory where samples are being prepared.

LAMP Variations

Since its development there have been several modifications to the original LAMP method. These modifications have usually involved integrating LAMP with other methods, such as rolling circle amplification (RCA) (Tian *et al*, 2019) and nucleic acid sequence-based amplification (NASBA) (Fukuda *et al*, 2020), to increase their reaction time however, reaction times for these techniques when combined with LAMP are often still more than several hours. Many of these variations also revolve around detection of target DNA with technologies such as fluorescence probes becoming more common in LAMP assays and still retaining high levels of specificity and sensitivity (Gadkar *et al*, 2018; Kubota *et al*, 2011; Mori *et al*, 2006)

LAMPORE is another recent example of technologies being integrated with LAMP. Designed by Oxford Nanopore the technique creates a workflow that integrates LAMP assays with their existing nanopore technology (Peto *et al*, 2021). Nanopore sequencing involves using flowcells with nanopores imbedded in an electro-resistant membrane. Each nanopore is equipped with a sensor that measures the electric current passing through it. The sensors then detect when this current is disrupted by a molecule and produce a signal unique to the molecule that has passed through. These signals can then be decoded using basecalling algorithms to produce a DNA or RNA sequence in real time. One of the limitations of nanopore is that the technique is reliant on DNA, or RNA, attaching to the nanopores to begin basecalling, which if a small initial volume of template is present can take a long time. Using LAMP to increase the volume of sequences to input into the nanopores is the primary objective of LAMPORE as adding more template increases the likelihood of template finding the nanopores.

Use of LAMP for OPM scenarios

Loop-mediated isothermal amplification (LAMP) would be ideal for this type of assay because of several factors:

1) LAMP is comparatively cheap compared to other amplification methods.
2) LAMP assays require less specialised knowledge to conduct.
3) LAMP has a quick turnaround, with results being available from 30-60 minutes depending on the variety of LAMP used.
4) Sensitivity and specificity remain comparable with other techniques.
5) LAMP assays are robust to matrix inhibitors produced by specimens.
6) Unlike many other isothermal nucleic acid amplification techniques LAMP is already well validated for use in the field.

In addition to the benefits listed, several LAMP assays have already been developed to detect pest species such as whitefly (Blaser *et al*, 2018), fruit fly (Huang *et al*, 2009; Sabahi *et al*, 2018), and *Aedes* spp. (Schenkel *et al*, 2019). These assays are routinely used at customs borders to detect the presence of their target pest on imports which allows for improved screening of high-risk products, ultimately leading to a reduced chance of pests being imported (Boonham, 2021).

Informing OPM management with LAMP would have a different overall aim. Instead of preventing a species from entering a specified area, the assay would be deployed to detect parasitism levels which would be linked to certain management outcomes e.g. high parasitism, low removal effort. And whilst the routine detection of parasitoids in pest species has not been used for IPM previously, the successful application of LAMP in similar scenarios supports the use of the approach here.

Multiplex LAMP

As with other nucleic amplification techniques it is possible to produce a multiplex assay. This refers to amplifying multiple DNA sequences simultaneously. With regards to the OPM parasitoid assay it could be argued that a multiplex LAMP assay would be “future-proofed” as the OPM parasitoid composition may change over time. Depending on how local non-specific parasitoids react to OPM populations *C. iliaca* may not continue to be the dominant larval parasitoids. This shift in parasitoid community composition has been observed in other pest

724 species such as the european corn borer (*Ostrinia nubilalis*) (Folcher *et al*, 2011). This shift in
725 community composition is impacted by typical drivers affecting non-parasitoids including
726 climate change, habitat fragmentation, urbanisation, and the use of pesticides and drugs in
727 the environment. However, parasitoids communities must also deal with the challenge of a
728 variation in host range. All of these drivers are likely to affect the OPM parasitoid community
729 so it is uncertain how stable this community will be.

730 If an assay were able to detect multiple species a more complex detection method would be
731 required to differentiate between the species. While this is feasible it adds cost to the assay
732 which potentially removes accessibility to some. There is also the question of whether a
733 multiplex assay would be cost effective. Whilst there are three other known parasitoids of
734 OPM in the UK there is only reliable biological information for *C. iliaca,* which based on current
735 knowledge is also responsible for most parasitism events. A wider knowledge of the other
736 parasitoids will be necessary to understand local site-based effects for better management.
737 However, in the short term the immediate need for data to inform management around *C.*
738 *iliaca* favours a more cost-effective assay that is simpler to develop and removes any expensive
739 post LAMP reaction analysis.

740 **Wider Applications of LAMP for invasive species and pest management**

741 OPM are part of the wider problem of invasive species which have a mean annual cost of
742 US$26.8billion to the global economy (Diagne *et al*, 2021). The management of invasive
743 species is complex and can range from aiming to eradicate a species from its invaded range to
744 doing nothing, depending on the costs involved (Epanchin-Niell, 2017; Prior *et al.*, 2018).
745 However, the ultimate goal of invasive species management is to prevent a species from
746 entering a designated area (Leung *et al.*, 2002). Whilst this is challenging there is a large body
747 of research that suggests that preventing an invasive species from establishing is more cost-
748 effective long term than controlling the same species if it established (Wittenberg and Cock,
749 2005; Hulme, 2009; Epanchin-Niell, 2017; Poland *et al.*, 2021). Prevention often involves large
750 bodies of predictive work, pathway mapping and strict checks of imports at border control
751 which is where molecular diagnostic techniques could prove to be a powerful tool (Hulme,
752 2009; Reaser, Frey and Meyers, 2020). A major flaw with these checks is that it sometimes
753 relies on security officers that would not necessarily have the expertise to identify invasive
754 species. Particularly if those species are being transported at an immature life stage which

often lack the distinct morphological characteristics of their adult forms or if the adult forms are morphologically cryptic. It is in these cases where LAMP has the potential to be most effective. The speed and simplicity of a LAMP assay means that a test could realistically be performed on site by non-specialists. One criticism of this approach is that a LAMP assay requires primers specific to the target so the target must be known prior to testing. However, many countries are already using horizon scanning to predict which species will most likely invade in the near future. High risk targets that are difficult for non-specialists to identify could have LAMP assays prepared and deployed at points of entry.

This approach is not new, a LAMP assay has been developed by Blaser *et al* (2018) to detect three important and frequently detected insect species groups at points of entry to Switzerland, a method that is still currently in use (Boonham, 2021).

Summary

This chapter has introduced a summary of the life history and ecology of OPM across northwestern Europe with a particular focus on the UK. It is clear that the increasing presence of the species is cause for concern both from a tree health and public safety perspective. Whilst traditional pest management startgies have worked in slowing the spread of OPM this is no longer sustainable long term with concerns around increasing cost and effect on non-target species. A move towards an IPM system is discussed although incorporating strategies such as biocontrol is still a challenge for land managers due to a lack of knowledge which is highlighted in this chapter. It is critical that land managers have a full suite of options available to them as many of these IPM tools will be context specific and may not be feasible for certain sites due to legislative reasons. As highlighted in this literature review, molecular tools have the capacity to revolutionise the way in which we acquire species information where this has previously been extremely difficult to achieve but they are largely overlooked in this context. This highlights the need to have accessible molecular diagnostic tools available so that land managers can i) make informed decisions, ii) add data to a larger pool of information that will allow for decision making over a larger space and a longer time frame. Therefore, the following chapters will apply industry standard validation techniques to develop a diagnostic assay followed by how this assay would be used to gather information and translate that into data that can inform management decisions.

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Chapter 3 Development of a LAMP protocol to identify the parasitoid *Carcelia iliaca* from Oak Processionary Moth (*Thaumetopoea processionae)* larval tissue to understand and enhance biocontrol management plans

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Abstract

1. Oak Processionary Moth (*Thaumetopoea processionea* - OPM) is a serious forestry pest and risk to public health in the UK. The economic and environmental cost of chemical pesticides in managing OPM has driven the need for sustainable, strategies which fit into integrated pest management frameworks, including the use of novel biocontrol methods such as conservation biocontrol.

2. *Carcelia iliaca,* a specific parasitoid of OPM, is currently the main biocontrol agent of the UK OPM population. However, basic information on *C. iliaca* life history and rates of parasitism are currently lacking, partly driven by the risks OPM pose to human health, making both study and incorporation of biocontrol into management plans difficult.

3. Here, we design and validate a molecular diagnostic assay based on loop-mediated isothermal amplification (LAMP) to detect *C. iliaca* from OPM larval tissue samples collected in the field, overcoming the challenges of studying problematic invasive species such as these.

4. To assess assay performance, diagnostic sensitivity, which was 91%, and specificity, which was 75%, are used alongside limit of detection (600 Pg). We discuss the wider applications for LAMP as a cost-effective tool for studying the natural enemies of insect pests which can be used to inform conservation biocontrol management strategies.

Key Words: molecular diagnostics, forest ecology, invasive species, Lepidoptera, Tachinid

Introduction

Oak Processionary moth (*Thaumetopoea processionea*) (OPM) is considered a serious pest across many European countries for two primary reasons: i) The threat it poses to the health of oak trees (Godefroid *et al.*, 2020) and ii) the pseudo-allergenic response by humans caused by the urticating hairs present on late instar larvae (Townsend, 2013; Marzano *et al.*, 2020).

Over the last fifty years OPM have been slowly spreading (possibly recolonising in some cases) into areas at its supposed northern range boundary, however they have also been introduced into new regions through human mediated pathways such as through trade of forestry products (Groenen and Meurisse, 2012). Across several north-western European countries, OPM populations are actively monitored and controlled through a combination of Cry protein based pesticide application (primarily Bacillus thuringiensis [*Bt*] derivatives), deltamethrin (which is only used where *Bt* is not suitable, due to its impacts on non-target species such as bees and aquatic life (Forest Research, 2022a)), and nest removal (Tomlinson, Potter and Bayliss, 2015; Forest Research, 2022a). However, the cost of conventional pest management and a desire to move towards more sustainable practises of controlling invasive species has led to calls for a more holistic approach in line with the general principles of integrated pest management (Barzman *et al.*, 2015a). Biocontrol is a key principle in integrated pest management with parasitoids being leveraged successfully to help manage Oriental Chestnut Gall Wasp (*Dryocosmus kuriphilus*) (Avtzis *et al.*, 2019), Great Spruce Bark Beetle (*Dendroctonus micans*) (Evans and Fielding, 1994), and Tomato Leafminer (*Tuta absoluta*) (Cascone *et al.*, 2015). Furthermore, biocontrol is also viewed as a vital tool for tackling ongoing and future invasions, however, lack of data around parasitoids and their host interactions still present a major roadblock (Miller, Polaszek and Evans, 2021)

In the UK, the discovery of a larval parasitoid in 2014, *Carcelia iliaca*, in a sample of OPM from Richmond Park in London prompted the potential to use this species as a biological control agent to aid in OPM management (Sands *et al.*, 2015). However, despite the long held understanding that *C. iliaca* interacts with OPM there is little known about the natural history of *C. iliaca*, nor how it interacts with the UK population of OPM, which makes it difficult to understand how to best exploit biocontrol via the process of apparent competition (Miller, Polaszek and Evans, 2021). This knowledge gap has previously been difficult to bridge as survey techniques required either the dissection of OPM nests and/or the sequencing of OPM

larvae to determine if *C. iliaca* are present (Sands *et al.*, 2015). Indeed, using a DNA-metabarcoding approach, Kitson *et al.,* (2019) found *C. iliaca* parasitism rates up to ~80% of individuals in a nest. But these techniques present significant roadblocks to accessibility and upscaling this work in that they are time consuming, require specialist facilities and training (due to the health and safety issues surrounding OPM), and present significant costs for sequencing. DNA-based diagnostic tools however have the potential to overcome these challenges and have rapidly become widespread in their usage in other scenarios, like identifying invasive pest species at the border for biosecurity and rapid diagnosis of crop pests, over a relatively short period of time (Armstrong and Ball, 2005; Darling and Blum, 2007; Darling and Mahon, 2011; Westfall, Therriault and Abbott, 2020).

There are several molecular diagnostic tests already in use that usually rely on PCR-based methods such as diagnostic PCR and sequencing for confirming species identification, and screening for target species (Zhang *et al.*, 2012; Piaggio *et al.*, 2014; Bott, 2015). However, loop mediated isothermal amplification (LAMP) has been growing in popularity as a diagnostic tool in ecology and biosecurity (Jenkins *et al.*, 2012; Agarwal *et al.*, 2020; Blacket *et al.*, 2020; Cuff *et al.*, 2023). LAMP can produce fast and accurate results, comparable to PCR, usually within 30-60 minutes, but with some assays taking as little as 15 minutes, and can be deployed in the field where necessary (Kurosaki *et al.*, 2016; Carlos *et al.*, 2017; Blacket *et al.*, 2020). LAMP achieves exponential amplification using a combination of four to six primers and a DNA polymerase with high strand displacement activity to produce structures with self-replicating sites (Notomi *et al.*, 2000). It is also possible to conduct LAMP reactions in the field using small, portable, heat blocks (such as the GENIE III (Optigene, Horsham)) as reactions only require a constant temperature (usually 65°C) to complete. Therefore, because LAMP is quick, accurate, and suitable for field-deployment, it has potential to be a powerful tool in the assessment of *C. iliaca* prevalence in OPM populations, with implications for how land managers make decisions around biocontrol. In this study, we design and validate a LAMP assay designed to detect *C. iliaca* in OPM tissue samples for the purpose of determining rates of parasitism. We do this by designing specific LAMP primers to detect *C. iliaca* and by using samples with confirmed parasitism status to validate the performance of the assay. Throughout, we discuss how this can inform management practices, with implications for other studies examining host-parasitoid interactions.

Methods

Test samples

The samples used in this study were collected as part of a pilot study conducted in Richmond Park, London, in 2018 (Kitson, J., Evans, D., Straw, N., 2019). This study froze nests at -20°C followed by dissection of nests to remove pupae and parasitoids which were then further stored at -20°C. From these samples a subset of 1000 caterpillars were used to perform DNA extractions, using the BOMB BIO TNA extraction protocol, and tested via tagged metabarcoding for the presence of parasitoids with 23% of these samples deemed parasitised (Kitson *et al.*, 2019; Oberacker *et al.*, 2019). A further subset of 88 of those DNA extractions were used in this study as inputs for the validation plate. Of these 88 samples, 70 were confirmed to be parasitised by metabarcoding and 18 were confirmed to not be parasitised. This reflects a 73% parasitism rate which is consistent with other estimates of infield parasitism rates based on nest dissections and morphological identification (Sands *et al.*, 2015; Kitson, J., Evans, D., Straw, N., 2019).

DNA was extracted from 10 *C. iliaca* larvae and 10 OPM pupae (collected as part of the pilot study) following the BOMB BIO TNA extraction protocol (the presence of the parasitoid in a larvae kills that individual before pupation therefore healthy pupae were deemed suitable negative controls) that were recovered from the nests for use as positive and negative controls (Oberacker *et al.*, 2019).

Whole Genome Amplification

Aliquots from the DNA extraction mentioned in the paragraph above were also used as inputs for a whole genome amplification (WGA) reaction. This was conducted to produce large quantities of DNA for use in the limit of detection plate. WGA was performed with the following reaction chemistry: phi29 10U/ µl, 10X phi29 reaction buffer (New England Biolabs, United States), 10X primer mix (New England Biolabs, United States), recombinant albumin 0.25 µl, 8mM dNTPs, and fill to the final reaction volume (50 µl) with nuclease-free water. Successfully amplified samples were used as the inputs for validation plates. Reactions were run in two stages, denaturing and amplification, on a PCRmax Alpha Cycler (PCRmax, Staffordshire). The denaturing stage was run for 3 minutes at 96°C followed by the amplification stage, which ran at 10°C for 4 hours and then at 65°C for 10 hours. Samples were

then quantified using a Qubit 4 (Invitrogen, Massachusetts) to determine the final DNA concentration.

Primer Design

Primers were developed using a multiple sequence alignment (MSA) approach and PrimerExplorerV5 ((C) FUJITSU LIMITED 1999-2005) (Eiken, 2012). In this study MSAs were constructed using ClustalW (Thompson, Higgins and Gibson, 1994) in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms program (Kumar *et al*, 2018). LAMP primers for this assay were designed based on alignments of published *C. iliaca* (Genbank: KT345964) and *T. processionae* (Genbank: KX049095) COI sequences. To avoid cross reaction with non-target species COI sequences from two tachinids (*Compsilura conccinata* (Genbank: OM037618) and *Zenillia libatrix* (Genbank: KX843781)) which are both confirmed in the UK and larval parasitoids of OPM were incorporated into the alignments. A third larval parasitoid, *Pales processioneae*, was allegedly discovered in the UK in 2020 based on morphological identification, however, no COI sequence was available to incorporate into the alignment (Raper, 2023). Instead, a sample of *P. processioneae* was tested using the developed primers to check for amplification. The MSAs produced in MEGA were used as inputs for PrimerExplorerV5 which generated several primer sets. These were then filtered by which sets produced successful Loop primers. Four full sets of primers were produced.

LAMP Assay

Each reaction was composed of 1 µl of 8,000 U/ml Warmstart Bst 2.0 (New England Biolabs, United States), 2.5 µl of 10x Isothermal Amplification Buffer II (New England Biolabs, United States), 1.5 µl of 100mM $MgSO_4$, 3.5 µl of 10 mM dNTP, 2.5 µl of the primer master mix (2 µm of the forward and reverse 3 primer, 8 µm of the forward and reverse IP primer, 4 µm of the forward and reverse Loop primer), 0.5 µl of 50x LAMP fluorescent dye (New England Biolabs, United States), 2 µl of template, and the final volume made up with nuclease free water. Positive controls included DNA extracted from either, *C. iliaca,* or a 1:20 dilution of OPM DNA extract that were previously confirmed to have been parasitised via Illumina MiSeq sequencing, whereas negative controls included one of a 1:20 dilution of DNA from OPM that had been confirmed as non-parasitised or nuclease-free water (Kitson, J., Evans, D., Straw, N., 2019)

Reactions were performed using the QuantStudio 5 Real-Time PCR System (Thermo Fischer Scientific, Massachusetts), with the program set to run for 60 minutes at 65°C measuring fluorescence on the Sybr channel every 15 seconds followed by an annealing phase running from 90°C to 70°C over a period of 10 minutes (total of 70-minute reaction time) to measure melt curves.

Limit of Detection

To assess limit of detection of the assay, a comparison was made between a diluted series of *C. iliaca* and a series of *C. iliaca* and OPM ratios (S4) (Forootan *et al.*, 2017). Ratios were set up with 10 ng/ µl of *C. iliaca* WGA DNA with the remainder filled with 10 ng/µl of OPM WGA DNA to replicate detecting *C. iliaca* in different quantities of OPM DNA, each well contained 20 µl. The following percentages of *C. iliaca* were used: 100%, 90%, 70%, 50%, 30%, 20%, 10%, with one row of nuclease-free DNA as a negative control (S1).

Validation

The approach taken in this study was to prepare validation samples to mimic the prevalence of *C. iliaca* that would be expected to be found across a site. The best estimates of *C. iliaca* prevalence comes from (Kitson, J., Evans, D., Straw, N., 2019), (Kitson *et al.*, 2019), and, Sands *et al* (2015), where parasitism rates have been measured in Richmond Park, London from 2014-2018. Over the course of this time period, *C. iliaca* parasitism rates were found to increase annually (2014: 31%, 2015: 36%, 2016: 59%, 2018: 63%) to the final measurement. Individual nests show parasitism levels of up to ~80% which has been taken here as the highest level of *C. iliaca* parasitism that would be expected to be present (Kitson *et al.*, 2019).

A 96 well validation plate was prepared with 88 random samples of the Richmond Park DNA extractions (70 samples confirmed as having *C. iliaca* DNA by sequencing, *C. iliaca* positive, and 18 that did not, *C. iliaca* negative) and 8 controls (4 positive (extracted *C. iliaca* DNA) and 4 negative (nuclease-free water)). The positions of samples were then randomised using base R functions v4.2.2 (R core Team, 2021) (this code is available at the following repository https://github.com/MillerK95/OPM-LAMP-assay-manuscript), to try to avoid any results being artefacts of plate set up. Plates were then tested using the final chemistry of the fluorometric assay described above.

A second validation plate was created (November 2022) to replicate the first plate. This was for three reasons: 1) to replace some depleted samples; 2) to avoid the problems of continued freeze/thaw cycles of remaining samples; and 3) to reduce the probability of contamination on the plate due to repeated handling.

Diagnostic sensitivity and specificity, positive and negative predictive values, and positive and negative likelihood ratios were then calculated to assess assay performance. These metrics are calculated using equations 1 – 6 (TP = true positive, FP = false positive, TN = true negative, FN = false negative).

Equation 1**:** Calculation of diagnostic sensitivity.

$$Sensitivity = TP \div (TP + FN)$$

Equation 2: Calculation of diagnostic specificity.

$$Specificity = TN \div (TN + FP)$$

Equation 3: Calculation of positive predictive values.

$$Positive\ Predictive\ Value = TP \div (TP + FP)$$

Equation 4: Calculation of negative predictive values.

$$Negative\ Predicitive\ Value = TN \div (TN + FN)$$

Equation 5: Calculation of positive likelihood ratio.

$$Positive\ Likelihood\ Ratio = Sensitivity \div (1 - Specificity)$$

Equation 6: Calculation of negative likelihood ratio.

$$Negative\ Likelihood\ Ratio = (1 - Sensitivity) \div Specificity$$

Two calculations of the above metrics are presented in the results. The first calculation assumes that the metabarcoding results of the validation samples is accurate. For the second calculation it was hypothesised that the metabarcoding may not have been able to detect DNA quantities below a certain threshold that a technique like LAMP could detect. Therefore, for the second calculation, any OPM DNA extracts that had been identified as negative but showed amplification in calculation one are treated as positive samples in calculation two.

This does not include negative controls that used nuclease-free water that amplified which were treated as contaminated.

Statistical Analysis

An analysis of variance (ANOVA) was performed to assess differences between mean cycle threshold (CT) for positive samples and mean melting temperature of LAMP products (MT) between validation plates, post-hoc Tukey tests were then performed to investigate groupings within the ANOVA results. This was conducted using R (v. 4.2.2) (R core Team, 2021). Both GenieIII and Quantstudio 5 outputs were analysed, tidied, and visualised using functions from Tidyverse (Wickham *et al.*, 2019) and ggplot2 (Wickham, 2016). Plotting scripts are available as a GitHub repository (https://github.com/MillerK95/OPM-LAMP-assay-manuscript).

Results

Primer Design

Four suitable primer sets were generated by PrimerExplorer V5 with three showing suitable amplification of *C. iliaca* DNA. One showed no amplification and was discontinued for further testing. Based on the results from initial testing, it was difficult to separate between the three successful primer sets so the decision was made to compare the primer sets to the multiple sequence alignment used for the input into PrimerExplorerV5 to determine which was theoretically more suitable.

Based on this comparison, the first two primer sets had primers at sites with the lowest sequence similarity across species (therefore potentially the highest specificity to *C. iliaca*) with the third primer set discarded due to higher sequence similarity. Of the remaining two primer sets, the F3 primer from the second set was hypothetically more specific than the F3 primer from the first set (S3). Therefore, these two primer sets were combined to create a final primer set called Primer Set F. The first primer set was also used as a comparison to Primer Set F, this set was called Primer Set 1. A table detailing the primers set sequences and a figure showing Primer Set F's placement in relation to the multiple sequence alignment can be found in S2 and S3.

Whole Genome Amplification

Whole genome amplification (WGA) produced eight successful reactions for both *C. iliaca* and OPM with quantities measured pre-clean-up (80.8 ng/µl - 283.2ng/µl) and post-clean-up (0.5 ng/µl – 106 ng/µl) by Qubit (Thermo Scientific, Massachusetts).

Limit of Detection Plate

The limit of detection plate (LOD plate) showed successful amplification of most of the expected wells within the assay timeframe with a range of endpoint results from 150 cycles to 225 cycles (37.5-56.25 minutes). No negative controls amplified during the run. On the LOD plate the lowest quantity of DNA to amplify within the assay duration was 600 pg. Wells F7 and G7, which contained 600 pg and 300 pg of DNA respectively, started to amplify but did not complete within the assay timeframe. This suggests that the limit of detection for this assay is around 600 pg of DNA (S4).

Validation plate

The results of three runs of the validation plates are presented here, two of the first validation plate using Primer Set F and Primer Set 1, and one on the replacement plate using Primer Set F. To ensure clarity to the reader, going forward the plates will be referred to as, i) Primer Set 1 - to refer to the first validation plate with the Primer Set 1 primers, ii) Primer Set F First – to refer to the first validation plate with Primer Set F primers and iii) Primer Set F Replacement – to refer to the replacement validation plate with Primer Set F primers.

Several samples that were previously identified as not containing *C. iliaca* (by Illumina sequencing) had *C. iliaca* amplification, with the majority amplifying later than known positives (Figure 3.1 and Figure 3.2)

Of the samples that amplified successfully, 93% amplified before 100 cycles (25 minutes) which represents an arbitrary threshold that determines whether a sample is considered a positive or not (Figure 3.2). There is a separation of samples based on melting temperature in the Primer Set F Replacement plate in Figure 3.2. Whilst this can be due to ununiform melting of DNA products (Abtahi *et al.*, 2011) the true cause was uncertain and thus these samples were removed from the diagnostic metric calculations. The plate is included in further results for comparison.

Mean CT varies across each plate although there is overlap between all three plates. This remains true even when only samples equal to or under the 100-cycle threshold are considered although standard deviation reduces. The mean CT and standard deviation is presented in Table 3.1 for all samples, and also for samples that only amplified within the 100 cycle threshold.

Positive control and OPM positive samples were consistent no matter which primer set was used, or whether samples above 100 CT were excluded (Table 3.1). This was also true for water negative controls that amplified. When all samples CT results are considered, results are less precise for OPM negative samples in CT for both Primer Set F plates. However, when the 100-cycle threshold is applied OPM negative results again show a similar level of precision to positive samples and water negative controls.

An analysis of variance (ANOVA) was conducted on the CT values after results had been filtered to contain only CTs equal to or below 100 ($p = 0.01$, $f = 42.53$ $df = 2$). A post-hoc Tukey

test showed that all three plates mean CT differed significantly from each other (p = <0.05, f = 42.22, df = 2).

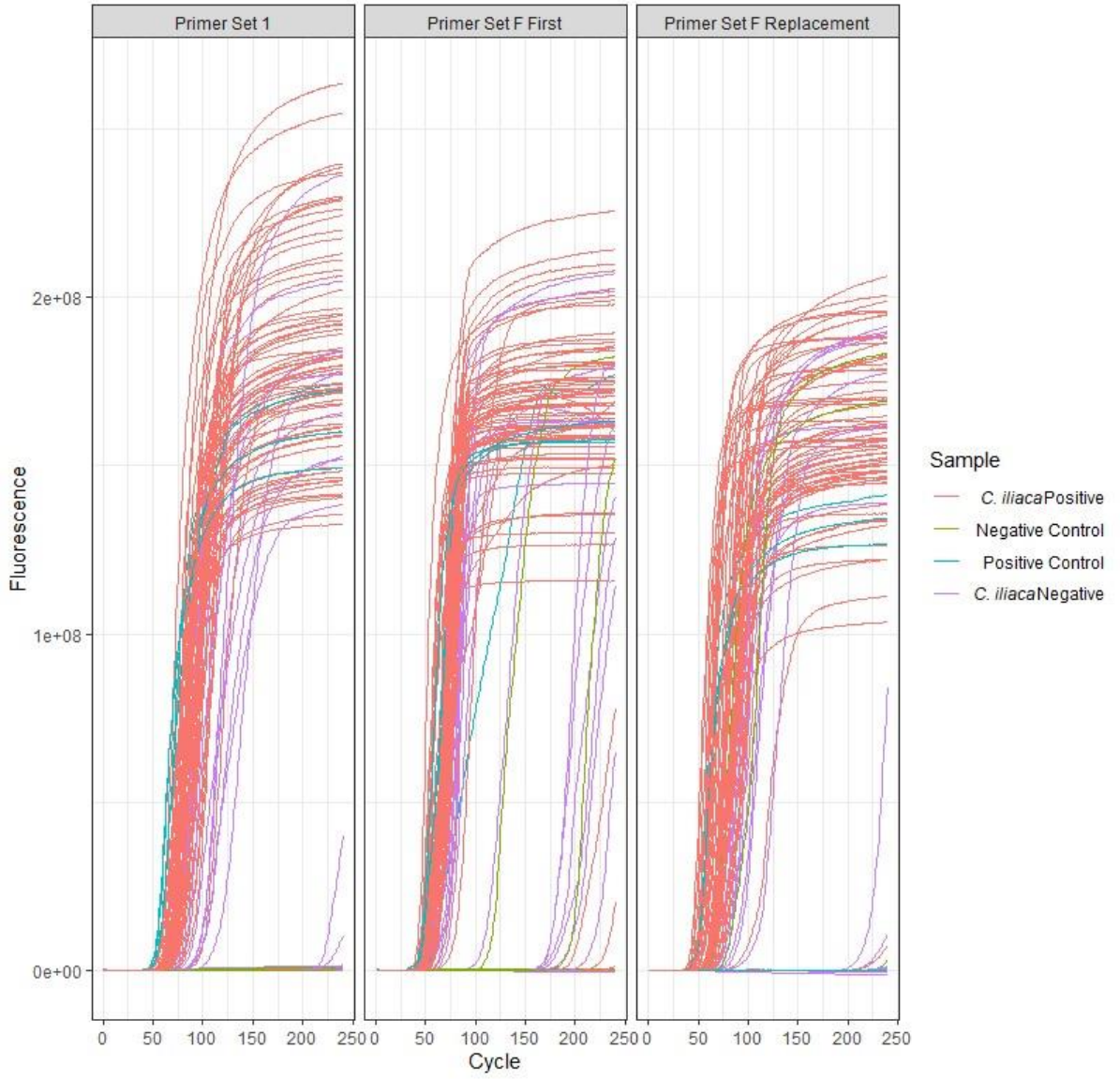

Figure 3.1: Amplification curves of the three validation plates with reaction time measured in cycles (1 cycle = 15 seconds). Red curves representing C. iliaca positive samples, green curves representing negative controls, blue curves representing positive controls, and purple curves representing C. iliaca negative samples.

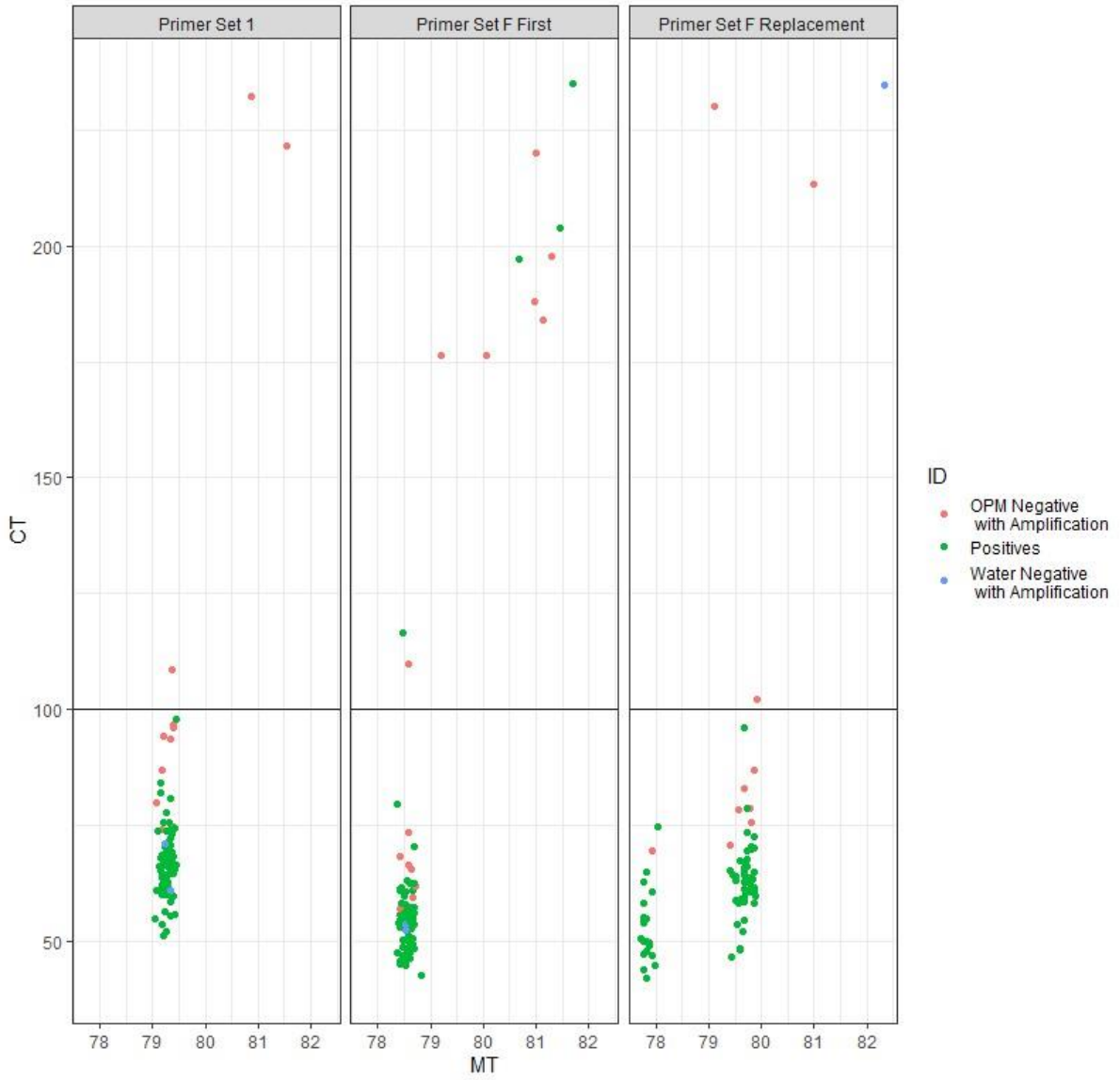

Figure 3.2: Validation plate time to positive (CT) plotted against melting temperature with a reaction threshold set at 100 cycles (25 minutes), green points represent any positive sample, red points represent OPM negative samples that showed partial or full amplification, blue points represent water negatives that showed partial or full amplification.

Table 3.1: Shows mean cycle threshold (CT) and mean melting temperature (MT) and standard deviations across the three validation plates as well as across the four sample types used in those plates.

Threshold	Sample	Primer	Mean CT	SD (±)	Mean MT	SD (±)
No 100-Cycle Threshold	Positive Control	Primer Set 1	53	1.8	79	0
		Primer Set F First	50.8	6.9	78.5	0.6
		Primer Set F Replacement	48	1	79.7	0.6
	OPM Positive	Primer Set 1	67.4	7.2	79	0
		Primer Set F First	62.5	34.2	78.8	0.7
		Primer Set F Replacement	60.9	8.7	79.4	0.9
	OPM Negative with Amplification	Primer Set 1	115	56.6	79.5	1
		Primer Set F First	122	63.7	79.5	1
		Primer Set F Replacement	109	60	79.7	0.8
	Water Negative with Amplification	Primer Set 1	66	7	79	0
		Primer Set F First	53.5	0.7	79	0
		Primer Set F Replacement	235	-	82	-
100-Cycle Threshold	Positive Control	Primer Set 1	53	1.8	79	0
		Primer Set F First	50.8	6.9	78.5	0.6
		Primer Set F Replacement	48	1	79.7	0.9
	OPM Positive	Primer Set 1	67.4	7.2	79	0
		Primer Set F First	54.5	6.2	78.7	0.5
		Primer Set F Replacement	61	8.7	79.4	0.9
	OPM Negative with Amplification	Primer Set 1	87.1	10	79	0
		Primer Set F First	64.6	5.6	78.7	0.5
		Primer Set F Replacement	77.7	6.1	79.6	0.8
	Water Negative with Amplification	Primer Set 1	66	7	79	-
		Primer Set F First	53.5	0.7	79	0
		Primer Set F Replacement	-	-	-	-

Amplification of positive samples (controls and OPM positives) for the Primer Set F First and Primer Set 1 were consistent both before and after the application of the 100-cycle threshold (Table 3.1). This is also true for the Primer Set F Replacement after the application of the 100-cycle threshold however, not prior to this. Here positives show a range of 77.7°C – 79.9°C. A similar pattern is observed in the OPM negative controls with amplification however, in this instance, it is the Primer Set F First with a range of 78.4°C – 81.3°C. Amplification observed in the water negatives is also consistent.

An analysis of variance (ANOVA) was conducted on the MT values after results had been filtered to contain only CTs equal to or below 100 ($p = 0.01$, $f = 31.27$, $df = 2$). A post-hoc Tukey test showed that the Primer Set F First differed from the other plates ($p = <0.05$, $f = 49.46$, $df = 2$). However, the Primer Set 1 and the Primer Set F Replacement did not differ ($p = 0.68$).

Repeated amplification below the 100-cycle threshold is observed in some samples despite not having *C. iliaca* present in sequencing results (S5). Most samples amplify consistently across the three plates however, there are some samples (B10, C2, D6, and G12) which show mismatches (S6).

Melting temperature of OPM negative samples is uniform within primer sets but varies across the three sets. This is shown well by the means which are 79.4°C (± 0.7°C, Primer Set F Replacement), 78.6°C (± 0.1°C, Primer Set F First), 79.3°C (± 0.1°C, Primer Set 1).

Sensitivity and Specificity

The results of the diagnostic metric calculations are presented here with Table 3.2 showing the results for both primer sets. Results are also provided for both unfiltered (all samples) and filtered (samples that amplify before the 100-cycle threshold). The Primer Set F Replacement results were not included due to the separation of Primer Set F Replacement samples in Figure 3.2 and uncertainty around the cause of this.

The Primer Set F assay shows a higher sensitivity (96%) than the Primer Set 1 assay (93%) but a lower specificity (27%) than the Primer Set 1 assay (40%). When results are filtered by the 100-cycle threshold the Primer Set 1 assay is both more sensitive (93%) and specific (47%) than the Primer Set F First assay (sensitivity 91%, specificity 40%). When samples are unfiltered the positive predictive value was higher for the Primer Set 1 assay (0.84) compared to the Primer Set F assay (0.81). However, the reverse is true when samples are filtered where the Primer Set F assay (0.88) has a higher positive predictive value compared to the Primer Set 1 assay (0.87). The opposite occurs with the negative predictive values where the the Primer Set F assay (0.67) has a higher NPV than the Primer Set 1 assay (0.64) when samples are unfiltered. However, when samples are filtered the Primer Set 1 assay (0.64) has a higher negative predictive value than the Primer Set F assay (0.50). The positive likelihood ratio is higher for the Primer Set 1 assay both when samples are unfiltered (1.55) and when they are filtered 1.80) compared to the Primer Set F assay (unfiltered = 1.34, filtered = 1.52). The negative likelihood ratio is lower for the Primer Set F assay (0.15) when samples are unfiltered compared to the Primer Set 1 assay (0.18). However, when samples are filtered the negative likelihood ratio is lower in the Primer Set 1 assay (0.15) compared to the Primer Set F assay (0.23).

Table 3.2: Comparison of Illumina Miseq results and LAMP carried out in the laboratory. Results for both primer sets on the old validation plate are presented, with Primer Set F Replacement removed due to uncertainties around buffer types. Unfiltered groups contain the full 96 samples whilst filtered results show only samples that amplified before the 100-cycle threshold. Within the results grid, upper left samples are true positives, lower left samples are false negatives, upper right samples are false positives, and lower right samples are true negatives.

Primer Set F First			Illumina MiSeq	
			+	-
LAMP	Unfiltered	+	71	16
		-	3	6
	Filtered	+	67	9
		-	7	6
Primer Set 1			Illumina MiSeq	
			+	-
LAMP	Unfiltered	+	69	13
		-	5	9
	Filtered	+	69	10
		-	5	9

Table 3.3**:** Diagnostic validation metrics for both primer sets. Unfiltered metrics are calculated from the the full 96 samples however, filtered metrics are only calculated from the samples that amplified before the 100-cycle threshold.

Validation Plate			Diagnostic Sensitivity (%)	Diagnostic Specificity (%)	Positive Predictive Value	Negative Predictive Value	Positive Likelihood Ratio	Negative Likelihood Ratio
Illumina Assumed Accurate	Primer Set F First	Unfiltered	96	27	0.81	0.67	1.34	0.15
		Filtered	91	40	0.88	0.50	1.52	0.23
	Primer Set 1	Unfiltered	93	40	0.84	0.64	1.55	0.18
		Filtered	93	47	0.87	0.64	1.80	0.15

Discussion

We present a novel LAMP assay that can rapidly detect *C. iliaca* from OPM larval samples with a high rate of accuracy for population monitoring and management in the field. The assay was demonstrated to be specific to *C. iliaca* producing no cross-rection with OPM. However, the lack of available sequences for *C. iliaca* means that it is uncertain whether the assay covers the whole range of genetic variation as only one sequence was available for primer design. As a quarter of the species of Insecta show intraspecific variation in the COI gene there is a high chance that the assay does not cover the whole genetic variation (Zhang and Bu, 2022), thus more research is necessary. Despite this, there were no perceived issues with assay performance during testing.

The LOD plate provided some useful insights into assay performance, the key outcomes being how DNA quantity is related to time to positive and the limit of detection of the assay. The real-time LAMP assay described here has an average amplification time of 10 minutes and can detect 600 Pg of *C. iliaca* DNA. The limit of detection is based on artificially prepared samples and the average amplification time is based on samples of caterpillars that were naturally parasitised and collected from Richmond Park during May and June 2018.

Regarding the limit of detection, the assay can reliably amplify 600 pg of DNA within the 25-minute cut off and shows potential to detect lower than this if amplification continues (e.g., ~300 pg). Whilst lower detection limits are usually more desirable, in this instance, having a cut off can be beneficial for interpretation of the assay (Jainonthee *et al.*, 2022). Parasitism attempts by *C. iliaca* will not always be successful but may still leave behind some trace of DNA on an OPM host. An assay that can amplify any amount of DNA may register this as a positive result and thus inflate what the user records as parasitism rate. Therefore, having a moderately sensitive assay makes the test more robust as this removes potential failed parasitism attempts and deceased *C. iliaca* larvae which would not kill their hosts. Whilst it is likely that the quantity of DNA left over from failed parasitism attempts would vary greatly, having some level of filtering will produce more accurate parasitism rate assessments and therefore, allow for more reliable decision making.

With 93% of samples on the validation plates amplifying on or before 100 cycles (25 minutes) there is a natural cut off for the assay run time (Figure 3.2). This matches current guidance

which advises for reaction times to be around 30 minutes for any NEB Bst as longer reaction times can lead to increased false positives (Aoki *et al.*, 2021; de Oliveira Coelho *et al.*, 2021; Alhamid, Tombuloglu and Al-Suhaimi, 2023). This is shown in the results presented here with several samples amplifying over 10 minutes after the threshold (Figure 3.2). As was suggested previously these results are likely false positives (Gonçalves *et al.*, 2014; Scheel *et al.*, 2014; Zou, Mason and Botella, 2020) or in the case of positive controls these samples may have lower concentrations of DNA than anticipated resulting in longer time to positive (Sherrill-Mix *et al.*, 2021). There is likely also some small occurrences of contamination due to four water negatives amplifying (Figure 3.2). This is a common occurrence in molecular laboratory work and is highlighted here for clarity rather than due to suspicion of compromised results (Weyrich *et al.*, 2019).

LAMP product melting temperature is uniform for both the Primer Set F First and Primer Set 1, however, this is not true for the Primer Set F Replacement (Figure 3.2). Whilst the variation in melting temperature is not outside of the range seen in other experiments (Tone *et al.*, 2017), the difference compared to the other primer sets was noteworthy. It is suspected that buffers used to store samples were not uniform across the plate resulting in two groups with marginally differing melting temperatures. Contamination or cross-reaction has been ruled out due to the specificity of the primers and also the similar pattern of amplification in terms of CT.

Whilst the sensitivity is high for both assays in Table 3.2 (96%, 91%, 93%, 93%) specificity is lower (27%, 40%, 40%, 47%) which is caused by the presence of several false positives. This is also reflected in the positive predictive values (0.81, 0.88, 0.84, 0.87) and negative predictive values (0.67, 0.50, 0.64, 0.64). These values suggest that a positive result for this assay is more likely to be a true value, whereas a negative result is less certain. When looking at the positive likelihood ratio (1.34, 1.52, 1.55, 1.88) and negative likelihood ratio (0.15, 0.23, 0.18, 0.15) both assays show values above one and below one respectively suggesting good assay performance (Ranganathan and Aggarwal, 2018). The differences in sensitivity and specificity will largely be inconsequential as minor changes in parasitism rate will not affect management outcomes. The major difference is that of the mean CT. The Primer Set F First set provided the fastest time to positive which will provide the most utility to landowners in producing quick results. Therefore, the Primer Set F primer set is presented as the final assay. Guidance for use

of this assay would suggest that positive results can be accepted whereas negative results may need to be followed by further testing.

The aforementioned false positives are shown in Figure 3.2 which shows samples that amplified before the 100-cycle threshold but were deemed negative for *C. iliaca* DNA during sequencing. There are three possible explanations for this, cross reaction of OPM DNA with the assay primers, contamination of *C. iliaca* DNA, or the metabarcoding was not as sensitive as the LAMP assay and missed *C. iliaca* DNA in some of the samples. Cross reaction is unlikely as these samples show the same profile, in terms of CT and melting temperature, as the samples that were deemed positive by sequencing. Amplification of another species, or primer artefact, would likely show differences in CT or MT (Ahmed *et al.*, 2017; de Oliveira Coelho *et al.*, 2021; Alhamid, Tombuloglu and Al-Suhaimi, 2023). Contamination is unlikely as false positives amplify consistently across the three plates apart from some mismatches (S6). Therefore, it is suspected that the metabarcoding samples did not accurately reflect the level of parasitism in all of the samples tested with a higher parasitism rate suspected.

Further work would need to be conducted to confirm this, however, the support for this hypothesis is that while LAMP is notoriously sensitive to small quantities of DNA, metabarcoding usually requires strict minimum DNA concentrations for reactions to be successful. High quantities of OPM DNA would mean samples would still pass the quality control necessary to pass these strict concentrations. However, it may be possible that the amount of *C. iliaca* DNA was not present in high enough concentrations in the false positives to be registered by metabarcoding. The rationale for unequal quantities of *C. iliaca* DNA between samples is that the samples were collected between May to July with Kitson *et al*, (2019) finding that the most likely time to detect *C. iliaca* in OPM was from samples between June and July. Therefore, samples with early *C. iliaca* attacks may not have sufficient DNA to be registered by metabarcoding but still be detected by a LAMP diagnostic assay. Again, further work would be needed to confirm this but if correct it would suggest that assay results are more accurate compared to the results presented here.

LAMP assays like the one presented here have the capability to provide previously inaccessible insights for integrated pest management plans, specifically biocontrol. Biocontrol is viewed as more sustainable in the hierarchy of control methods, however, its impact is often seen as unreliable due to difficulty in measuring outcomes (Barzman *et al.*, 2015b). This is particularly

prevalent with OPM and *C. iliaca* where not only parasitism rates are poorly understood but distribution of *C. iliaca* is also poorly understood. The application of a LAMP diagnostic assay would allow for clarity around both *C. iliaca* distribution and parasitism rates. Following this better understanding of parasitism rates land managers would then be able to make decisions tailored to their site, with higher parasitism rates potentially allowing land managers to maintain their current practises. Medium parasitism rates signalling that land managers should protect overwintering nests where *C. iliaca* overwinter to boost next years population. And low parasitism rates signalling that long term interventions are needed to boost parasitism rates while alternative measures are needed in the short term to control OPM populations. Further research is needed to determine exactly where the exact separation exists between low, medium, and high parasitism rates for this system. However, having any form of guidance is an improvement to the current situation land managers currently face when it comes to biocontrol of OPM. Furthermore, this situation is not unique to OPM. As sustainable pest management practises, like biocontrol, become more desirable to tackle current and future pests there is a need for more data to improve management outcomes and also give land managers more agency over the decisions they make. As we have shown here, molecular tools like LAMP have the ability to facilitate both of those needs and will be vital for tackling future invasive forestry pests.

This study provides a new molecular tool to assess *C. iliaca* parasitism rates of OPM larvae for use in biocontrol strategies. This is a first step towards generating widespread, reliable, conservation biocontrol data of a highly invasive forestry pest and public health concern. Furthermore, while LAMP has already become a regular tool in medical diagnosis (Kurosaki *et al.*, 2016), food authentication testing (Tasrip *et al.*, 2019), and invasive pest identification on imported goods (Blaser *et al.*, 2018), this work shows that there is scope for this technique to provide a similar role in conservation biocontrol. Not only would LAMP be able to provide easily accessible identification of important parasitoids it would also be able to quantify levels of parasitism through affordable large-scale testing which could feed into decision making frameworks. This data, while immediately useful from a land management perspective could also be used for a variety of functions. Primarily, to fill in knowledge gaps surrounding parasitoids and their interactions, and, to improve models of how invasive species spread once they have established (Miller, Polaszek and Evans, 2021; Wadkin *et al.*, 2022). There are a

variety of invasive species that are predicted to enter the UK and provide a threat to native tree species and trees of forestry importance (Pine Processionary Moth (*Thaumetopoea pityocampa*) and Emerald Ash Borer (*Agrilus planipennis*) (Forest Research, 2022b; Woodland Trust, 2024)) and with conservation biocontrol an increasing priority for policy makers the need for reliable data to create evidence-based strategies is more prevalent today than ever. Techniques like LAMP that can be deployed simply and cost effectively, will be integral to making these strategies work.

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Chapter 4 Application of a Novel Molecular Diagnostic Method to Detect a Larval Parasitoid in Oak Processionary Moth (*Thaumetopoea processionea*)

Introduction

Oak Processionary moth (*Thaumetopoea processionea* - OPM) is a serious forest pest across many northern European countries where it is both a defoliator of oak trees and a public health issue due to the presence of urticating hairs on late instar larvae (Townsend, 2013; Tomlinson, Potter and Bayliss, 2015; Marzano *et al.*, 2020). OPM was estimated to have established in the UK in 2005 after a breeding population was discovered in 2006 near Kew Gardens, London where it began to spread at a rate of ~2km per year until 2015 where it began to spread at a rate of ~6km per year (Suprunenko *et al.*, 2022). This expansion has seen OPM turn from a local problem into an issue for land managers across the entire Greater London area and, has in turn caused the cost of management to increase to unsustainable levels. Typically, management of OPM is achieved through the application of pesticides such as Dipel containing *Bacillus thuringiensis* var. kurstaki (*Bt*) and the physical removal of nests prior to emergence of adult moths in late summer. Both methods are expensive and as OPM continues to spread this cost continues to rise. These conventional management approaches have now been scaled back to apply a more risk-based approach which evaluates whether individual nests need to be controlled based on their proximity to public facing areas (for example children's play parks and recreational areas). However, to keep wider OPM populations at relatively low numbers and to keep in line with EU integrated pest management commitments, the UK government has sought cost effective, sustainable, alternative approaches to OPM management. One option has been through conservation biocontrol which became a viable option with the discovery of the OPM specific parasitoid *Carcelia iliaca* in Richmond Park, London, in 2015 (Sands *et al.*, 2015). *C. iliaca* is a known prominent parasitoid of OPM on the European continent however the species is data deficient in the UK with little understood about its role in UK OPM biocontrol. A report by Kitson, Evans, and, Straw (2019) details that *C. iliaca* likely lays eggs on OPM in the instar prior to pupation (around June/July in the UK) and that the parasitism rate of *C. iliaca* in Richmond Park has fluctuated from 31% in 2014 to 61% in 2018. However, to better incorporate *C. iliaca* into decision making frameworks an increased knowledge of *C. iliaca* is needed, namely how is *C. iliaca* distributed in the UK? And at what rate does *C. iliaca* parasitise OPM in the UK?

The primary barriers to answering these questions have been due to sampling issues. As *C. iliaca* is an endoparasitoid it is impossible to conduct a visual assessment of parasitism status.

Additionally, dissection of OPM larvae to confirm parasitism status is complicated by the presence of the urticating hairs which results in the need for specialist laboratory facilities and extensive protective gear for researchers. Nested metabarcoding has been used previously to investigate parasitism rates in UK OPM (Kitson *et al.*, 2019) although, the cost per sample was relatively expensive and meant it was unfeasible for large scale monitoring of parasitism. However, a recently developed assay based on loop-mediated isothermal amplification (LAMP) is cost-effective and would allow for scaled-up parasitism rate assessments across the whole of the Greater London area (Miller *et al.* 2024). It would also allow for sampling of OPM larvae outdoors with reasonable PPE rather than the specialist facilities required for nest dissection. In Chapter 3 the author detailed the development and validation of a diagnostic assay to detect *C. iliaca* DNA from whole *T. processionea* larval samples. This chapter will detail the collection of *T. processionea* samples to test with the aforementioned assay to test for the presence of *C. iliaca.* The aim of this chapter is to address Objective 2:

Objective Two: *Apply the assay developed in Chapter 3 to real world samples collected in field seasons 2021 and 2022 to assess the rate of C. iliaca parasitism for those years across London, UK.*

The specific sub-objectives of this chapter are:

- Collect *T. processionea* samples from different sites across London, UK and diagnose parasitism with the assay developed in Chapter 3.
- Determine parasitism rates for different sites across London, UK.

The purpose of this chapter is to identify the distribution of *C. iliaca* in relation to OPM and to determine parasitism rates in the UK OPM population across the Greater London area.

Materials and Methods

Samples were collected in June 2021 and in June and July in 2022. This was to obtain a set of samples that had unknown parasitism status to demonstrate the suitability of the validated assay with field samples and also to be able to assess parasitism rates from a range of sites across London. These months were chosen as previous work (Kitson, J., Evans, D., Straw, N., 2019) has shown that just prior to June and July is when most *C. iliaca* attacks take place.

Giving *C. iliaca* larvae a short time to develop would reduce the risk of the assay detecting shed DNA or failed parasitism attempts rather than a successful *C. iliaca* larvae.

In year 1 (2021) samples were collected from 10 sites across London within the M25 ring road which has been a core area of OPM infestation since the arrival of the species. A target of 200 larvae from each site, totalling 2000 larvae for year 1. In year 2 (2022) sites from year 1 were contacted for repeatability. In total 5 sites were able to provide OPM samples for a second year. To gain access to more sites, several back up sites were selected from year 1 and new sites were contacted. In addition to this, several sites were asked whether they would be able to collect samples voluntarily. This provided 7 new sites, so year 2 had a total of 12 sites including the repeated sites. Again, a target was set for this year, aiming for around 4000 with roughly ~350 larvae collected from each site where possible. Sample targets were different between years due to COVID-19 restrictions during year 1 and the want to access a wider range of sites in year 2. All sites can be viewed in Figure 4.1 and a full description of each site can be found in Appendix 1.

Figure 4.1: All sites where samples were collected as part of fieldwork highlighted by year. Contains OS data © Crown Copyright and database right 2023. Contains data from OS Open Greenspace and OS Open Built.

Sample Collection

At each location nests were identified on trees through either visual assessment or through previous knowledge of site managers. What3words was used to take a reliable location measurement for each nest which was later converted to GPS using What3words converter (what3words, 2023). History of OPM on site, control measures used, number of OPM nests, and dominant land cover were also recorded.

Sampling was conducted in AlphaTec 1500 PLUS Model 111 microgard suits with two layers of nitrile gloves that were taped at the wrists, and a face respirator to minimise exposure to hairs (Figure 4.2).

Caterpillars were removed from processions or resting clusters using plastic tweezers and placed in to individual 15 ml screw cap sterile tubes that were filled with 2 ml of GITC lysis buffer (4 M GITC, 50mM Tris HCl, 2% sarkosyl, 20 mM EDTA, 0.1% antifoam) (Oberacker *et al.*, 2019) and a 3 mm steel ball bearing, and pre-labelled using Avery resistant labels that were printed with an individual ID (Avery Products Corporation, United States) (Figure 4.3). Samples were then frozen in a portable freezer, at -20°C, prior to transportation back to Newcastle University.

Year 2 Changes

In year two the sampling procedure and methods were changed slightly due to availability of resources. Sampling in year 2 replaced 15 ml screw cap sterile tubes with 5 ml screw cap Eppendorf's (Eppendorf, Hamburg) to save space during transportation. This was not previously done as the availability of 5 ml tubes was limited during the Covid-19 pandemic. The GITC lysis buffer was also replaced with 10mM Tris-HCL of the same quantity. This was informed by amplification results presented in this chapter however, the change was also spurred by an increase in availability of resources. To obtain the amplification results several potential buffers (10mM Tris HCL, TNES, TE buffer, Tanner buffer (Appendix 2), H_2O, and the previous lysis buffer), were used to store *Carcelia* larvae (obtained from the 2018 nest dissections described in Chapter 3), at -20°C to replicate storage conditions for in transport larvae. These larvae were stored for six weeks before being defrosted and ground following the steel bead grinding protocol described in Chapter 3. Lysate produced from this grinding was then tested using the assay. Results from this experiment can be found in Appendix 3.

Finally, several sites were sampled by volunteer collectors who sampled using the same methodology set out for year 2. This was to access a wider range of sites and to get more samples than would be possible with a lone sampler. Volunteers were given 5 ml tubes in batches of 50 (depending on how many they thought they would be able to sample), which was considered the minimum number of larvae to generate robust site data whilst also being acceptable to volunteers.

Missing Samples

During transportation of samples, some samples labels became detached due to the freezing process. These samples were not tested to avoid contributing random samples to sites.

Figure 4.2: Author sampling oak processionary moth larvae in full PPE from outside of a nest in Richmond Park, June 2021.

Figure 4.3: Tubes with samples. steel beads, and frozen buffer. Samples collected from first year (Left) using larger tubes compared to samples collected in second year (Right) in smaller tube.

Data Analysis

Parasitism Rates

Prior to processing, tubes containing samples were mixed in a bag and randomly drawn out in batches of 93 for year one and 92 for year two to randomise sites across extraction runs. This randomisation was to avoid any biases occurring due to site, or plate amplification. Samples were then processed and tested using the steel bead grinding methodology and LAMP assay described in Chapter 3. Parasitism rates were calculated by using positive results to calculate the percentage of parasitism on site. These were then visualised with box and whisker plots that were generated in R (R Core Team, 2023) using *Tidyverse* (Wickham *et al.*, 2019) and *ggplot2* (Wickham, 2016) packages.

Results

Sample Collection

A total of 17 sites were visited and 3900 caterpillars were tested over two years of sampling.

Table 4.1: Sites where caterpillars were collected, dates of collection and No. of caterpillars taken from each site. – denotes where samples were unable to be taken either due to a lack of availability or because samples were not collected there that year.

Site	**No. of Caterpillars Year 1**	**No. of nests per site Year 1**	**No. of Caterpillars Year 2**	**No. of nests per site Year 2**	**Total Larvae**	**Total Nests**
Belhus Woods	173	1	93	3	266	4
Broxbourne Woods	-	-	27	1	27	1
Bullswater Common	-	-	3	1	3	1
Bushy Park	44	1	40	1	84	2
Claygate	-	-	202	5	202	5
Danson Park	25	1	-	-	25	1
Effingham Common	-	-	62	2	62	2
Epsom Common	100	1	-	-	100	1
Hampstead Heath	746	9	957	8	1703	17
Lee Valley	74	1	63	1	137	2
Merrow Common	-	-	50	1	50	1
Oaksmere Park	81	1	-	-	81	1
Osterley Park	-	-	382	6	382	6
Richmond Park	303	3	217	3	520	6
Riverside Nature Reserve	-	-	50	1	50	1
Tempsford Park	108	1	-	-	108	1
Wisely Common	100	1	-	-	100	1
Total	**1754**	**20**	**2146**	**33**	**3900**	**53**

Parasitism rate statistical analysis

T-Test

Parasitism rates for year one varied between 50%-69% with the mean parasitism across the sampled area being 60.3%. For year two parasitism rates varied between 26%-56% with the mean parasitism rate being 34.1 %.

A two-sample t-test was performed to the compare whether there was a significant difference between parasitism rates in year 1 and year 2. A significant difference was observed with the results present in Table 4.2.

Table 4.2: Mean parasitism rate across all sites for both year one and two, including standard deviation.

Year	Mean Parasitism Rate (%)	SD(±)	t	df	p-value
1	60.3	6.2	7.7374	16	<0.05
2	34.1	7			

1742 Table 4.3: Parasitism rates per site for both years of fieldwork.

Site	**Year 1 Parasitism Rate (%)**	**Year 2 Parasitism Rate (%)**
Belhus Woods	69	34
Broxbourne Woods	-	36
Bullswater Common	-	33
Bushy Park	55	38
Claygate	-	53
Danson Park	68	-
Effingham Common	-	29
Epsom Common	62	-
Hampstead Heath	57	34
Lee Valley	59	26
Merrow Common	-	29
Oakmere Park	69	-
Osterley Park	-	37
Richmond Park	56	35
Riverside Nature Reserve	-	26
Tempsford Park	58	-
Wisely Common	50	-

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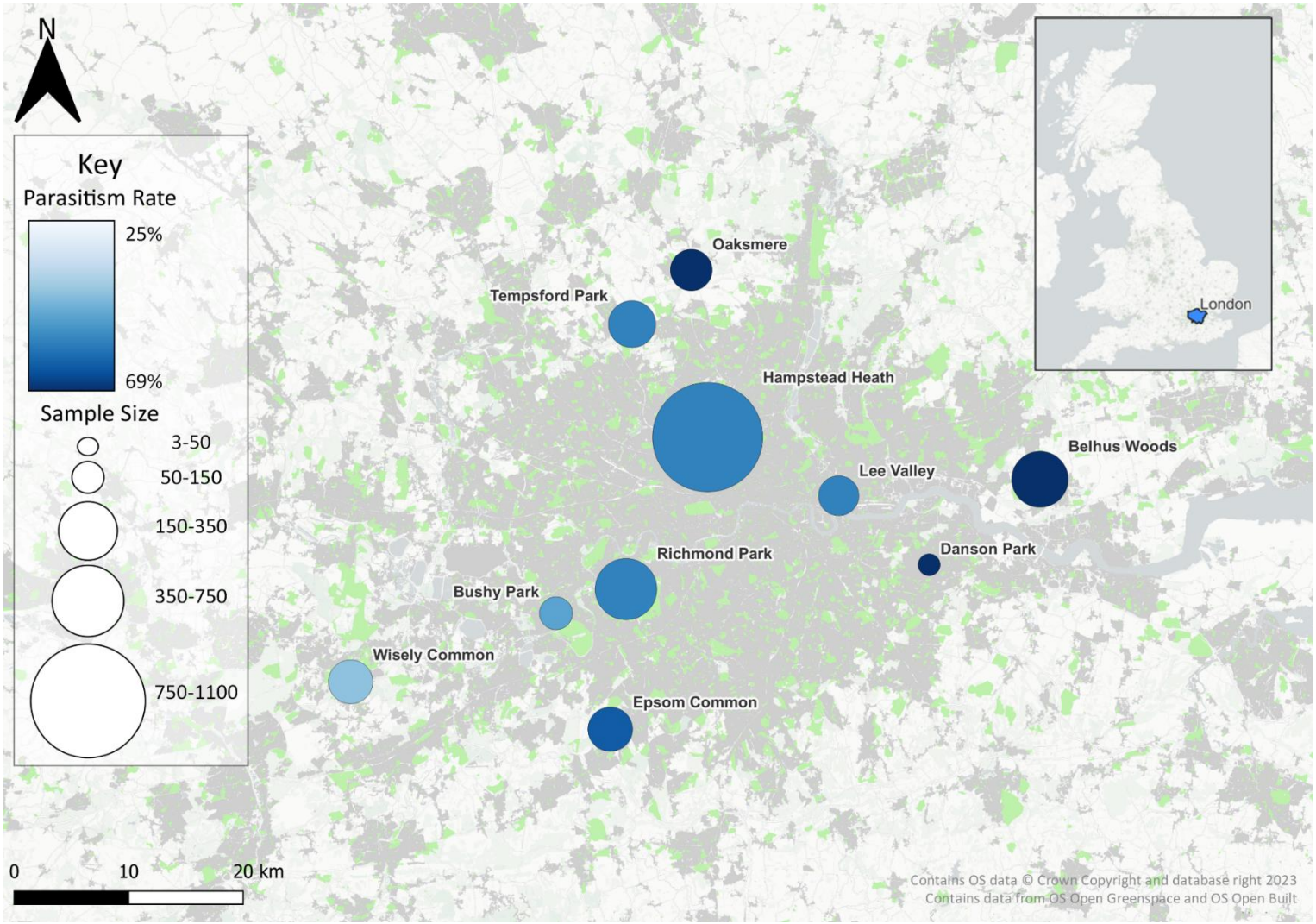

1744

1745 Figure 4.4: Map of all sites sampled in the first year (2021) with site indicator weighted by sample size and colour ramp weighted by parasitism rate. Contains

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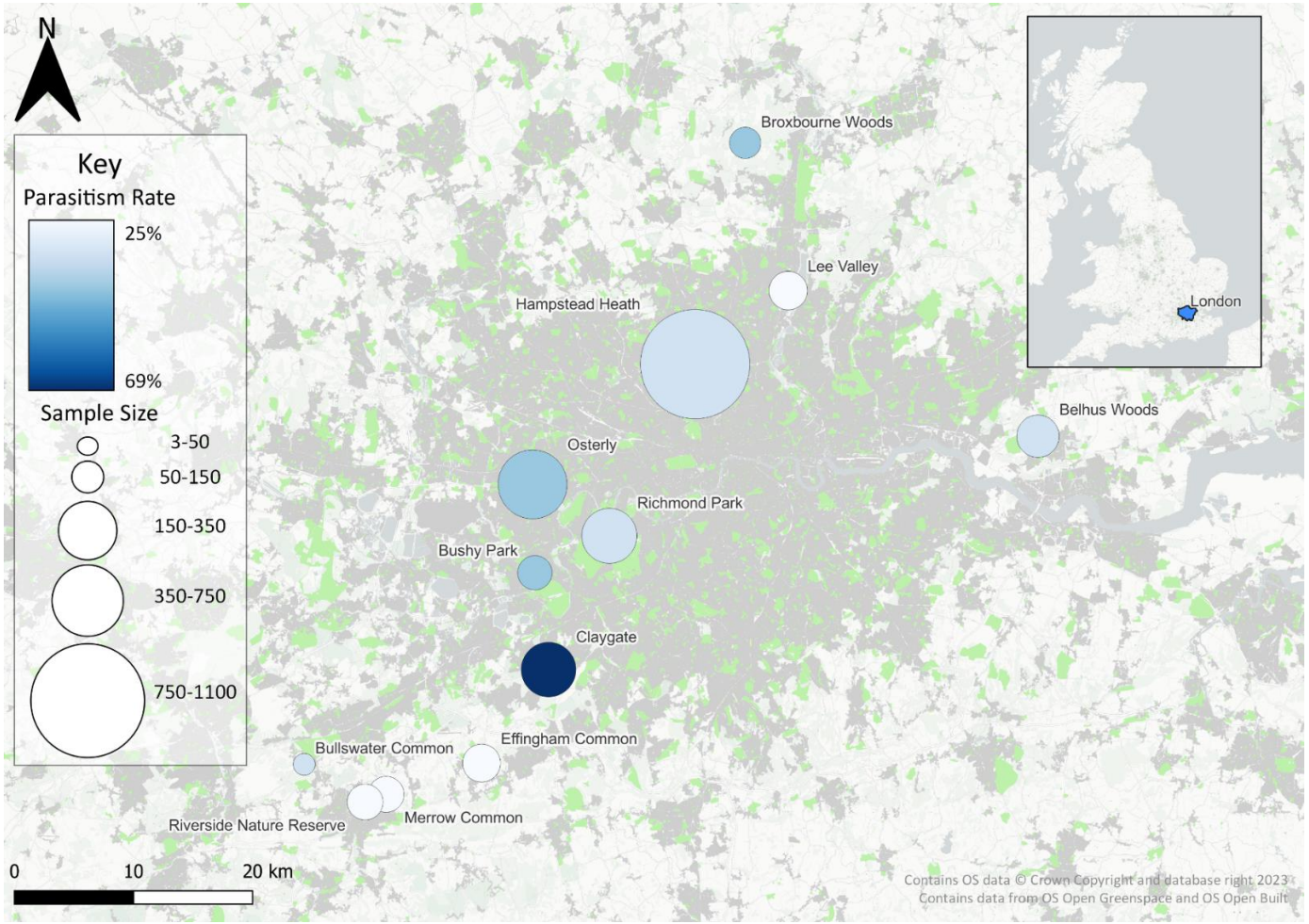

Figure 4.5: Map of all sites sampled in the second year (2022) with site indicator weighted by sample size and colour ramp weighted by parasitism rate. Contains OS data © Crown Copyright and database right 2023. Contains data from OS Open Greenspace and OS Open Built.

In year one (Figure 4.4) the highest parasitism rates (69%) can be found in Belhus Woods and Oaksmere which are sites furthest to the east and north respectively. The lowest site is Wisely Common (50%) which is the site furthest west. The most southern site, Epsom Common, has a higher-than-average parasitism rate (62%) and is further east than Wisely Common. This shows a general trend of parasitism increasing the further east a site is.

Danson Park also has a high parasitism rate (68%) however, this site has the lowest samples collected (25) for the first year and only had samples collected from one nest.

In year two (Figure 4.5) the highest parasitism rate (58%) can be found in Claygate with the remaining sites ranging from 26%-38%. There is no spatial trend in the second year with the higher parasitism rates associated with sites in the east no longer occurring. Instead Claygate has a more central positioning compared to the other sampled sites and being slightly southwest of the Greater London area.

Discussion

This study provides the first evidence of the presence of *C. iliaca* outside of Richmond and Bushy Park. Also shown are parasitism rate assessment from these sites which are again the first outside of Richmond Park. Whilst the results provide an insight into parasitism rates across a range of sites in the Greater London area there are only five sites where parasitism rates were repeated across both years. It should also be noted that one of these sites (Lee Valley), the nests sampled were taken from greater distances between each other compared to other sites (Figure 4.1). Therefore, it is uncertain how reflective of a repeat measure this truly is.

Across the five repeated sites (Belhus Woods, Bushy Park, Hampstead Heath, Lee Valley, and Richmond Park) parasitism rates decreased between years 1 and 2 (Table 4.3). Whilst there are no immediate explanations for this there are some hypotheses that might provide an answer. Firstly, OPM populations are cyclical with periods of high abundance and "outbreaks", and periods of low abundance (Townsend, 2013; Sands, 2017). Based on anecdotal evidence (conversations with stakeholders around numbers of caterpillars observed and nests removed) OPM populations peaked across 2017-2019. With similar conversations suggesting lower populations between 2020-2022. If what is being observed is the natural cycles of OPM then it is possible that there is a natural reduction of OPM populations across this time period. The reduction may also be exacerbated by the application of control measures and pressure from natural enemies as well. This population decrease will reduce the available number of hosts for *C. iliaca* which could potentially impact the reproductive success of the parasitoid.

C. iliaca Distribution

One of the initial aims at the start of this work was to explore how *C. iliaca* was distributed across the OPM established area. Initial hypothesis predicted that *C. iliaca* parasitism rates would increase with time since OPM establishment and that parasitism rates would decrease further from the initial core zone. However, these data show that this is not the case, with the highest parasitism rates being in some of the sites furthest from the core zone (Belhus Woods (69%, Year 1), Danson Park (68%, Year 1), Claygate (53%, Year 2) Table 4.3) and some of the sites closest to the core zone having average parasitism rates (Richmond Park (56%, Year 1), Bushy Park (55%, Year 1), Hampstead Heath (34%, Year 2)). What these data clearly show is that *C. iliaca* is present across all of the sampled areas of the OPM established zone. However,

it is unclear how this came to be. It is possible that lower rates further from the initial core may have occurred during the early stages of the OPM invasion and that since measurement efforts have taken place *C. iliaca* has had enough time to distribute across the OPM establishment zone. OPM females are known to disperse slowly, and tachinids are a mobile group which suggests that *C. iliaca* outpacing the spread of OPM could be a possibility. Furthermore, specialist parasitoids are well known for being able to exploit a range of cues from coevolved hosts which means they are particularly efficient at finding hosts in an environment. Although, it is uncertain what factors may influence the dynamic between parasitoid and host when both species are present in a new environment (Giunti *et al.*, 2015; van Oudenhove, Mailleret and Fauvergue, 2017). It is also possible that *C. iliaca* arrived earlier than previously thought perhaps even predating the arrival of OPM having previously been unrecorded or being a native species that shows host-dependant phenotypic differences and has just taken advantage of a new host. Investigating this was beyond the scope of this thesis however, this research is vital to understanding whether prioritising *C. iliaca* as a biological control agent could have non-target impacts.

Richmond Park Long Term Data

Richmond Park stands out as an important case study amongst the other sites as this is the site with the most years where parasitism rate has been assessed. Whilst a range of methods to assess parasitism rates have been used (nest dissection, metabarcoding and diagnostic assay) it is interesting to note that there has been an increase in parasitism to 2018, when OPM populations anecdotally peaked and the measurements after this date have sequentially decreased. The final parasitism rate assessment in 2022 (35%) stands out as this is a similar recording to the first two parasitism rate measurements in 2014 (31%) and 2015 (36%) (Kitson *et al.*, 2019; Kitson, J., Evans, D., Straw, N., 2019). It should be noted that data are missing for 2019 and 2020 due to no measurements being taken during these years so it is uncertain whether the observed decrease in parasitism rate was gradual or rapid.

This pattern of parasitism rate increasing from 2014 to 2018 and then decreasing over the next two measured years lines up with general observations around OPM, and other processionary moth, population dynamics. That being, where moths of this group will experience an increasing population for up to 10 years before suffering a population collapse (Battisti *et al.*, 2015; Blaser *et al.*, 2022). This pattern has been noted in the UK also where

anecdotally, OPM abundance has fluctuated in line with the parasitism rates presented here. However, while this data has answered questions on *C. iliaca* distribution and continued to show how parasitism rates change in Richmond Park, it is still largely unclear how the dynamics between OPM and *C. iliaca* play out across the whole OPM established zone, particularly at the expansion front.

As the management of OPM is shifting from relying solely on pesticide application and nest removal to a more holistic strategy, it is important that these tools remain evidence based. The data here has clearly shown that *C. iliaca* parasitism rates vary geographically and temporally which creates uncertainty for land managers who are being asked to rely on biocontrol. To truly utilise *C. iliaca* and other parasitoids a further understanding of OPM/*C. iliaca* dynamics is necessary to build a comprehensive understanding of when conservation biocontrol will have the most impact on OPM populations and when other interventions may be necessary. The first stage to addressing this would be long term monitoring of *C. iliaca* and other OPM parasitoids, such as *Pales processionea,* on sentinel sites where differences in management and control practises can be accounted for. Further work would likely need to incorporate the wider network of interactions surrounding OPM and its parasitoids in order to fully understand this system. Sites such as Richmond Park and Hampstead Heath that have the beginnings of long-term data would be ideal to begin answer these questions and should also further highlight the importance of long-term data in ecology when attempting to develop evidence-based and effective conservation biocontrol strategies.

Habitat Availability and Manging for Nature

Tachinids are well known for using floral resources, particularly Apiaceae, for nutrition (Skaldina, 2020). We hypothesised that this would also be true of adult *C. iliaca* based on the knowledge that adult *C. iliaca* emerge from OPM nests during the early spring, before OPM larvae begin to hatch in late spring, and well outside the range in which most *C. iliaca* attacks have been noted on OPM larvae (June-July), and thus must require another resource to survive (Sands, 2017). Therefore, we expected that sites with a higher cover of wildflowers would have higher parasitism. However, when tested via GLM (S1) there was no link between parasitism rate or any form of dominant habitat cover. There were also no links between other variables measured. While these data were unable to identify any pattern between *C. iliaca* and its habitat variables, a study that could unravel these would be important due to the

potential management implications. Apiaceae are particularly common in the UK and are also commonly used in restoration projects meaning they are an abundant resource (Skaldina, 2020). (Evans and Kitson, 2020) showed that metabarcoding both adult pollinators and flowers can provide complementary information for a complete understanding of the dietary breadth of *C. iliaca.* This information would help land managers develop strategies to boost *C. iliaca* survival post emergence, it would also have the knock-on effect of helping other pollinator species which are notoriously in decline (Potts *et al.*, 2010).

Another factor that should be considered in management plans is the effects of control measures on parasitoid populations. There is little data surrounding this and while pesticide application may occur early enough (larvae are sprayed in the first two instar) to not directly affect parasitoids the reduction in available hosts for breeding may indirectly reduce populations of parasitoid. The effect of nest removal is even less clear although it could be hypothesised to have a negative effect if nests are removed at the end of the summer as parasitoids are known to overwinter in nests before emerging in the spring (Sands *et al.*, 2015)

Natural Enemy Behavioural Response

Understanding how adaptable OPM is in responding to natural enemies is important for a variety of reasons, however in a biocontrol context it is vital in understanding how sustainable some options are long term. Whilst answering this was beyond the scope of this study there are several anecdotal points that are worth preserving in the literature to highlight where future work on this topic could be directed.

In both 2021 and 2022 OPM was considered to have “good years” with monitoring teams at Richmond Park, and Hampstead Heath, all reporting lower nest and procession sizes, and fewer nests and processions overall (Royal Parks, pers comm, 2022; City of London, pers comm, 2022). This was hypothesised to be linked to the poor spring weather, with 2021 having a colder than average spring (Met Office, 2022d), and 2022 having late spring frosts (Met Office, 2022e), both of which are linked to poor fitness of OPM larvae (Groenen and Meurisse, 2012; Godefroid *et al.*, 2020). However, a previously undiscussed hypothesis, in this scenario, is the effect of both parasitism and predation affecting what was previously a naïve population that had not encountered *C. iliaca* since its establishment. This work has presented data which shows *C. iliaca* is present and parasitising UK populations and previous work by Kitson *et al*

(2019) shows that this has been the case since 2014 based on our understanding of UK Tachinid taxonomy. There are also a number of known avian predators of OPM which have previously been recorded in Europe (Sobczyk, 2014). Some of which are now anecdotally attacking OPM processions and nests in the UK. This reported predation comes from a range of species including several members of the Paridae family (although reports are primarily great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) and the greater spotted woodpecker (*Dendrocopos major*) (City of London, pers comm, 2022). The behaviour noted is similar across both Europe and the UK with species predating by either taking larvae directly from processions or pecking holes in nests to grab larvae (Sobczyk, 2014; Nicoll, 2022).

Whilst there has been no direct evidence to prove that OPM are changing their behaviour in response to these new pressures it is possible that smaller populations and smaller nest sizes are an attempt to avoid being detected by natural enemies. There is lack of data across Europe surrounding this which makes comparison difficult however, this has been noted in other systems where population sizes can be reduced due to impacts on fitness and migration (Peacor *et al.*, 2013; Sheriff and Thaler, 2014). As previously mentioned, answering this was beyond the scope of this study however, understanding how OPM is responding to natural enemies is important for multiple reasons. This includes, how effective certain biocontrol agents will be long term, how important will OPM become in the food network at a local and landscape scale, and will OPM facilitate the settling of other species that would prey on OPM in their native range that are becoming increasingly common in the UK such as the Eurasian Hoopoe (*Upupa epops*).

Caveats and Future Work

There are several caveats that need to be considered as part of this work. Several sites have taken samples from one nest and thus also only from one tree. There are potential spatial biases occurring here particularly if large sites have OPM across the entire range meaning parasitism across the whole site may differ from that one recording. Some sites also have low sample sizes that reduce the confidence in the accuracy of the parasitism rate. This is the result of some of the sampling limitations mentioned prior and could not be mitigated for in some circumstances so should be considered when interpreting results.

Whilst this work has started to provide insights in how *C. iliaca*/OPM dynamics there are still fundamental questions surrounding these species that need answering. For instance, understanding,

- How these species fluctuate on a long-term basis?
- Can *C. iliaca* populations can be influenced by management practises?
- What do host-parasitoid dynamics look like at the OPM expansion front?
- Will the potential arrival of other parasitoids from OPM's native range displace *C. iliaca* as the predominant parasitoid of OPM?

Conclusion

This chapter has demonstrated the application of a specific LAMP assay to gather distribution data of an OPM parasitoid from field samples of OPM larvae. *C. iliaca* were found to be distributed across a large portion of the OPM established zone, however, it was not possible to access sites at the very boundary where OPM will be expanding from. Sampling took place across two years with five out of seventeen sites being repeated across both years. Parasitism rates decreased significantly from the first year's sampling to the second although it is uncertain why this has been the case.

The phenomena of positive and negative gradation periods in UK OPM population dynamics appears to be backed up by the limited data although this is far from conclusive and further work is required to truly understand the mechanisms behind OPM, and subsequently *C. iliaca,* population fluxes. What is clear however, is that natural biocontrol is currently not being facilitated by the habitat available. This is yet another signal that woodland health remains fragile despite ongoing efforts to allow our ecosystems to function in their natural manner.

The work conducted with this chapter has met objective two which was laid out at the start of Chapter 1.

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Appendix

Appendix 1 – Site Descriptions

Richmond Park

Richmond Park is a 2500-acre Royal Park located in the London borough Richmond-upon-Thames. The park is designated as a SSSI and SAC, and is made up of a variety of habitats however the dominant habitat types are broad-leaved deciduous woodland, scrub, improved grassland and dry grassland. There are also small water bodies present on site as well as a 40-acre Victorian woodland plantation. The park has a long history of use for livestock grazing and collecting firewood, which has shaped the current habitats present in the park. Grazing pressure is still present in with large populations of Fallow and Red deer keeping many areas open alongside mowing from the managing authority. Pollarding also still occurs as part of the Royal Parks management. The combination of pollarding and grazing results in several areas across the park that can be categorised as wood pasture.

The site has several tree species however, Oak species (*Robur, rubra and cerris*) and Birch (*Betula*) are dominant across its woodlands and wood pasture.

OPM have been present at Richmond Park since… and no current estimation of their population is currently available. Due to the SSSI status of the park there has been minimal pesticide application therefore, management of OPM has primarily been through nest removal. However, despite intervention OPM are still common throughout the park.

Carcelia were confirmed on site in 2015 through molecular identification (Sands *et al*, 2015). Very little is understood about the distribution and dynamics of *Carcelia* throughout the park. However, nest dissections have been used to record parasitism rates in 2014 (31%), 2015 (36%), 2016 (59%), and 2018 (67%) (Kitson, J., Evans, D., Straw, N., 2019). Meaning this is the only site with a data set resembling a time series.

Bushy Park

Bushy Park is Londons second largest Royal park after Richmond covering 1000 acres. Similar to Richmond park, Bushy Park shows historical evidence of use as pasture and herds of Fallow and Red deer continue to apply grazing pressures. The habitat composition of Bushy Park is fairly similar to Richmond with areas of broadleaf deciduous woodland and several important

grassland habitats common. Areas between woodland and grassland show tree density consistent with that of wood pasture. Many of the paths through Bushy park are lined with Oak avenues, a feature which is not present at Richmond Park. A large number of trees across the park are considered veteran or are large sources of deadwood which contributes towards its SSSI status. Bushy Park also has a series of waterways that connect several bodies of water and facilitate the presence of wet grasslands and reed beds.

Because of close proximity to Richmond Park OPM have been present for a similar length of time. Also because of its SSSI status similar management approaches have been taken. Again, despite these interventions OPM are still prevalent on site. It is currently uncertain whether *Carcelia* are present at Bushy Park.

Chobham Common Roundabout Car Park

Chobham Common is the largest National Nature Reserve in the south east of England and is managed by Surry Wildlife Trust. The site is also designated as a SSSI, SAC, and, SPA. The area is dominated by lowland heath with large areas of lowland fen interspersed between, however the area immediately surrounding the Roundabout Car Park is more characteristic of semi improved grassland with several large trees of varying species (*quercus*, *fagus*, *Betula*).

OPM have been present on Chobham Common since 2019 with nest removal being the only form of control used. Little is known about the dynamics of OPM at Chobham Common in part due to their recent habitation of the site, this also lends uncertainty as to whether *Carcelia* are present on site with no visual records of Tachinids attacking processions available.

Epsom Common

Epsom Common is a 176 hectare Local Nature Reserve managed by Epsom and Ewell Borough Council. The Common is comprised of a mosaic of habitats including open grassland, relict heathland, open waterbodies, scrub and woodland, which contribute towards its SSSI status. Grazing had been absent from the site prior to 1996 which had led to successional development covering many of the previously open areas. Due to fears of biodiversity loss a grazing project was implemented in 1997 which restored many of the open areas now present.

Control at Epsom Common has been limited to nest removal only. The SSSI status of the area has meant that applications to apply pesticides have been denied. Recently the management

authority at Epsom Common have taken the decision to reduce the level of OPM control they do, focussing primarily on low hanging nests close to paths, benches, and other areas with close proximity to the public.

OPM have been present on Epsom Common for around 7 years and despite interventions are still common. There has been no official confirmation of *Carcelia* however, there has been anecdotal evidence of Tachinid-like flies hovering and "attacking" processions which may indicate the presence of *Carcelia* or another parasitoid.

Hampstead Heath

Hampstead Heath is 275-hectare open space 4 miles outside of the centre of London, managed by the City of London Corporation (The City). The site is a mosaic of mown grasslands, remnant heath, open water and woodland. Two separate sections of woodland (Ken Wood and North Wood) are designated as one SSSI (Hampstead Heath Woods) based on the maturity of the woodland and suitability for a wide range of insects species. The woodlands on site are dominated by Sessile oak (*Quercus petraea*) and Beech (*Fagus sylvatica*) however, *Quercus robur* is also common across site.

A variety of control measures have been implemented however, the primary method of control has been the removal of nests by contractors. The other measure

OPM have been present for around 9 years and continue to persist despite interventions, *C. iliaca* have been confirmed at Hampstead Heath for a similar time frame.

Oaksmere Park

Oaksmere park is the former grounds of Grade II listed Oaksmere House (now The Oaksmere Restaurant Pub). The park covers 6.85 hectares, is described as having a "classic 19th century layout" and is primarily mown lawn with specimen trees lining pathways. There are also two large artificial lakes in the centre, a childrens play area, various outdoor sports (football/basketball) equipment and a picnic area. Due to the historical use of the park there are a wide variety of ornamental tree species on site however, *Quercus robar* is abundant on site with several individuals lining pathways or standing in small groups.

At the time of the 2021 sampling OPM had only just been reported at Oakmere therefore there have been no previous control measures implemented. However, the managing

authority confirmed nests discovered this year would be removed rather than sprayed with pesticide. In addition to no prior control measures being used it is uncertain is *Carcelia* is present in the recent Oakmere OPM population.

Tempsford Green

Tempsford Green is an urban outdoor sports facility that is primarily made up of football pitches. There is however, a planted barrier, that blocks a view of the A1 motorway, with both mature and recently planted young trees. The composition of this barrier is mixed with oak, beech, birch, and hawthorn

OPM have been present at Tempsford Green for 2 years at the time of sampling. Due to the low numbers found by surveyors only nest removal has been used as a control measure on site. Due to the short presence of OPM on site it is uncertain if *Carcelia* is present.

Belhus Woods

Belhus Woods is a country park located in Aveley, South Ockendon and managed by the Thames Chase Trust. Formerly the grounds of the stately home Belhus Woods are now part of the Thames Chase Community Forest which was founded in 1990. Belhus Woods itself covers 300 hectares and is a mixture of working and ancient woodland, wildflower meadows, open grassland and lakes. The woodlands are mainly dominated by pedunculate oak, field maple, hazel and hornbeam.

The management authority at Belhus Woods have employed both spraying and nest removal to control OPM.

OPM have been present at Belhus Woods for around 7 years and continue to persist despite interventions. There has been no confirmation of *Carcelia* on site at the time of writing.

Lee Valley

The Lee Valley site is a small urban green space between Wharfside Road, London and the River Lea, just north of the Bow Creek Ecology Park. The site is managed by the Lee Valley Regional Park Authority and is used for a variety of outreach activities. Only a handful of semi-mature oak trees are present at this site with the remaining non-artificial surfaces a small sections of wildflower meadow.

As of 2021 OPM had been present on site for around 5 years with pesticide application the main form of control used on site. Due to the lack of suitable resources the population is unlikely to grow on this site and rather use the site as a corridor to move through London. Anecdotal evidence suggest that tachinid-like flies have been seen attacking processions however, no formal assessment has been conducted to confirm the presence of *Carcelia*.

Danson Park

Danson Park is a public park in the London Borough of Bexley, located between Welling and Bexleyheath. The park is 75 hectares and is the second largest public park in the borough

Danson Park is primarily mown grassland with trees present either as small copse or as linings along paths. There is a mix of tree species on site including *quercus robar.*

As of 2021 OPM have been present on site for around a year. Both pesticide application and nest removal have been used on site and the population has remained low despite the suitable habitat present. No formal assessment of *Carcelia* has been conducted on site.

Appendix 2 – Tanner Buffer Ingredients

Table 4.4: Reagent list for tanner buffer preparation.

Reagent	**Concentration**
(NH4)2SO4	10mM
KCl	50mM
MgSO4	8mM
Tween-20	0.10%

Appendix 3 – Buffer Test

Methods

Out of the six potential buffers only two (Tris HCL and TE) showed signs of amplification, with a third (TNES) showing some level of amplification but not what is considered a complete reaction.

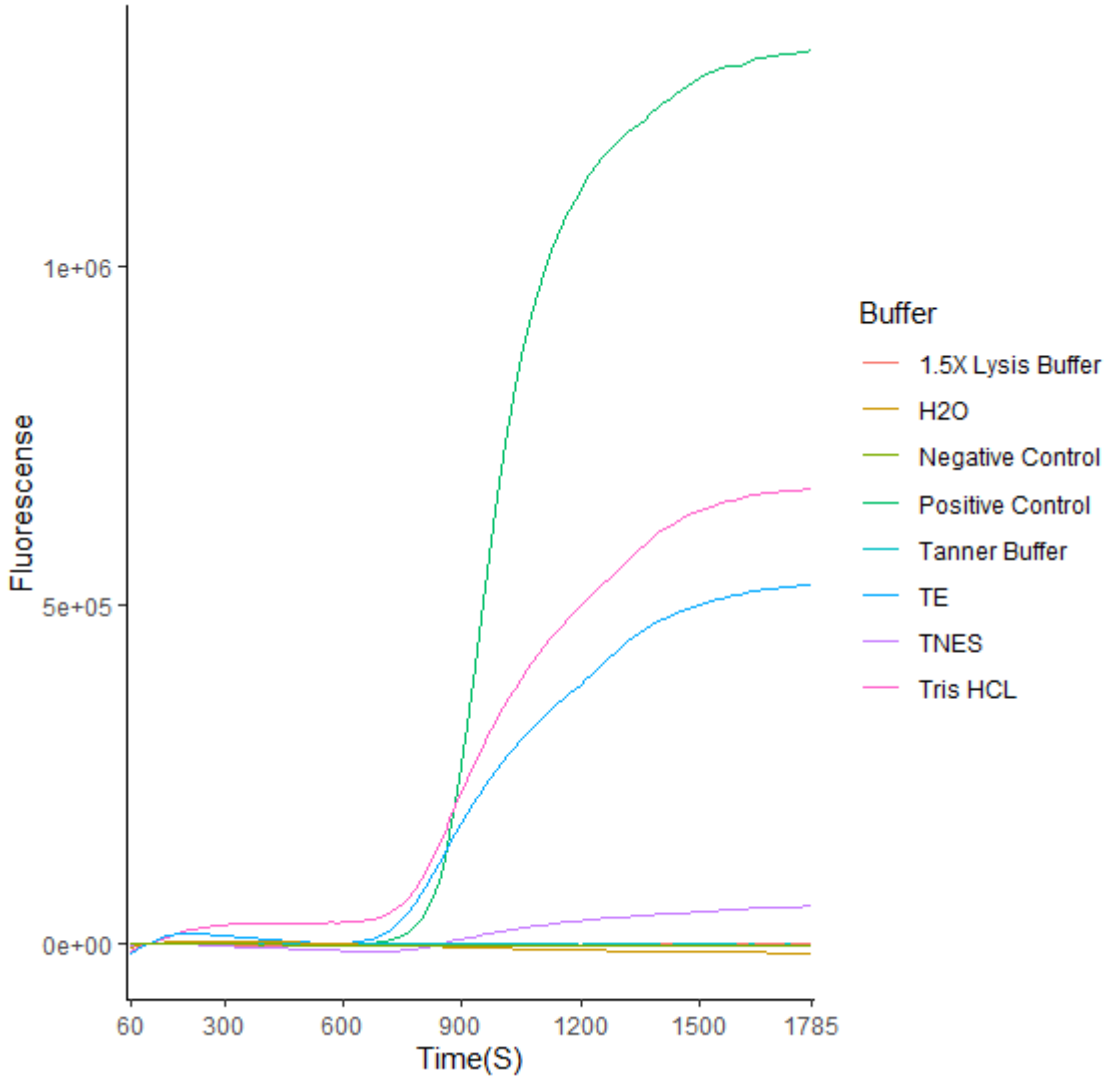


Figure 4.6**:** Amplification curves of six potential buffers to be used for storing field samples.

Discussion

The aim of this experiment was to understand which would be most suitable to retrieve DNA from after a period of freezing. The samples stored in this experiment were stored for six

weeks which was longer than would typically be anticipated. However, a longer time period was chosen to ensure each buffer was suitable in case samples remained frozen for longer than anticipated. Both 10mM Tris HCL and TE showed full amplification of products after being defrosted, whilst TNES showed some partial amplification. However, in this instance 10mM Tris HCL and TE appeared most suitable, with 10mM Tris HCL being chosen for the second years samples due to lab availability (Figure 4.6).

Chapter 5 Using Tagged Nested Metabarcoding to Classify Microbiome Differences in Healthy and Diseased Oak Processionary Moth (*Thaumetopoea processionea*) Lavae

Acknowledgements

Extracted DNA samples used in this chapter were prepared by Dr. James Kitson as part of a previous project investigating the fungal microbiomes of OPM larvae (Kitson, J., Evans, D., Straw, N., 2019). This chapter contains further molecular work to these samples as well as a new bioinformatics analysis of this work and a re-analysis of the fungal data conducted by Dr James Kitson.

Introduction

The damage caused by invasive pest species to the global economy is estimated to cost around US$423 billion and this cost is predicted to rise as there appears to be no saturation of invasive species worldwide (Seebens *et al.*, 2017; IPBES, 2019; Diagne *et al.*, 2021). This rising economical cost and pressure on resource production systems means that invasive pest species are an increasing priority for management. While pest species have been managed in one form or another for thousands of years, the typical form of management for these species currently resides in the form of chemical pesticide application. Whilst effective, there have been two primary drivers that have resulted in an ever-decreasing arsenal of pesticides for farmers and land managers. Firstly, regulation and thus removal of products that have been deemed to be environmentally unsafe and, secondly, the cost of discovery of new products has increased drastically resulting in fewer new products making it to market (Hillocks, 2012; Jess *et al.*, 2014; Matthews, 2015). This combination of factors has resulted in prolonged application of a small suite of pesticides which increases the risk of resistance forming to these products (Hillocks, 2012; Kumar, 2012).

This concern has prompted calls for a diversification of control methods with pushes for more effective biocontrol options amongst this. Biocontrol is often thought of as the use of parasitoids and predators however, leveraging microbial entomopathogens to control species also falls into this bracket. This approach is not novel, with many major products utilising entomopathogenic fungi, bacteria, and viruses however, the most sought after commercial microbial products are those based on the gram-positive bacteria in the genus *Bacillus* (Rastegari, Yadav and Yadav, 2020; Kumar *et al.*, 2021). *Bacillus thuringiensis,* abbreviated to *Bt*, has provided the most successful microbial pesticide to date with over 130 products that account for around 90% of biopesticides sold which target Lepidopterans, Dipterans, Coleopterans, Hymenopterans, Homopterans, and Mallophaga (Sanchis, 2012; Glare, Jurat-Fuentes and O'Callaghan, 2017; Jallouli *et al.*, 2020).

However, as with traditional chemical pesticides the over reliance on certain strains of entomopathogens adds to concerns around resistance to the products. For instance, the first reported example of resistance occurring in *Bt* products occurred in the early 1990s. In this instance, *Plutella xylostella* larvae (Lepidoptera: Plutellidae) showed resistance to Dipel 2X (Abbot Laboratories, Ilinois), which was one of the first commercial formulations of the HD-

012 strain of *Bt* (Melo, Soccol and Soccol, 2016). Since then, there have been several cases of increased resistance across a range of insects, with measures attempting to prevent resistance ranging from limiting application (Hillocks, 2012) to creating plant cultivars that produce toxins from two different strains (Bates *et al.*, 2005).

Despite this, resistance management has had limited results which means there are now constant efforts to find novel species and strains of already effective entomopathogens. In spite of *Bt* being the most prominent control method there are concerns around the specificity of *Bt* var. *kurstaki* as whilst the pesticide is Lepidopteran specific, OPM share similar habitats in the UK with species of conservation concern such as purple hairstreak (*Favonius quercus*), and, purple emperor (*Apatura iris*), whose larvae could suffer lethal effects due to *Bt* application. This identifies a need for a species specific biopesticides to maintain current levels of control without the potential impacts on other species.

During prospecting for biocontrol agents at Richmond Park, London, in 2018 several diseased caterpillars were discovered (Kitson, J., Evans, D., Straw, N., 2019). These caterpillars were characterised by black necrotic tissue and were consistently found outside of pupal chambers. The lack of healthy caterpillars discovered at the time of nest collection implies that either all caterpillars within the nest succumbed to the disease or the remaining caterpillars developed into pupae and subsequently adult moths. It was proposed that the cause of the disease status was entomopathogenic fungi. To assess whether fungal pathogens were the cause, DNA was extracted from both diseased and healthy caterpillars using the protocol of (Oberacker *et al.*, 2019). DNA was then amplified using a protocol based on the work in (Kitson *et al.*, 2019). Samples were sequenced at the NUomics facility at Northumbria University using Illumina MiSeq (v3 chemistry). Assessment of the fungal communities between diseased and healthy caterpillars showed no difference in community composition. This suggests that either a different group of pathogens is causing the diseased status or there is a coinfection occurring that wasn't identified by analysing fungal communities alone.

The most likely cause of the diseased status is an entomopathogenic bacterium which presents two scenarios. Either the diseased caterpillars are caterpillars that have been treated with *Bt,* which is the only biopesticide licensed to control OPM, or a novel entomopathogenic bacteria is present. In the case of the latter scenario, it is important to identify the species to

determine whether it can be leveraged as a biopesticide to control OPM. The aim of this chapter is to address thesis Objective Four:

Objective Four: *Use DNA metabarcoding to better understand the fungal and bacterial communities associated with healthy and diseased OPM larvae to determine whether there are novel entomopathogens that could be used for OPM management.*

The specific sub-objectives for this chapter are:

- Optimise a PCR workflow to amplify bacterial DNA extracted from diseased OPM caterpillars.
- Analyse the bacterial communities associated with diseased OPM caterpillars.
- Compare the bacterial communities with an existing data of fungal communities associated with diseased OPM caterpillars.

The purpose of this chapter is to analyse bacterial communities associated with diseased and healthy OPM caterpillars and pupae, and to compare these results with a previous fungal analysis. This would show whether the diseased status in these caterpillars is caused by *Bt* or whether there is a novel cause to this status. This analysis might also elucidate whether there is a coinfection between bacterial and fungal pathogens, with fungal species potentially increasing the pathogenicity of entomopathogenic bacteria.

The results of this chapter will be used to determine if there are novel entomopathogenic bacteria that are available with potential for controlling OPM, or whether there are fungal communities that can increase the pathogenicity of bacterial based pesticides currently used to control OPM.

Methods

Sample Collection

Samples were collected as part of work by Kitson, Evans, and Straw, (2019) which was conducted across May, June, and July in 2016. A total of 120 OPM nests were collected from north, south, east and west sections of Richmond Park, London. Nests were stored at -20°C before being dissected at Newcastle University between October, November and December 2017, and April and May 2019. During dissection 94 diseased larvae and 84 healthy pupae were retained from 61 nests for sequencing. No healthy caterpillars were found suggesting that at the time of nest collection, all individuals had either succumbed to infection or had pupated successfully. Samples were extracted using an SPRI extraction protocol ((Oberacker *et al.*, 2019). It should be noted that there have been several studies that have documented differences in the microbiome of lepidopteran larvae and their pupae (Hammer, McMillan and Fierer, 2014; Gao *et al.*, 2019; Mereghetti *et al.*, 2019; Wang *et al.*, 2020). To mitigate this impact, only known entomopathogenic genera were chosen for analysis based on the assumption species of these genera are less likely to be influenced by life stage.

PCR

Libraries were prepared following a nested tagged metabarcoding approach (Kitson *et al.*, 2019). The primers used were tagged versions of the 515F and 806 primers which target the 16S rRNA locus and have been shown to successfully amplify DNA from bacteria in previous metabarcoding studies (Caporaso *et al.*, 2011; Shi *et al.*, 2020). Extractions were amplified in a 20 µl reaction using 5 µl of input DNA. Reactions used the following PCR chemistry; 2X Myfi master mix solution (Bioline, Essex), 2 µM of forward and reverse primer, 1% Ficoll solution, 0.5% Tartrazine solution, 0.1% Xylene Cyanol solution, with the remaining volume was made up with nuclease free water. PCRs were amplified using the following protocol, 3 minutes at 95°C followed by 5 cycles of 40 seconds at 95°C, 40 seconds at 45°C and 1 minute at 72°C, followed by 35 cycles of 40 seconds at 95°C, 40 seconds at 51°C and 1 minute at 72°C, followed by a final extension step of 5 minutes at 72°C. Individual wells were sealed with mineral oil (administered with a 1 ml plastic dropper pipette) to prevent cross contamination between plates.

For PCR2 reactions used 5 µl of cleaned PCR product as input DNA. Reactions were again 20 µl and used the following chemistry: 2X Myfi master mix solution (Bioline, Essex), 10 µM of forward and reverse primer, with the remaining volume filled with nuclease free water. The PCRs amplified using the following protocol, 5 minutes at 95°C, followed by 20 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 1 minute at 72°C, followed by a final extension step of 10 minutes at 72°C. Again, individual wells were sealed with mineral oil.

Normalisation of PCR products was performed following a modified version of the protocol set out in (Hosomichi *et al.*, 2014). Briefly, a 1:1 ratio of PCR product to silica-coated beads in PEG buffer, is mixed by pipetting and incubated at room temperature for 5 minutes. Product/bead mix were placed on a magnetic plate and left until solution was clear (the plate containing mix was not removed from the magnetic plate until stated). Supernatant was pipetted off and samples were washed for 60 seconds using 200 µl of 80% ethanol. The ethanol was then pipetted off and the previous wash step was then repeated. This wash was also pipetted off and the beads were dried for 10-15 minutes to ensure complete evaporation of ethanol but prior to cracks forming in the bead pellet. Next, 20 µl of nuclease-free water was added to dried beads and mixed using a vortex spinner. This mix was then left to elute at room temperature for 10 minutes. The beads were then placed back on the magnetic plate and left until the solution was clear. Supernatant containing normalised PCR product was then transferred to a holding plate.

Sequencing libraries were prepared by normalising by the lowest ng/µl and then concentrating samples into 20 µl volumes using the bead clean up described above. Two columns were pooled per library which resulted in one plate containing six libraries. A total of twelve libraries were sent for sequencing. Sequencing was performed by the Genomics Core Facility at Newcastle University using Illumina MiSeq sequencing (V3 chemistry).

All DNA concentration quantification was performed using Qubit 4 fluorometer (Thermofisher, Massachusetts) and a subsequent dsDNA Quantitation High Sensitivity kit (Thermofisher, Massachusetts). Tapestation (Agilent Technologies, California) d1000 reagents were, used to analyse the length of PCR products to ensure peaks had shifted between PCR1 and PCR2, DNA concentrations were assessed here but not recorded.

Bioinformatics

Samples were demultiplexed using Cutadapt (Martin, 2011) on Newcastle Universities high powered computing cluster "Rocket". The remaining downstream analysis was conducted using *R* (v. 4.2.2) (R core Team, 2021). Demultiplexed data was processed following the standard *DADA2* protocol, laid out in the tutorial (v1.26) (Callahan, B., McMurdie, P., Rosen, P., Han, A., Johnson, A., and Holmes, S., 2016) which was developed to provide sample inference from amplicon data, and *Insect* (Wilkinson *et al.*, 2018). Briefly, this involved filtering and trimming sequences based on read quality, merging paired-end reads, removing chimeras, assigning Amplicon Sequence Variants (ASVs CallahanXXX 201) and assigning taxonomy using the SILVA (v123) training set for 16S data and UNITE (v0.8) for retrained fungal data. Empty rows were removed and the samples with less than 10 reads were filtered out. Further data analysis and visualised was conducted using *Vegan* (Oksanen *et al.*, 2008) and *GGPlot2* (Wickham, 2016).

Statistical Analysis

Nonmetric multidimensional scaling analysis, using Bray-Curtis distance, were performed with Vegan (Oksanen *et al.*, 2008) to assess differences in community structure. The structure of the NMDS was also formally analysed with PERMANOVA with adonis used to test for community composition differences and betadisper used to test for homogeneity of variances. Vegan was also used to calculate species richness and Shannon index to measure diversity. These are not true calculations but used Genera as a proxy as species level data were not present.

T-tests were performed to test for differences between diseased and healthy communities for bacterial, fungal, and combined communities.

Read depth was normalised by dividing each read count by the total. Total read depth by status was then compared for each community and Mann-Whitney U tests were used to compare differences.

Chi square test was also used to test for differences in the proportion of read depth entomopathogenic genera made up in samples identified as diseased and healthy. All analysis was conducted using R (v. 4.2.2) (R core Team, 2021). Plots were visualised using functions from Tidyverse (Wickham *et al.*, 2019) and ggplot2 (Wickham, 2016).

Results

Sequencing Results

For the bacterial sequencing, sequencing produced 15.1 million reads, 11.2 million reads remained after filtering, denoising, merging paired ends and chimeral removal. After removing short reads, positive and negative samples and low occurrence ASVs a final count of 10.4 million reads remained for the final dataset. For the reanalysed fungal data, sequencing produced 11 million reads, 9.7 million reads remained after filtering, denoising, merging paired ends and chimeral removal. After removing short reads, positive and negative samples and low occurrence ASVs a final count of 9.1 million reads remained for the final dataset.

Genera

A total of 568 genera were found of which, 202 were bacterial and 366 were fungal. Of these, 9 bacterial genera and 20 fungal genera are known to contain entomopathogens (Table *5.1*: Table of identified entomopathogenic generaTable 5.1). A full list of genera can be found in Table 5.5 in Appendix 1.

Table 5.1: Table of identified entomopathogenic genera

Bacteria	**Fungi**
Acinetobacter	Acremonium
Bacillus	Akanthomyces
Moraxella	Alfaria
Pseudomonas	Aspergillus
Psychrobacte	Beauveria
Rickettsiella	Cordyceps
Sanguibacter	Erythrobasidium
Wolbachia	Fusarium
Yersnia	Geomyces
	Meira
	Myriangium
	Penicillium
	Pyrenophora
	Samsoniella
	Scopulariopsis
	Septobasidium
	Simplicillium
	Tolypocladium
	Trichoderma

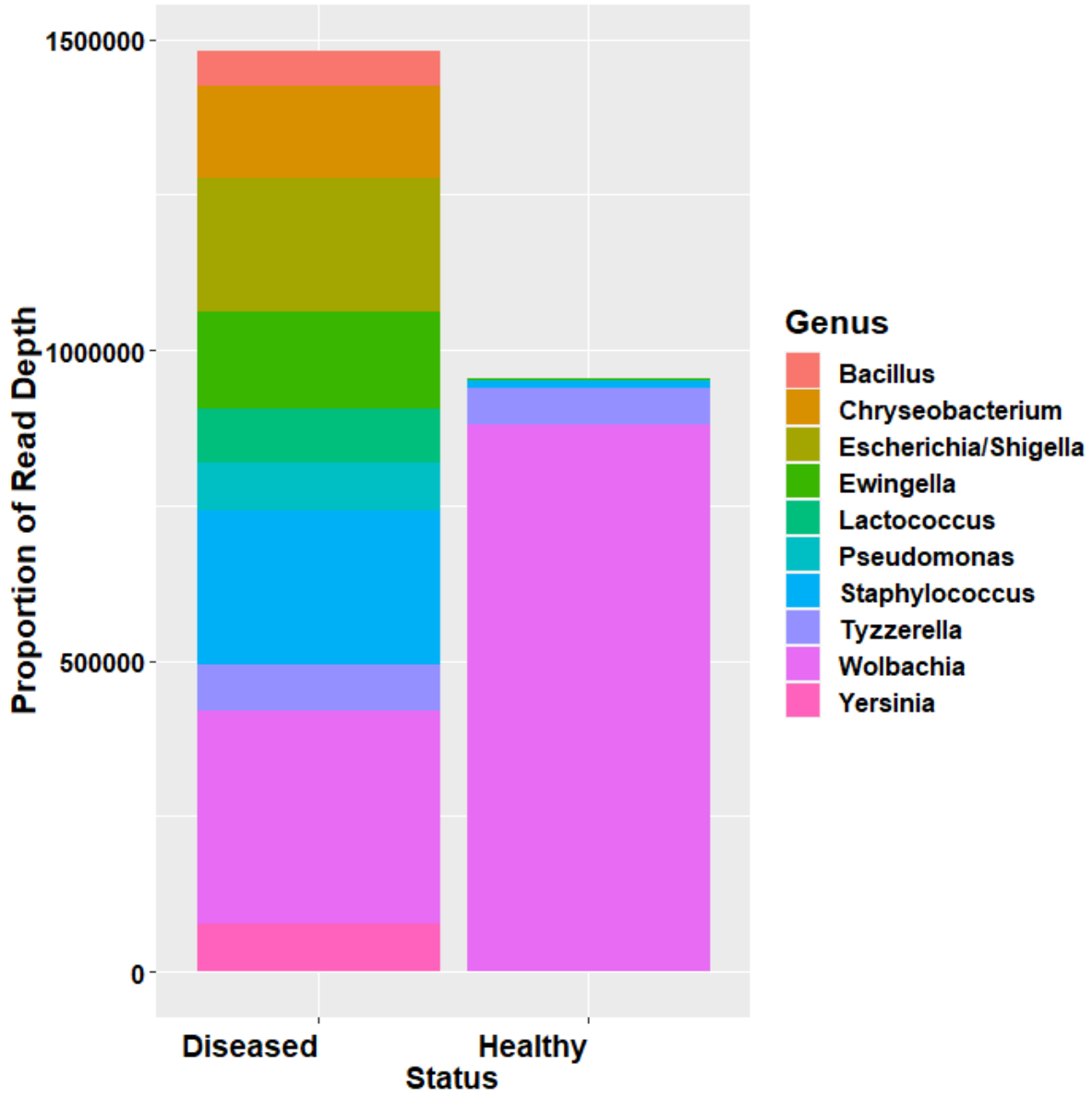


Figure 5.1: Stacked bar chart showing the proportion of read depth accounted for by the top 10 most common genera in the bacterial community.

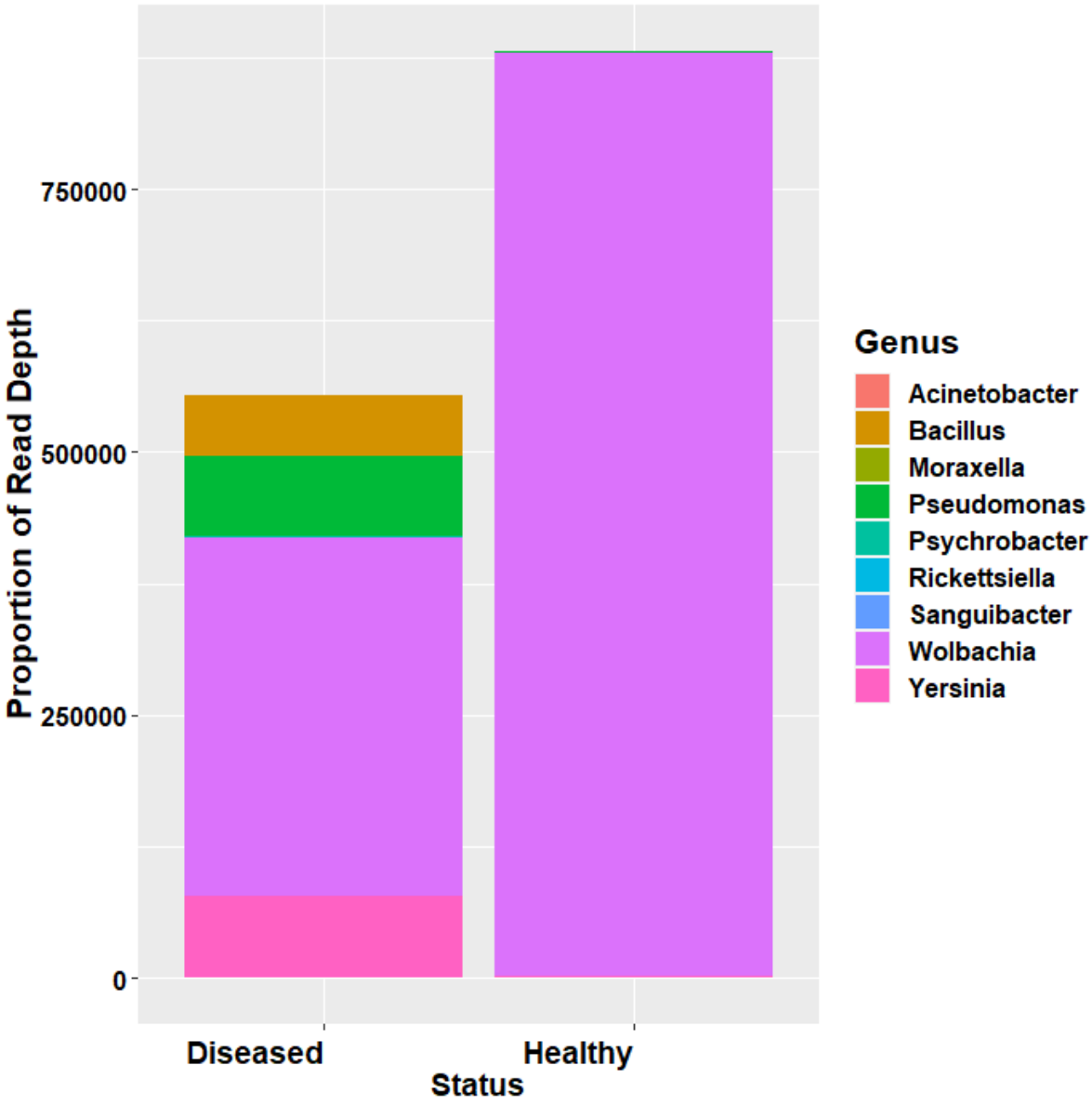


Figure 5.2: Stacked bar chart showing the proportion of read depth accounted for by the entomopathogenic genera in the bacterial community.

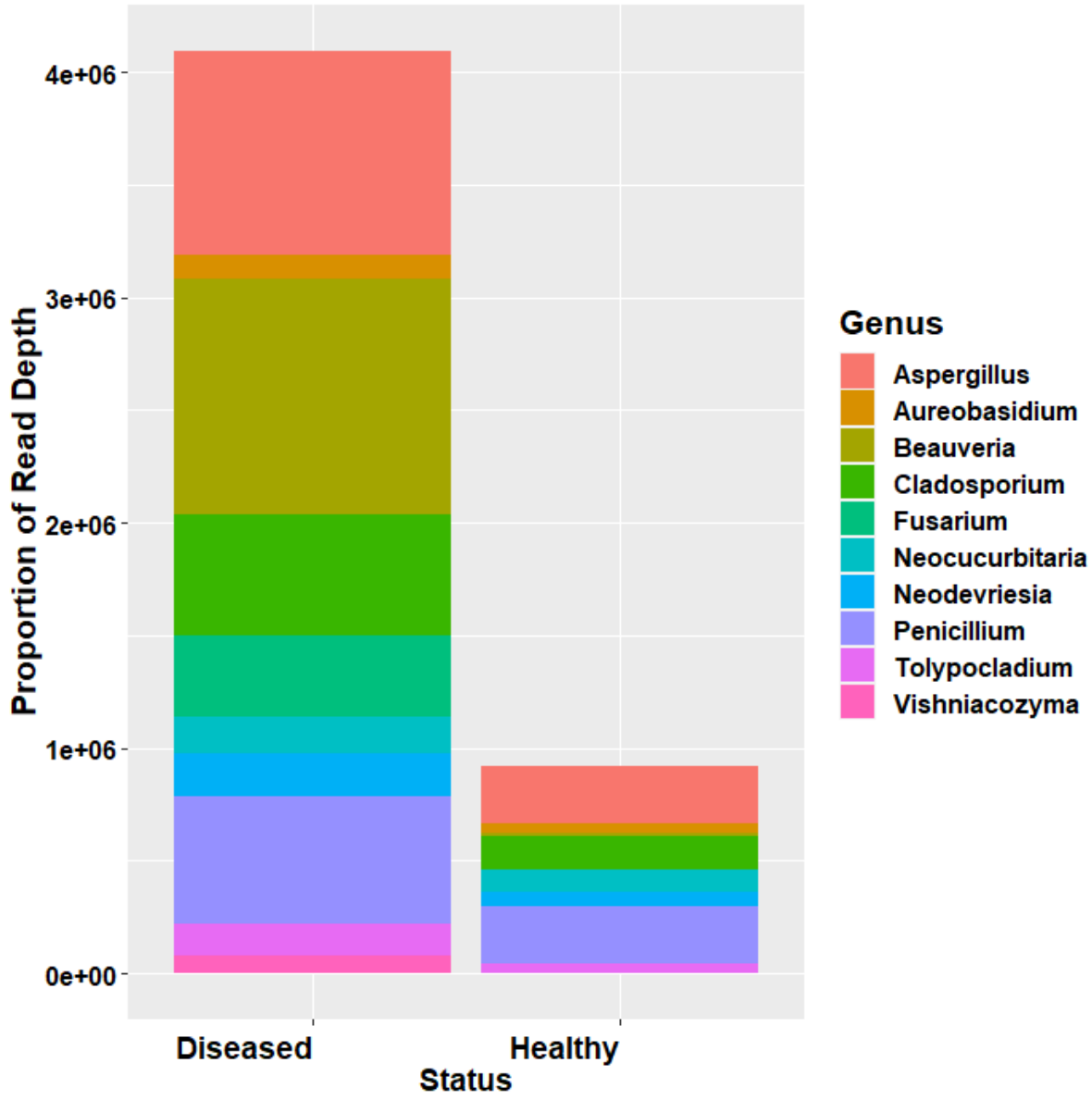


Figure 5.3 Stacked bar chart showing the proportion of read depth accounted for by the top 10 most common genera in the fungal community.

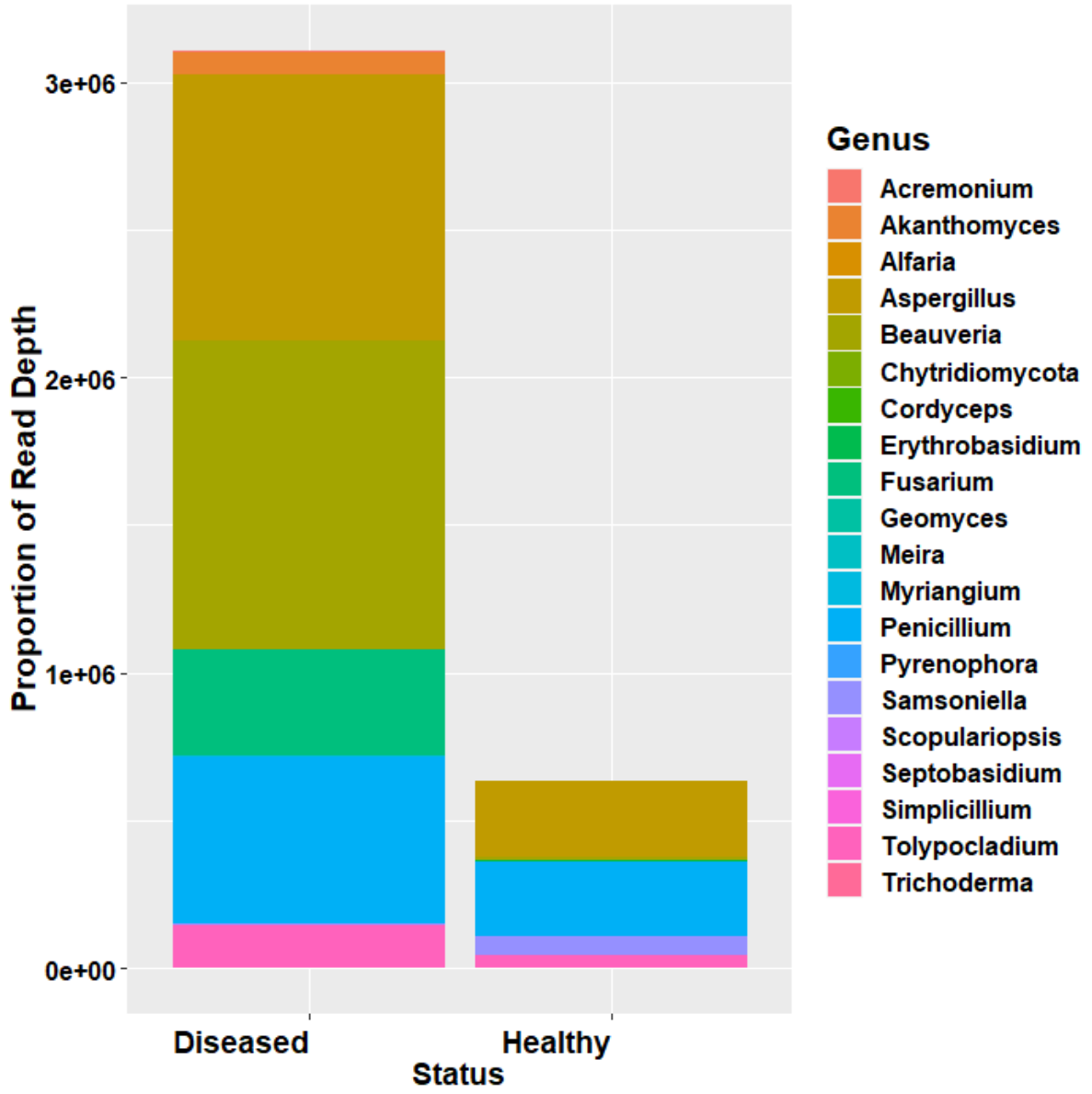


Figure 5.4: Stacked bar chart showing the proportion of read depth accounted for by the entomopathogenic genera in the fungal community.

Species Richness

Species richness was calculated for bacterial, fungal, and combined communities (Figure 5.5) with bacterial samples being more species rich compared to fungal samples. Mean richness was higher in diseased samples for all communities whilst also being more variable compared to healthy samples. A T-test was also used to compare the difference in disease status for all three communities with all three showing significant differences between their healthy and diseased samples (Table 5.2).

Table 5.2: Mean species richness across diseased and healthy samples for all three communities and results of comparison.

Community	Mean Diseased Species Richness	Mean Healthy Species Richness	P-value	df
Bacterial	79.54	33.84	< 0.001	117.07
Fungal	76.72	31	< 0.001	108.42
Combined	156.71	65.76	< 0.001	126.56

Species Diversity

Shannon diversity was calculated for both the bacterial and fungal communities (Figure 5.6). Mean species diversity was higher for bacterial communities and between diseased bacterial samples and healthy bacterial samples, diseased samples had a higher mean diversity (Table 5.3). When compared with a t-test this difference was significantly different. This was not the case with fungal samples however, where healthy fungal samples had a higher mean diversity than diseased samples. However, when subject to a t-test this difference was non-significant.

Table 5.3: Mean species diversity across diseased and healthy samples for bacterial and fungal communities and results of comparison.

Community	Mean Diseased Species Diversity	Mean Healthy Species Diversity	P-value	df
Bacterial	3.47	2.64	< 0.001	117.07
Fungal	2.26	2.40	0.3516	145.5

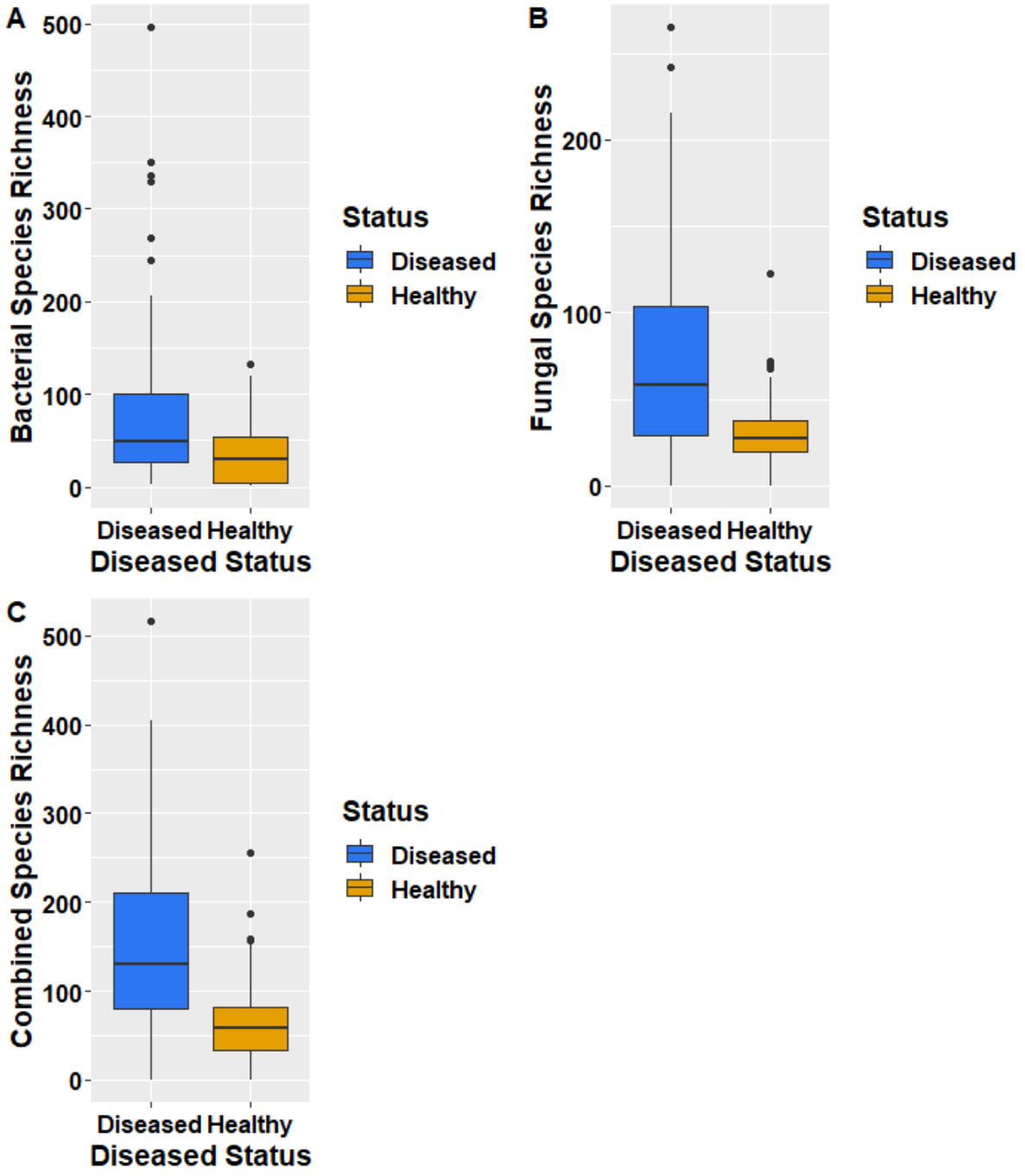
NMDS

NMDS analysis was performed on both the full bacterial (Figure 5.7), fungal (Figure 5.9), and combined (Figure 5.11) communities and also only the communities of entomopathogens (Figure 5.8, Figure 5.10, Figure 5.12). This was performed using proportional read depth of each sample as the input.

For the full bacterial community there is separation across both NMDS1 and NMDS2 with a cluster of diseased samples occurring lower on NMDS2 and centrally on NMDS1. When the structure is formally tested via PERMANOVA neither adonis nor betadisper are significant. For the entomopathogenic bacterial community separation across NMDS1 is largely driven by one sample. When the structure is formally tested via PERMANOVA neither adonis nor betadisper are significant.

For the full fungal community the main separation occurs across NMDS1. Two clusters of diseased samples are present further left on NMDS1 then the main cluster (Figure 5.9). When the structure is formally tested via PERMANOVA adonis is significant (p-value = 0.005) however betadisper is not. For the entomopathogenic fungal community there is a large spread of all samples across NMDS1 and NMDS2 however neither adonis nor betadisper are significant.

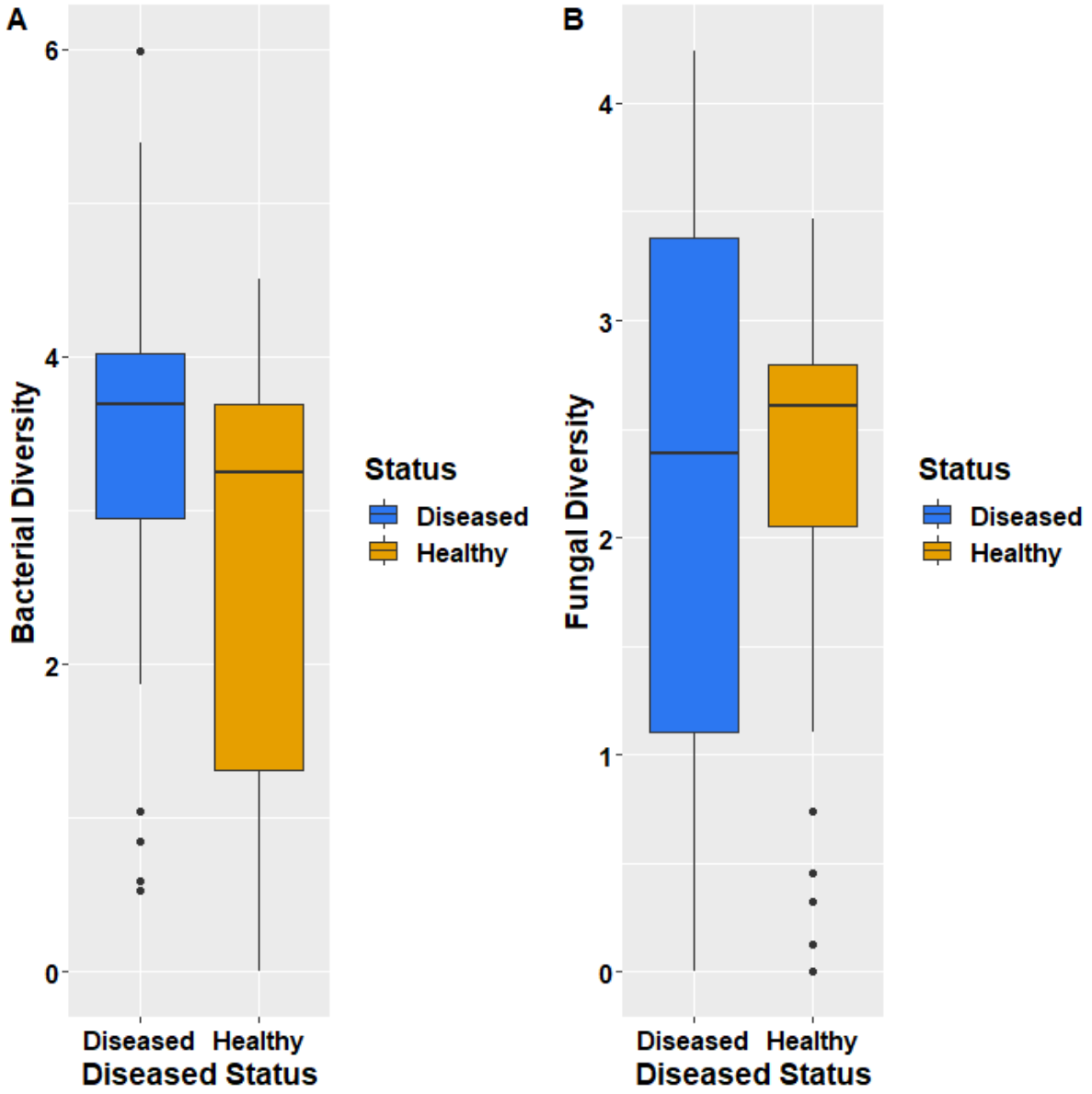
For the full combined community there is separation across both NMDS1 and NMDS2 (Figure 5.11). When the structure is formally tested via PERMANOVA adonis is significant (p-value = 0.003) however betadisper is not. The same is true for the entomopathogenic combined community where adonis is significant (p-value 0.037) however betadisper is not.



2402

2403 Figure 5.5: Species richness compared between diseased and healthy samples for bacterial (A), fungal

2404 (B), and combined (C) communities.



2405

2406 Figure 5.6: Species diversity compared between diseased and healthy samples for bacterial (A) and
2407 fungal (B) communities.

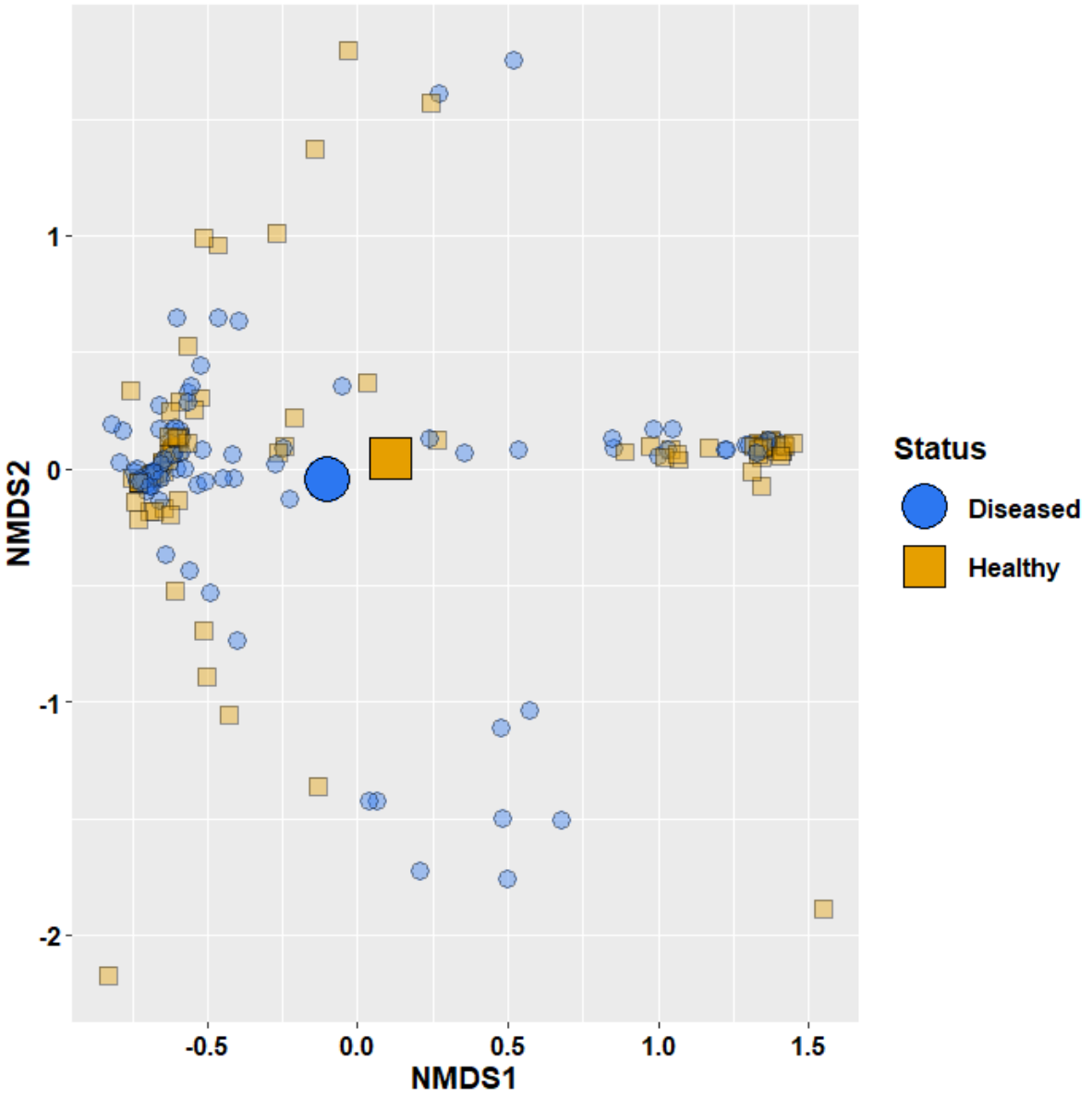
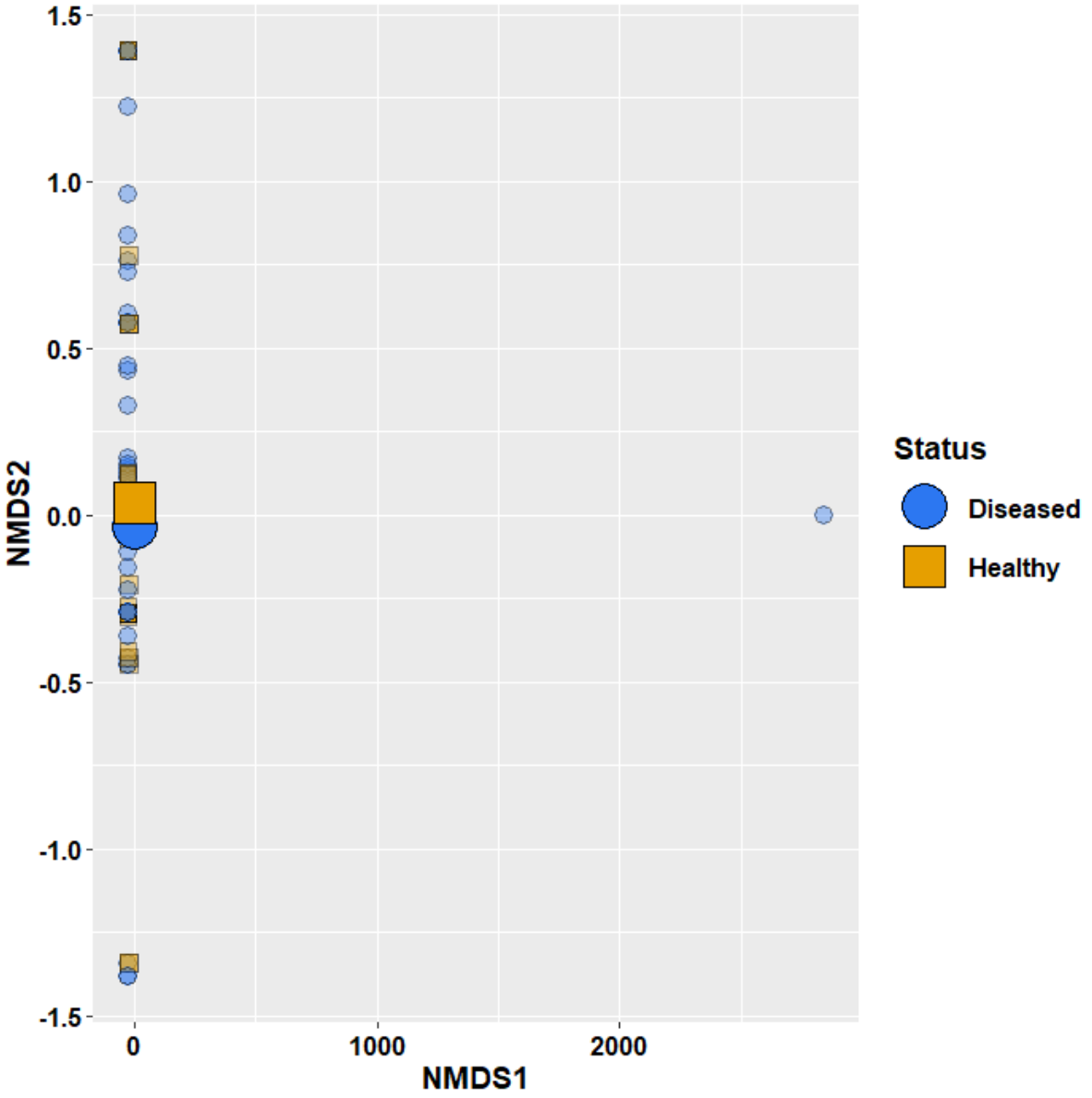


Figure 5.7: Plot of NMDS analysis of the full bacterial community with diseased samples in blue and healthy sample in yellow. The average centroid position of diseased and healthy samples is shown with full opacity and larger than individual samples.



2413

2414 Figure 5.8: Plot of NMDS analysis of the entomopathogenic bacterial community with diseased
2415 samples in blue and healthy sample in yellow. The average centroid position of diseased and healthy
2416 samples is shown with full opacity and larger than individual samples.

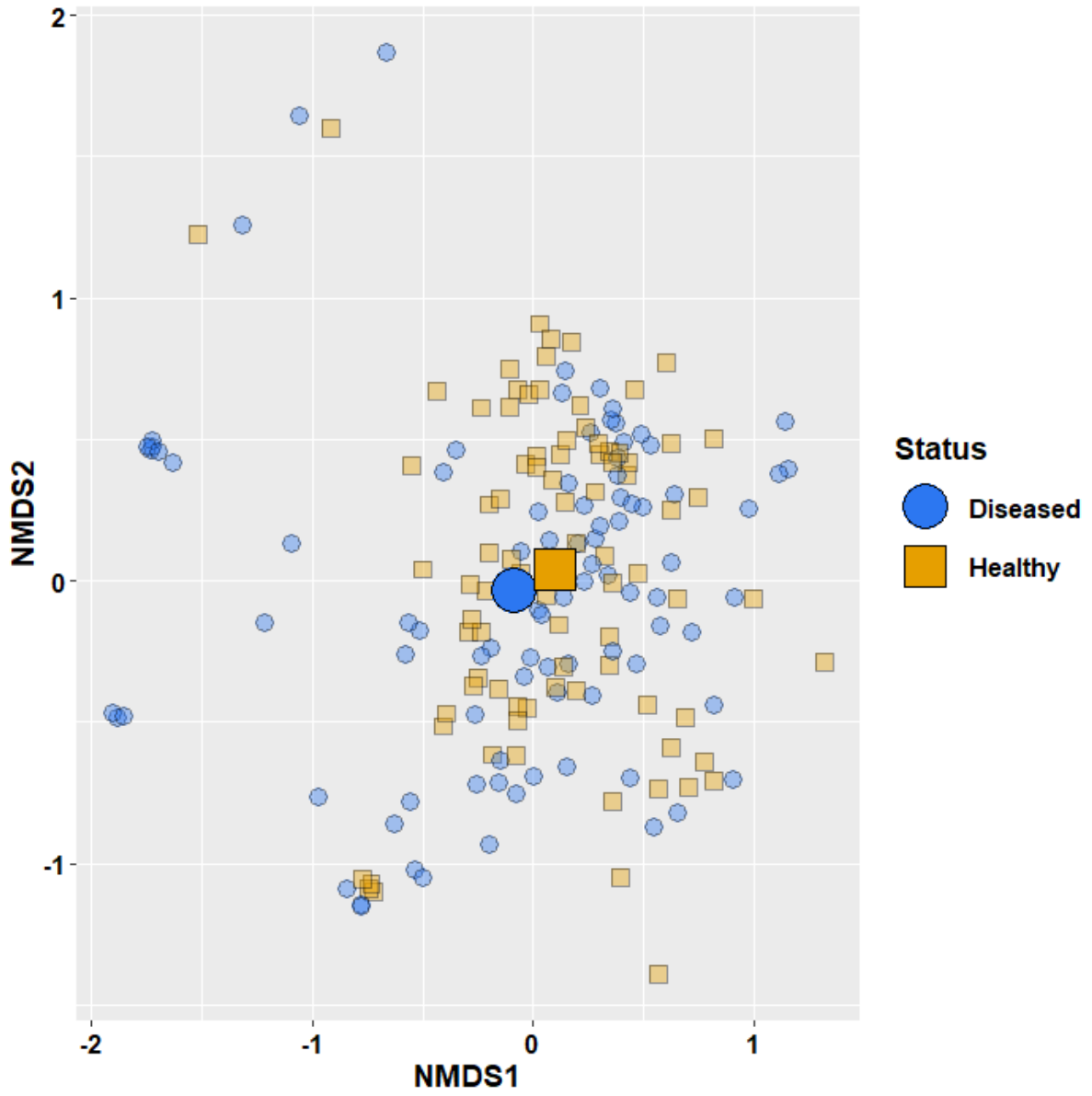


Figure 5.9: Plot of NMDS analysis of the full fungal community with diseased samples in blue and healthy sample in yellow. The average centroid position of diseased and healthy samples is shown with full opacity and larger than individual samples.

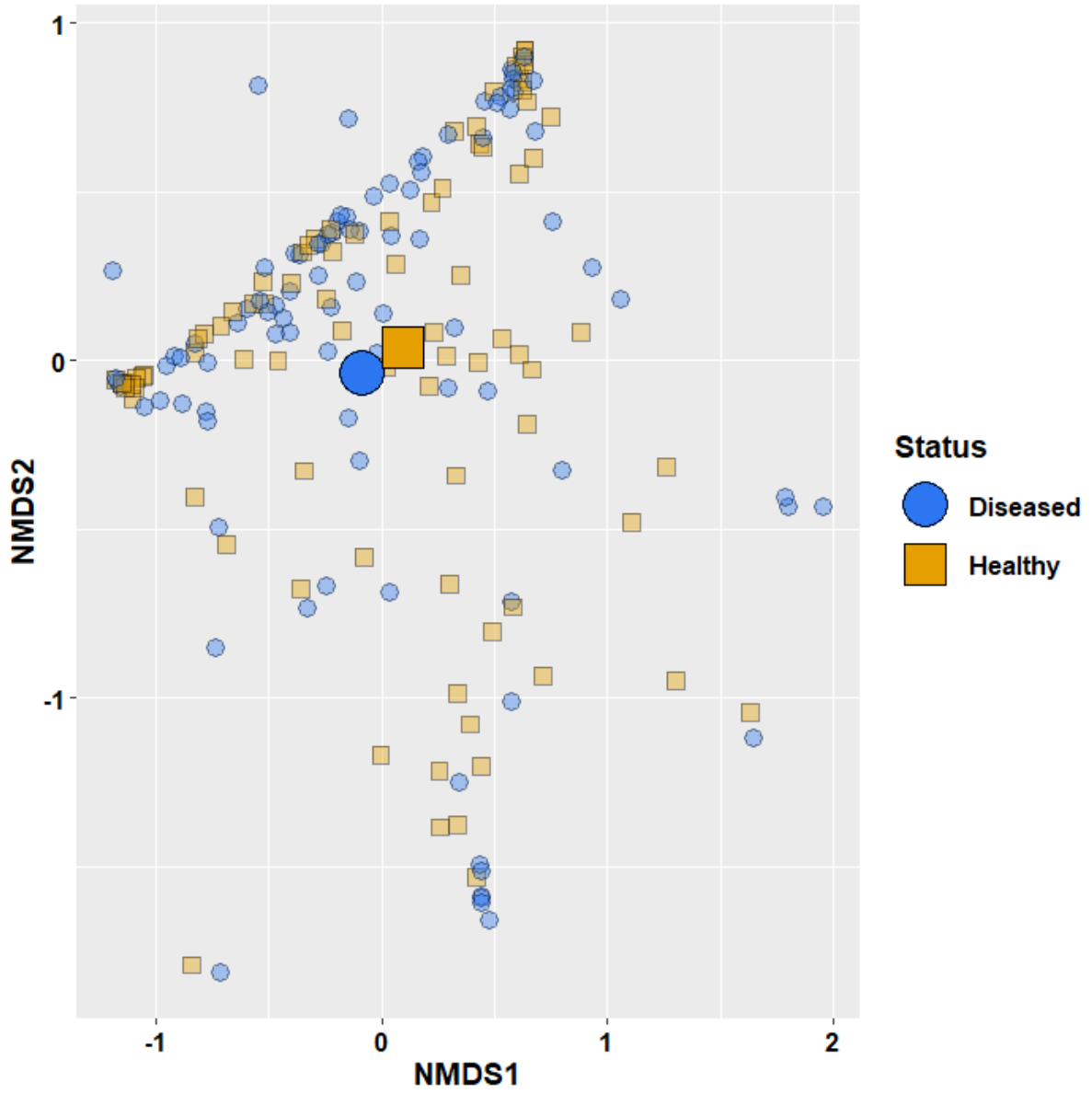


Figure 5.10: Plot of NMDS analysis of the entomopathogenic fungal community with diseased samples in blue and healthy sample in yellow. The average centroid position of diseased and healthy samples is shown with full opacity and larger than individual samples.

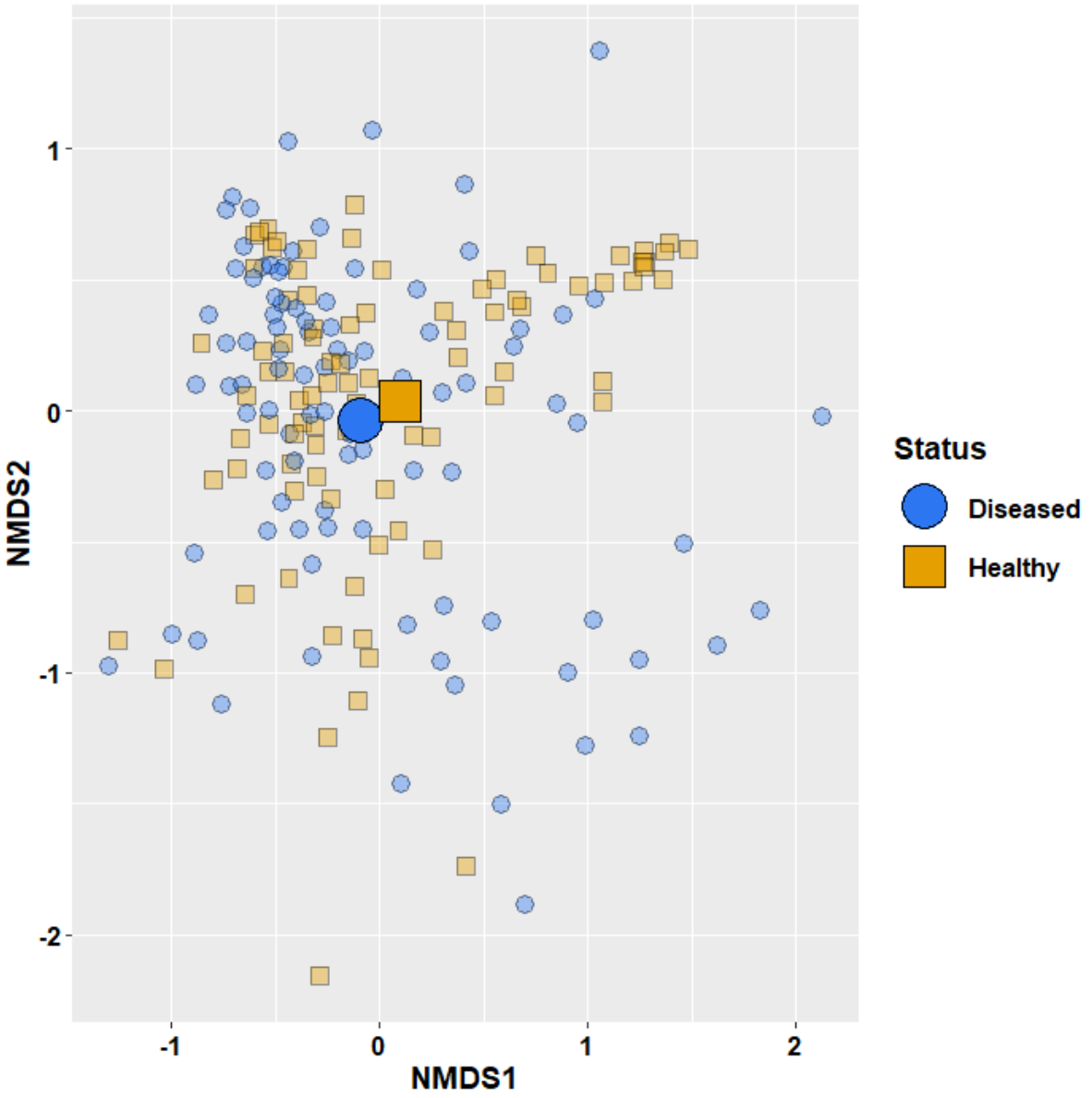
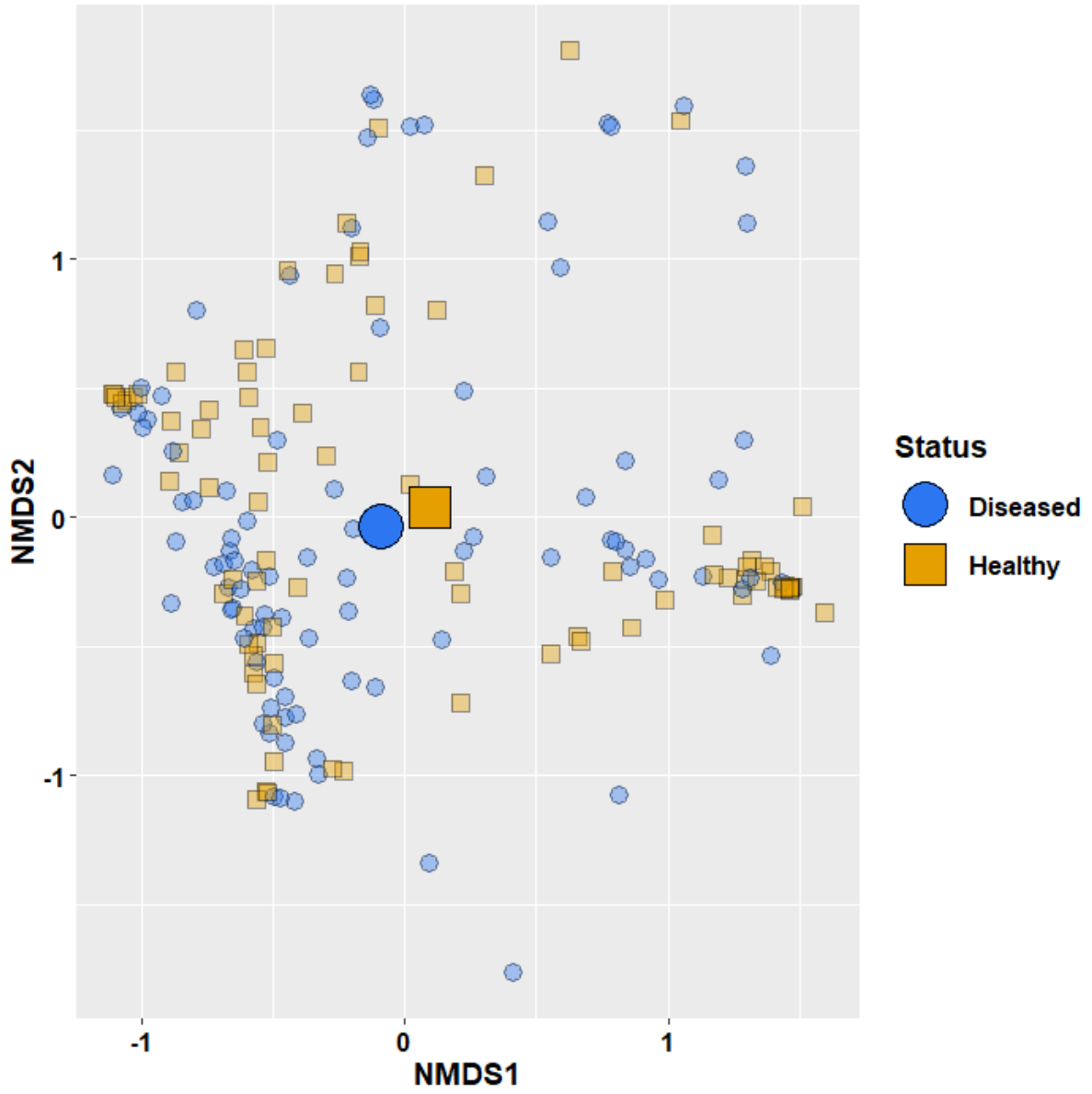


Figure 5.11: Plot of NMDS analysis of the full combined community with diseased samples in blue and healthy sample in yellow. The average centroid position of diseased and healthy samples is shown with full opacity and larger than individual samples.



2429

2430 Figure 5.12: Plot of NMDS analysis of the entomopathogenic combined community with diseased
2431 samples in blue and healthy sample in yellow. The average centroid position of diseased and healthy
2432 samples is shown with full opacity and larger than individual samples.

2433 Table 5.4: Results of Vegan analysis of NMDS communities with adonis used to test differences in
2434 structure and betadisper used to test homogeneity of variance.

NMDS	Test	Df	SumOfSqs	R2	Mean sq	F	Pr(>F)
Bacterial	Adonis	1	0.648	0.01471	-	2.6718	0.059
	betadisper	1	$1.6200e^{-08}$	$1.6235e^{-08}$	0.17	-	0.6806
Fungal	Adonis	1	0.893	0.01813		3.3045	**0.005**
	betadisper	1	0.0004955	0.00049550	3.8533	-	0.0512
Combined	Adonis	1	0.944	0.01772	-	3.2287	**0.003**
	betadisper	1	0.00002617	$2.6174e^{-05}$	2.6557	-	0.1049
Bact_ento	Adonis	1	0.0928	0.017	-	1.8505	0.375
	betadisper	1	0.00015	-	0.0001512	0.0065	0.936
Fungal_ento	Adonis	1	0.113	0.00285	-	0.506	0.739
	betadisper	1	0.00526	0.0052611	0.5505	-	0.4591
Combined_ento	Adonis	1	0.756	0.01405	-	2.536	**0.037**
	betadisper	1	0.01197	-	0.0119732	1.8899	0.1709

2435

Read Count

Normalised read count and proportional read counts were calculated and can be found in Appendix 3.

Discussion

Despite the importance of the bacterial and fungal microbiomes in determining individual health and both inter and intra species interactions there is a lack of information in Lepidopterans with less than 0.1% of species having been screened (Paniagua Voirol *et al.*, 2018). This is also true of OPM which is a voracious forestry pest that is also an issue for human health. The discovery of diseased OPM larvae in 2018 provided the opportunity to study both the bacterial and fungal microbiome associated with healthy and diseased OPM larvae and to determine whether there were any differences present. In this chapter we use tagged nested metabarcoding techniques to provide the first look at OPM bacterial and fungal microbial communities with downstream analysis to determine whether there are entomopathogens present that were associated with diseased status.

Genera

Whilst a total of 568 genera were found it is unsurprising that only a small number of genera were entomopathogens as the majority will be associated with the caterpillar gut microbiome or a mixture of saprophytes and detritivores. Several notable entomopathogenic genera were identified, such as *Bacillus, Beauveria,* and *Pseudomonas,*

Richness and Diversity

Species richness and Shannon diversity were calculated for each community and differences were tested between healthy and diseased samples. For species richness all three communities showed differences between healthy and diseased samples with diseased samples all having higher means than healthy samples (Table 5.2). For Shannon diversity, only the bacterial community showed significant difference (Table 5.3) although again the diseased samples showed a higher diversity than healthy samples. When looking at the community composition of both the entomopathogenic bacterial (Figure 5.2) and fungal (Figure 5.4) community it is clear to see these differences. However, it is uncertain what is driving these differences. It is possible that the primary cause of the diseased state in these larvae has reduced their immune response so that other entomopathogenic species have been able to establish where previously they couldn't. It is also possible, as the larvae were collected after they had died, that many of the genera are saprophytes and detritivores that are taking advantage of the deceased host.

It should also be mentioned here that the lack of diversity in the healthy bacterial samples appears to be due to a large proportion of this being made up by *Wolbachia* which may have skewed the evenness (Figure 5.1, Figure 5.2). However*, Wolbachia* is a genus of interest as depending on the strain *Wolbachia* can have, mutualistic, parasitic, or commensal relationships with its host (Werren, Baldo and Clark, 2008). The literature around *Wolbachia* is also vast as the bacterium is incredibly widespread infecting around 80% of Lepidoptera with 90 strains identified so far (Ahmed *et al.*, 2015; Ahmed, Breinholt and Kawahara, 2016; Sazama, Ouellette and Wesner, 2019).

From a biocontrol perspective the inhibitory effects shown by some *Wolbachia* have potential with effects including reproductive incompatibility, feminization of genetic males, and embryonic male killing (Werren and Windsor, 2000; Stouthamer, Hurst and Breeuwer, 2002). However as mentioned prior *Wolbachia* is heavily present in healthy samples, not diseased. There are several *Wolbachia* strains that are known to provide benefits to its host including resistance to pathogens (Panteleev *et al.*, 2007; Teixeira, Ferreira and Ashburner, 2008; Osborne *et al.*, 2009; Walker *et al.*, 2011; Bian *et al.*, 2013; Cattel *et al.*, 2016), pesticides (Berticat *et al.*, 2002), and parasitoids (Martinez *et al.*, 2012). While this study provides no definitive evidence of any kind of effect from *Wolbachia* it is still important to consider as several studies that have shown *Wolbachia*-conveyed resistance have noted similar results, where *Wolbachia* is recorded altering host microbiomes, usually causing decreases in both bacterial diversity and bacterial species richness (Duan *et al.*, 2020; Li *et al.*, 2022).

Despite a lack of definitive proof here the large proportion of *Wolbachia* in healthy samples should prompt further investigation to ensure there is no risk of resistance to the main pesticide *Bt.* This phenomena is a known possibility as resistance has already evolved in several species of Lepidoptera with prevailing theories suggesting gut bacteria may provide a crucial role in this process (McGaughey, 1985; Tabashnik *et al.*, 1990; Shelton *et al.*, 1993; Janmaat and Myers, 2003; Raymond *et al.*, 2009; Xia *et al.*, 2013; Paramasiva, Sharma and Krishnayya, 2014). This also once again highlights the vulnerability of current OPM management plans that largely rely on *Bt* to control outbreaks in pest free areas and underlines the requirement for holistic management strategies that reduce the over reliance on one form of control and for healthy forest ecosystems that can provide services, like biocontrol, indefinitely.

Community Composition

Community composition for all three communities, both full communities and only entomopathogenic communities, was visualised using NMDS and formally tested using PERMANOVA. Despite differences in richness and diversity, there appeared to be little difference in actual composition between healthy and diseased samples. Three communities appear to have significant differences when tested with adonis, (full fungal (p-value = 0.005), full combined (p-value = 0.003), and only entomopathogen combined (p-value = 0.037)). However, when tested with betadisper to determine if community differences are driven by variances in data all three communities are insignificant meaning the results of the adonis analysis are unreliable. This is unsurprising as the taxonomic assignment was only reliable down to genus level. Several notable entomopathogenic genera were identified, such as *Bacillus, Beauveria,* and *Pseudomonas,* however within these genera there are both species that are part of the regular gut microbiome and species that are potential entomopathogens. Therefore, it is difficult to understand whether the lack of community difference is due to a real lack of difference or due to poor data resolution.

Future work

Despite the presence of some prominent entomopathogens it is still inconclusive as to what was the cause of mortality in the diseased samples. There are two possible causes that still need investigating. Either the diseased status is caused by viruses which were not tested for and was out of the scope of this study. Or the diseased status was caused by the application of *Bt* pesticide.

The potential effects of *Wolbachia* presented in this study also brings up several hypothesise that need testing. The healthy samples that are associated with the presence of *Wolbachia* should be further investigated to determine whether a resistance to *Bt* pesticide is being conferred by *Wolbachia*. This has potential management implications with *Bt* being the predominant pesticide used to control OPM and any resistance being conferred risks the efficacy of current management strategies which have been shown to be effective at slowing the spread at OPM. Another factor to consider for future study is the potential for *Wolbachia* to be conferring resistance to parasitoids. There is precedence for this in the literature and with the move towards nature-based solutions for managing OPM it is important to understand how effective parasitoids may be in the long term (Martinez *et al.*, 2012).

Conclusion

To reduce the over reliance on chemical pesticides the study of pest microbiomes is incredibly important for discovering novel entomopathogens that might be utilised in integrated pest management programs. Metabarcoding technologies are increasingly becoming more cost effective which makes this explorative work feasible for a wider range of pest species. This chapter has demonstrated the importance of applying these technologies to understand how dynamics between pest species and their microbiomes might be changing or be utilised to better inform management strategies. For instance, in this chapter an association between healthy OPM and *Wolbachia*, which is known for conferring resistance to insecticides, has been uncovered which could have significant knock-on effects for OPM management if resistance to *Bt* was found to be conferred in this system. This ultimately meets objective four which was described at the start of this chapter. As for the specific sub-objectives the first is met in sections 2.1, and the second and third are met in sections, 2.2, 3.1, 3.2, 3.3, 3.4., and 3.5.

Whilst the objectives of this chapter have been met, ultimately, it is still uncertain what the definitive cause of mortality for the caterpillars is. The three prominent entomopathogenic genera found in this work (*Bacillus, Beauveria, Pseudomonas*) could be the subjects of further work to observe how species within these genera interact with OPM and how useful they might be for integrated pest management strategies. Another avenue for investigation would be viruses and how they relate to the healthy samples and diseased samples. However, the primary focus of any future work should be the link between healthy samples and *Wolbachia*. Untangling whether there is a net positive relationship between OPM and the endosymbiont is critical to understanding how sustainable our current management practises are. If *Bt* usage for OPM control is not sustainable, then it is vital to know so that research efforts can be prioritised to diversify current control methods. This also may prompt a review of current control strategies for other pest species with limited control methods to better understand how sustainable practises are at a national level.

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Appendix

Appendix 1 – Full list of genera

Table 5.5: Full list of genera found after demultiplexing.

Kingdom	Genus
Fungi	Abrothallus
Bacteria	Acidibacter
Bacteria	Acidiphilium
Bacteria	Acidothermus
Bacteria	Acinetobacter
Fungi	Acremonium
Fungi	Acrodontium
Bacteria	Actimicrobium
Bacteria	Actinomycetospora
Bacteria	Actinoplanes
Bacteria	Aeromonas
Bacteria	Agreia
Fungi	Akanthomyces
Fungi	Alatosessilispora
Fungi	Aleurodiscus
Fungi	Alfaria
Bacteria	Algoriphagus
Bacteria	Aliihoeflea
Bacteria	Alloprevotella
Fungi	Alternaria
Fungi	Amandinea
Bacteria	Aminobacter
Bacteria	Amnibacterium
Fungi	Ampelomyces
Fungi	Amphisphaeriaceae
Fungi	Amphosoma
Bacteria	Anaerococcus
Fungi	Angustimassarina
Fungi	Apiognomonia
Fungi	Apiospora
Bacteria	Aquabacterium
Bacteria	Aquicella
Bacteria	Aquincola
Bacteria	Arcticibacter
Fungi	Arthoniales
Bacteria	Arthrobacter
Fungi	Arthrocatena
Fungi	Articulospora
Fungi	Ascobolus

Fungi	Ascochyta
Fungi	Aspergillus
Bacteria	Asticcacaulis
Fungi	Atrocalyx
Fungi	Aureobasidium
Fungi	Auricularia
Bacteria	Azomonas
Fungi	Bacidia
Fungi	Bacidina
Bacteria	Bacillus
Fungi	Bannozyma
Fungi	Bartalinia
Bacteria	Bdellovibrio
Fungi	Beauveria
Bacteria	Beijerinckia
Fungi	Bellamyces
Fungi	Bensingtonia
Fungi	Bhatiellae
Fungi	Biatora
Bacteria	Bifidobacterium
Fungi	Blastobotrys
Bacteria	Blastocatella
Bacteria	Blastomonas
Fungi	Blumeria
Fungi	Boeremia
Bacteria	Bosea
Fungi	Botryobasidium
Fungi	Botryosphaeria
Fungi	Botryotrichum
Fungi	Botrytis
Bacteria	Brachybacterium
Bacteria	Bradyrhizobium
Bacteria	Brevibacterium
Bacteria	Brevundimonas
Fungi	Brycekendrickomyces
Bacteria	Bryobacter
Bacteria	Bryocella
Bacteria	Buchnera
Fungi	Buckleyzyma
Bacteria	Budvicia
Fungi	Bullera
Bacteria	Burkholderia
Bacteria	Buttiauxella
Bacteria	Byssovorax

Fungi	Caliciopsis
Fungi	Calophoma
Fungi	Caloplaca
Fungi	Camposporium
Bacteria	Campylobacter
Fungi	Candelaria
Fungi	Candelariella
Fungi	Candida
Bacteria	Candidatus_Gigarickettsia
Bacteria	Candidatus_Koribacter
Bacteria	Candidatus_Solibacter
Fungi	Cantharellales
Fungi	Capronia
Bacteria	Carnobacterium
Fungi	Catenulostroma
Bacteria	Caulobacter
Bacteria	Cedecea
Bacteria	Cellulomonas
Bacteria	Cellvibrio
Fungi	Cephalotheca
Fungi	Ceratobasidium
Fungi	Ceratocystis
Fungi	Cercospora
Fungi	Chaenotheca
Fungi	Chaenothecopsis
Fungi	Chaetomium
Fungi	Chaetosphaeronema
Bacteria	Chryseobacterium
Fungi	Chrysozyma
Bacteria	Chthoniobacter
Bacteria	Chthonomonas
Fungi	Chytridiomycota
Fungi	Cippumomyces
Fungi	Citeromyces
Fungi	Cladoniaceae
Fungi	Cladophialophora
Fungi	Cladosporium
Fungi	Claviceps
Fungi	Cliostomum
Fungi	Clitopilus
Fungi	Colacogloea
Fungi	Colpoma
Fungi	Conioscypha
Fungi	Constantinomyces

Fungi	Coprinellus
Fungi	Coprinopsis
Fungi	Cordyceps
Fungi	Coriolopsis
Fungi	Coronicium
Fungi	Corticium
Fungi	Corynascus
Bacteria	Corynebacterium
Bacteria	Corynebacterium_1
Bacteria	Crocinitomix
Fungi	Cryptodiaporthe
Fungi	Cryptosphaeria
Fungi	Cuniculitremaceae
Bacteria	Cupriavidus
Bacteria	Curtobacterium
Fungi	Curvibasidium
Fungi	Curvularia
Fungi	Cutaneotrichosporon
Fungi	Cyathicula
Fungi	Cyberlindnera
Fungi	Cylindrium
Fungi	Cylindromonium
Fungi	Cyphellophora
Fungi	Cyphellophoraceae
Fungi	Cyphobasidiales
Fungi	Cystobasidiomycetes
Fungi	Cystobasidium
Fungi	Cytosporella
Bacteria	Dactylosporangium
Fungi	Daldinia
Fungi	Datronia
Fungi	Debaryomyces
Bacteria	Deinococcus
Fungi	Dendrophoma
Fungi	Dendryphion
Bacteria	Dermacoccus
Bacteria	Devosia
Fungi	Diaporthe
Fungi	Diatrype
Fungi	Diatrypella
Fungi	Dichomitus
Fungi	Didymella
Fungi	Didymocyrtis
Fungi	Dinemasporium

Fungi	Diplodia
Fungi	Dissoconium
Fungi	Distoseptisporaceae
Bacteria	Dokdonella
Fungi	Dothiora
Bacteria	Duganella
Bacteria	Dyadobacter
Bacteria	Echinicola
Bacteria	Edaphobacter
Fungi	Elsinoe
Bacteria	Endobacter
Fungi	Endoconidioma
Fungi	Endosporium
Bacteria	Enhydrobacter
Bacteria	Enterococcus
Fungi	Entomortierella
Fungi	Epichloe
Fungi	Epicoccum
Bacteria	Epilithonimonas
Bacteria	Erysipelatoclostridium
Fungi	Erysiphe
Fungi	Erythrobasidiales
Fungi	Erythrobasidium
Bacteria	Escherichia/Shigella
Fungi	Eutypa
Bacteria	Euzebya
Bacteria	Ewingella
Fungi	Exidia
Fungi	Exobasidium
Fungi	Exophiala
Fungi	Extremus
Fungi	Fellomyces
Bacteria	Ferruginibacter
Fungi	Filobasidiaceae
Fungi	Filobasidium
Bacteria	Finegoldia
Bacteria	Flavobacterium
Fungi	Flavoparmelia
Bacteria	Flexivirga
Bacteria	Fluviicola
Fungi	Fomes
Fungi	Fonsecazyma
Bacteria	Frankia
Bacteria	Frondihabitans

Fungi	Fungi_gen_Incertae_sedis
Fungi	Funiliomyces
Fungi	Furcasterigmium
Fungi	Fusarium
Fungi	Fuscoporia
Fungi	Ganoderma
Bacteria	Gemmata
Bacteria	Gemmatimonas
Fungi	Genolevuria
Fungi	Geomyces
Fungi	Geranomyces
Fungi	Gnomoniopsis
Fungi	Golovinomyces
Bacteria	Granulicella
Fungi	Gremmenia
Fungi	Gyoerffyella
Fungi	Gyrographa
Bacteria	Haemophilus
Bacteria	Haliangium
Fungi	Hansfordia
Fungi	Haudseptoria
Fungi	Helicosporium
Fungi	Helminthosporium
Bacteria	Hephaestia
Fungi	Heterocephalacria
Fungi	Hohenbuehelia
Bacteria	Humibacter
Fungi	Humicola
Fungi	Hyalorbilia
Bacteria	Hymenobacter
Fungi	Hyphoderma
Fungi	Hypholoma
Fungi	Hypotrachyna
Fungi	Incertomyces
Bacteria	Indibacter
Bacteria	Inquilinus
Bacteria	Isoptericola
Bacteria	Isosphaera
Fungi	Itersonilia
Bacteria	Jatrophihabitans
Fungi	Jeremyomyces
Fungi	Kalmusia
Fungi	Keissleriella
Fungi	Kernia

Bacteria	Kineococcus
Bacteria	Kineosporia
Bacteria	Kitasatospora
Fungi	Knufia
Fungi	Kockovaella
Bacteria	Kocuria
Fungi	Kondoa
Fungi	Kondoaceae
Bacteria	Kurthia
Fungi	Kurtzmanomyces
Fungi	Kwoniella
Fungi	Lachancea
Fungi	Lachnum
Bacteria	Lacibacter
Fungi	Lacrymaria
Bacteria	Lactobacillus
Bacteria	Lactococcus
Fungi	Laetiporus
Fungi	Lasiodiplodia
Bacteria	Leadbetterella
Fungi	Lecanora
Fungi	Lecophagus
Bacteria	Leminorella
Fungi	Lentinus
Fungi	Lepraria
Fungi	Leptosillia
Fungi	Leptosphaeria
Fungi	Leptospora
Bacteria	Leuconostoc
Fungi	Leucosporidium
Fungi	Lichenostigmatales
Fungi	Lichtheimia
Fungi	Linnemannia
Fungi	Lophiostoma
Fungi	Lophiotrema
Fungi	Lophium
Bacteria	Luteibacter
Bacteria	Luteimonas
Bacteria	Luteolibacter
Bacteria	Lysinimonas
Fungi	Malassezia
Fungi	Marchandiobasidium
Bacteria	Marmoricola
Bacteria	Massilia

Fungi	Meira
Fungi	Melanchlenus
Fungi	Melanelixia
Fungi	Meristemomyces
Bacteria	Mesorhizobium
Fungi	Metarhiziopsis
Bacteria	Methylobacterium
Bacteria	Methylorosula
Bacteria	Methylovirgula
Fungi	Microascus
Fungi	Microcera
Fungi	Microcyclospora
Fungi	Microdochium
Bacteria	Micromonospora
Fungi	Microsporomyces
Fungi	Microsporomycetaceae
Fungi	Miniancora
Bacteria	Mitsuaria
Fungi	Montagnula
Bacteria	Moraxella
Fungi	Moristroma
Fungi	Mortierella
Fungi	Mortierellomycetes
Bacteria	Mucilaginibacter
Bacteria	Mumia
Fungi	Muriphaeosphaeria
Fungi	Mycocentrospora
Fungi	Mycoleptodiscus
Fungi	Myriangiaceae
Fungi	Myriangiales
Fungi	Myriangium
Fungi	Myrmecridium
Fungi	NA
Bacteria	NA
NA	NA
Eukaryota	NA
Fungi	Naevala
Fungi	Naganishia
Bacteria	Nakamurella
Fungi	Nectria
Fungi	Nemania
Fungi	Neoascochyta
Fungi	Neocatenulostroma
Fungi	Neocladophialophora

Fungi	Neocucurbitaria
Fungi	Neodevriesia
Fungi	Neoerysiphe
Fungi	Neoheleiosa
Fungi	Neophaeococcomyces
Fungi	Neopseudolachnella
Fungi	Neopyrenochaeta
Bacteria	Neorhizobium
Fungi	Neosetophoma
Fungi	Neostagonospora
Fungi	Neptunomyces
Fungi	Niesslia
Fungi	Nigrospora
Bacteria	Nitrobacter
Bacteria	Nitrospira
Bacteria	Nocardioides
Bacteria	Novosphingobium
Bacteria	Nubsella
Bacteria	Oceanicella
Fungi	Oidiodendron
Bacteria	Opitutus
Fungi	Orbilia
Fungi	Ovicillium
Bacteria	Oxalicibacterium
Bacteria	P131-4
Fungi	Paecilomyces
Bacteria	Pantoea
Fungi	Papiliotrema
Fungi	Paraconiothyrium
Bacteria	Parafilimonas
Bacteria	Parapedobacter
Fungi	Parapyrenochaeta
Fungi	Parastagonospora
Fungi	Parmelia
Fungi	Parmotrema
Bacteria	Patulibacter
Bacteria	Pedobacter
Bacteria	Pedomicrobium
Fungi	Penicillium
Fungi	Peniophora
Fungi	Peniophorella
Fungi	Periconia
Fungi	Petrophila
Fungi	Phacidium

Fungi	Phaeoannellomyces
Fungi	Phaeococcomyces
Fungi	Phaeoisaria
Fungi	Phaeosphaeria
Bacteria	Phaselicystis
Bacteria	Phenylobacterium
Fungi	Phialemonium
Fungi	Phialocephala
Fungi	Phoma
Fungi	Phomatospora
Fungi	Phragmocamarosporium
Fungi	Physcia
Bacteria	Phytohabitans
Bacteria	Pir4_lineage
Bacteria	Piscinibacter
Fungi	Plagiostoma
Bacteria	Planctomyces
Fungi	Plectosphaerella
Fungi	Podosphaera
Fungi	Podospora
Bacteria	Polaromonas
Fungi	Polycauliona
Fungi	Polylobatispora
Fungi	Preussia
Bacteria	Prevotella
Bacteria	Prevotella_7
Fungi	Pringsheimia
Fungi	Pseudobensingtonia
Fungi	Pseudocamarosporium
Fungi	Pseudocosmospora
Bacteria	Pseudofulvimonas
Bacteria	Pseudomonas
Fungi	Pseudoophiobolus
Fungi	Pseudophloeospora
Fungi	Pseudopithomyces
Fungi	Pseudosoloacrosporiella
Bacteria	Pseudospirillum
Bacteria	Pseudoxanthomonas
Bacteria	Psychrobacter
Fungi	Punctelia
Fungi	Pyrenochaetopsis
Fungi	Pyrenophora
Fungi	Pyrigemmula
Bacteria	Quadrisphaera

Fungi	Rachicladosporium
Fungi	Radulomyces
Bacteria	Rahnella
Bacteria	Ralstonia
Fungi	Ramularia
Fungi	Resinicium
Fungi	Retiarius
Fungi	Rhamphoria
Fungi	Rhamphoriopsis
Bacteria	Rheinheimera
Fungi	Rhinocladiella
Bacteria	Rhizobacter
Bacteria	Rhizobium
Bacteria	Rhizomicrobium
Bacteria	Rhodanobacter
Bacteria	Rhodopila
Bacteria	Rhodopseudomonas
Fungi	Rhodosporidiobolus
Fungi	Rhodotorula
Bacteria	Rhodovastum
Fungi	Rhodoveronaea
Fungi	Rhytisma
Bacteria	Rickettsiella
Bacteria	Roseimaritima
Bacteria	Roseococcus
Bacteria	Rothia
Bacteria	Rubrivirga
Fungi	Ruptoseptoria
Fungi	Rutstroemia
Fungi	Saccothecium
Fungi	Samsoniella
Bacteria	Sanguibacter
Fungi	Sarocladium
Fungi	Sawadaea
Fungi	Scleromitrula
Fungi	Scoliciosporum
Fungi	Scopulariopsis
Fungi	Scytalidium
Bacteria	Sediminibacterium
Fungi	Seimatosporium
Fungi	Septobasidium
Fungi	Septoriella
Fungi	Serendipita
Bacteria	Shinella

Bacteria	Simiduia
Fungi	Simplicillium
Bacteria	Singulisphaera
Fungi	Sistotrema
Bacteria	SM1A02
Bacteria	Smaragdicoccus
Bacteria	Solibacillus
Fungi	Solicoccozyma
Bacteria	Sorangium
Fungi	Sordaria
Fungi	Spencermartinsiella
Fungi	Sphaerulina
Bacteria	Sphingobacterium
Bacteria	Sphingomonas
Bacteria	Spirosoma
Fungi	Spizellomycetales
Fungi	Sporidesmium
Fungi	Sporobolomyces
Fungi	Sporormiella
Fungi	Stagonospora
Bacteria	Stakelama
Bacteria	Staphylococcus
Fungi	Steccherinum
Fungi	Stemphylium
Bacteria	Stenotrophomonas
Fungi	Stereum
Bacteria	Streptococcus
Fungi	Stypella
Fungi	Symbiotaphrina
Fungi	Symmetrospora
Fungi	Sympoventuriaceae
Bacteria	Taibaiella
Fungi	Taphrina
Fungi	Taphrinaceae
Bacteria	Tardiphaga
Bacteria	Tatumella
Fungi	Teichospora
Bacteria	Telluria
Fungi	Teratosphaeria
Bacteria	Terriglobus
Fungi	Teunia
Bacteria	Thalassobacillus
Fungi	Thanatephorus
Fungi	Thelebolus

Bacteria	Thermomonas
Fungi	Thoreauomyces
Fungi	Tilachlidium
Fungi	Tolypocladium
Fungi	Torula
Fungi	Toxicocladosporium
Bacteria	Trabulsiella
Fungi	Trechispora
Fungi	Tremella
Fungi	Tremellodendropsidales
Fungi	Trichaptum
Fungi	Trichobolus
Fungi	Trichoderma
Fungi	Trichomerium
Fungi	Trichosporon
Bacteria	Truepera
Fungi	Tubakia
Bacteria	Tyzzerella
Fungi	Udeniomyces
Fungi	Umbelopsis
Fungi	Uredinophila
Fungi	Ustilago
Bacteria	Variovorax
Bacteria	Vasilyevaea
Bacteria	Veillonella
Fungi	Verrucoccum
Fungi	Vexillomyces
Fungi	Vibrissea
Bacteria	Viridibacillus
Fungi	Vishniacozyma
Fungi	Volucrispora
Fungi	Vuilleminia
Fungi	Wallemia
Bacteria	Weissella
Bacteria	Williamsia
Bacteria	Wolbachia
Fungi	Xanthoria
Fungi	Xenoacremonium
Fungi	Xenodevriesia
Fungi	Xenophoma
Fungi	Xenopyrenochaetopsis
Fungi	Xenoseimatosporium
Bacteria	Yersinia
Fungi	Yunzhangia

Fungi	Zeloasperisporium
Fungi	Zymoseptoria
Fungi	Zyzygomyces

2715

Appendix 2 – Important Entomopathogenic Genera

Bacillus

Bacillus is well studied in terms of insecticidal effects with many strains of *Bacillus* used worldwide as a microbial insecticide. The most prominent form is that of *Bt* with several strains in use depending on the target group. Its presence is unsurprising as *Bacillus* occurs naturally in the gut microbiome of many species, including lepidopterans. However, external *Bt* applied as pesticide cannot be ruled out as the cause of caterpillar mortality as the insecticide is only comprised of the Cry toxins that the bacteria produce which are then consumed by the caterpillars ultimately causing death.

Beauveria

Beauveria, is a well-studied genus, and species have found uses in biocontrol of pests (e.g. *Beauveria bassiana*), bioremediation of industrial effluent and heavy-metal pollution (Singh *et al.*, 2015). *Beauveria* is a common choice for the biocontrol of pests with products synthesised from this fungi including *Metarhizium* which comprises nearly 70% of all mycoinsecticides (Mascarin and Jaronski, 2016). Typically, products have been applied through a spray directly to target species, although this has limitations. New approaches are looking to use *Beauveria* as an endophyte, with evidence that plants that have been endophytically colonised by *Beauveria* results in pests feeding on those plants having reduced fitness (Ownley, Gwinn and Vega, 2010; Singh *et al.*, 2015). This method would ultimately be favourable for defending against pests, as it would require minimal treatments and would be long lasting, however, this type of blanket protection that persists in the environment can also vastly increases the risk of non-target effects.

What is interesting however, is that *Beauveria* is not typically used to control OPM in the UK with the sole biopesticide used being *Bt* despite knowledge that the fungi can infect processionary moths (de Boer and Harvey, 2020). Taking into account the risk of resistance forming when a small number of pesticides are used to control a species, *Beauveria* should be seriously considered for the management of OPM as a link to mortality is shown here.

Pseudomonas

Pseudomonas are a genus of gram negative bacteria with some species being well known insect pathogens that have been studied extensively for their insecticidal activity (Mashtoly *et*

al., 2011; Sarkhandia *et al.*, 2023). In particular, *Pseudomonas aeruginosa* has shown to lead to high mortality rates in insects, with some Lepidoptera suffering up to 93% mortality due to the bacterium (Osborn *et al.*, 2002). Furthermore, some *Pseudomonas* sp. were found to increase the toxicity of both *Bt kurstaki* and *Bt aizawai* (Mashtoly *et al.*, 2011). However, despite this, the species of the genus have not been used to produce microbial pesticides in a similar fashion to *Bacillus* and *Bt*. The primary reason for this is that *Pseudomonas,* particularly *P. aeruginosa*, is known to infect humans and cause disease (de Bentzmann and Plésiat, 2011). In both its insect and human hosts *P. aeruginosa* is an opportunistic pathogen, as it mainly infects humans in hospital settings, with post-operation patients, or patients with devices that breach our outer immune system (catheters, respirators), most at risk (Harrison *et al.*, 2006; de Bentzmann and Plésiat, 2011). *P. aeruginosa* is also commonly associated with microbial resistance (Diggle and Whiteley, 2020). This combination of factors makes this genus an unsuitable candidate for microbial biocontrol.

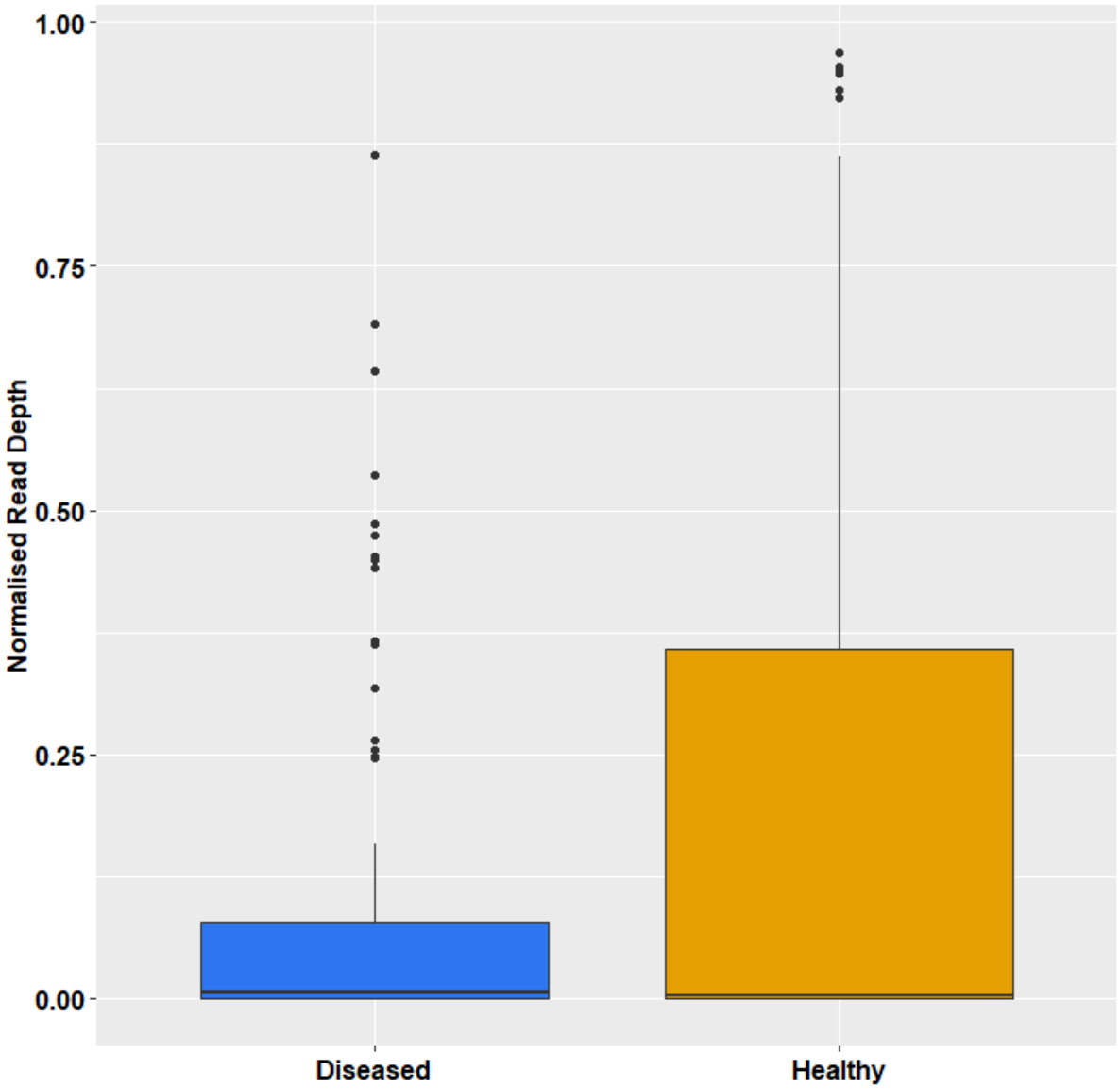
Appendix 3 – Read Count

Normalised Read Count

Normalised read count for each full community is presented in Figure 5.13, Figure 5.14, and Figure 5.15 and the differences between healthy and diseased samples is tested using Mann-Whitney U tests TbleXXX. For the bacterial community the difference between normalised red count of healthy and diseased samples is non-significant (p-value = 0.098). For the fungal (p-value = < 0.001) and combined (< 0.001) communities there is significant difference.

Proportion of Read Depth

The proportion of read depth that is made up by entomopathogenic genera in both healthy and diseased samples for both bacterial and fungal communities is presented in FIG and FIG. Differences between entomopathogenic proportional read depth were tested using Chi Square presented in TBL. Neither community showed a significant difference with both communities presenting p-values = 1.



2772

2773 Figure 5.13: Bar chart showing normalised read depth difference in diseased and healthy bacterial
2774 communities.

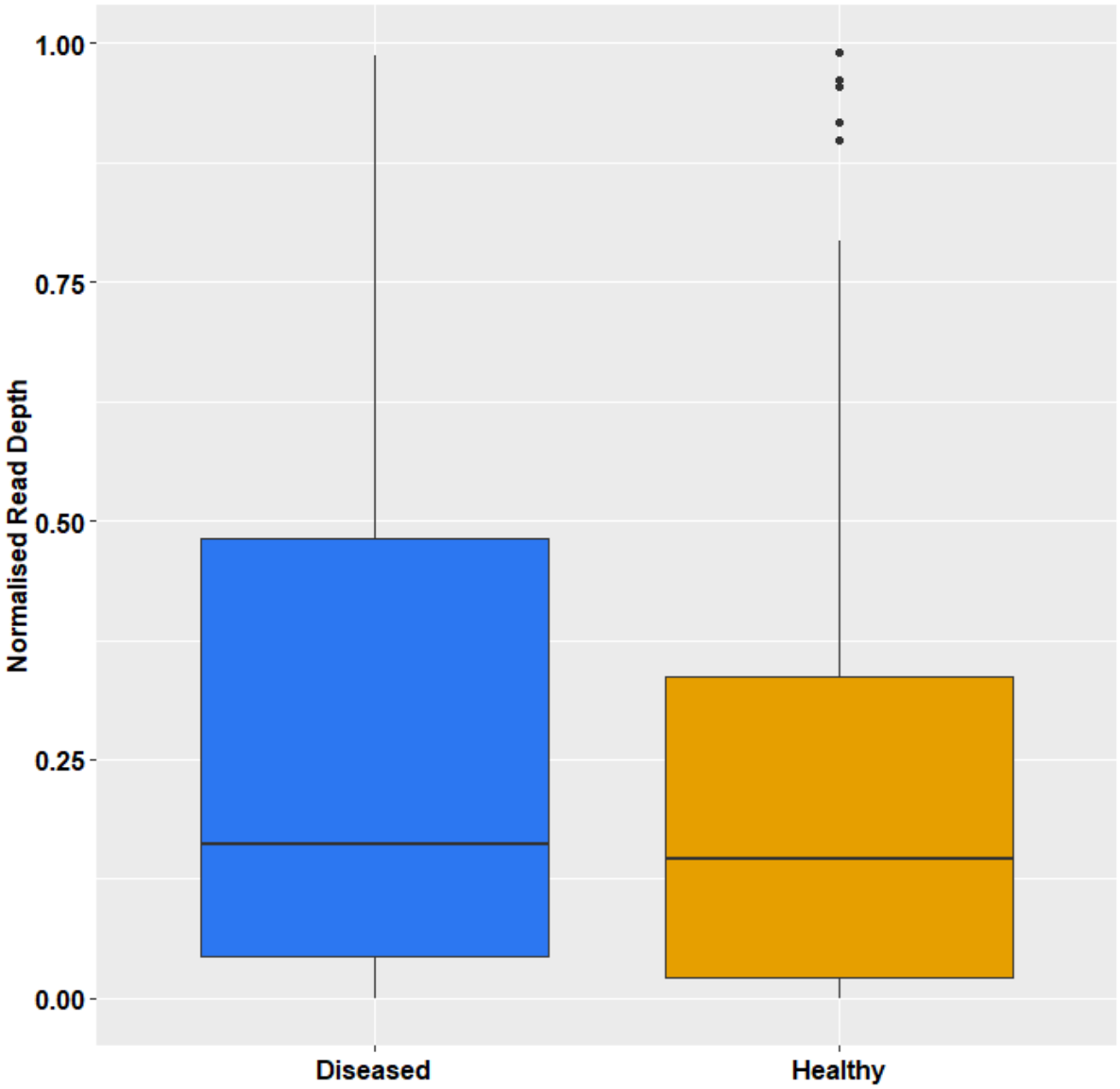


Figure 5.14: Bar chart showing normalised read depth difference in diseased and healthy fungal communities.

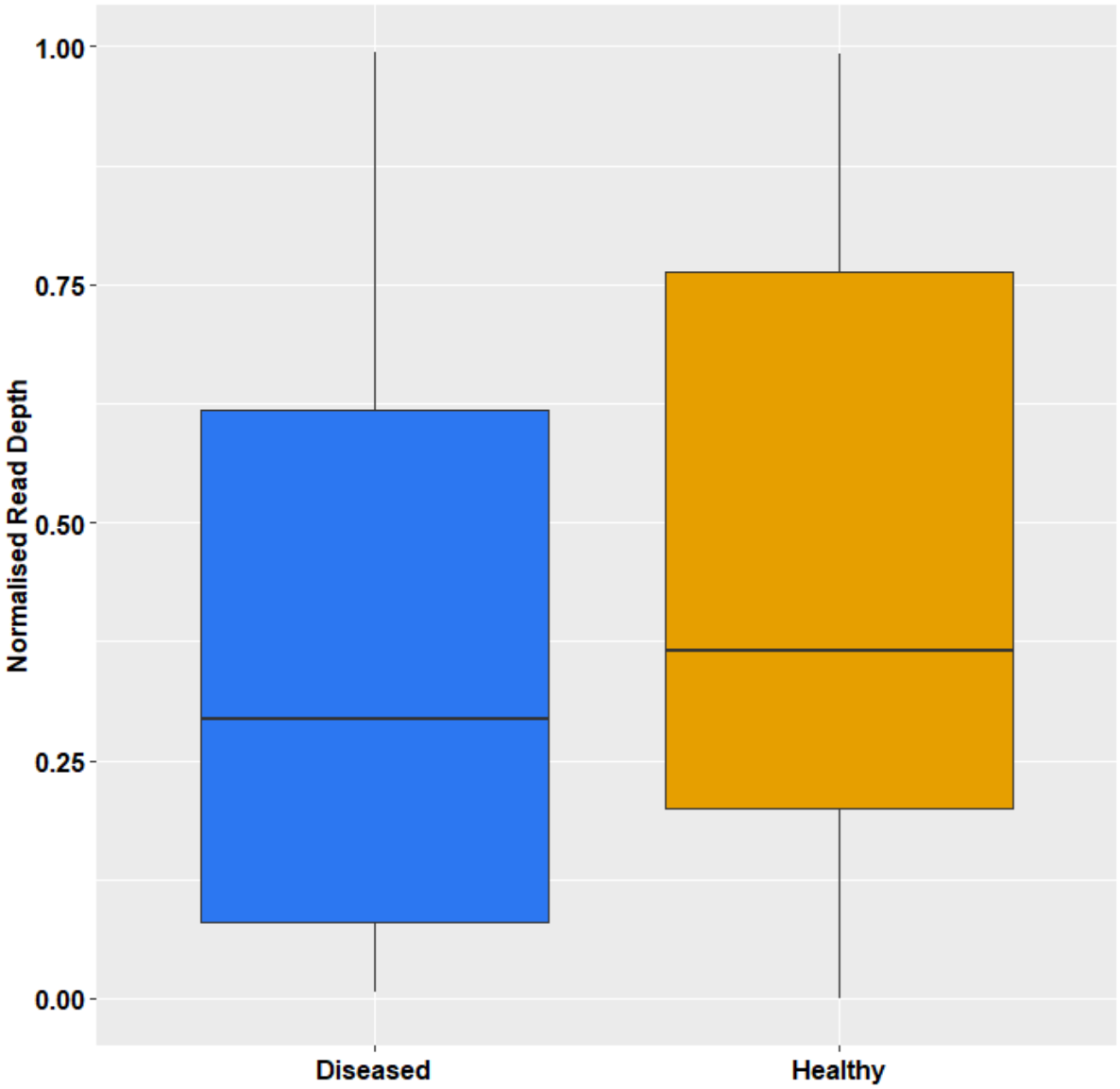


Figure 5.15: Bar chart showing normalised read depth difference in diseased and healthy combined communities.

Table 5.6: Results of Mann-Whitney U test, testing for differences in normalised read depth between diseased and healthy samples for different communities.

	W	**p-value**
Bacterial	165358210	0.098
Fungal	604945296	< 0.001
Combined	1147072752	< 0.001

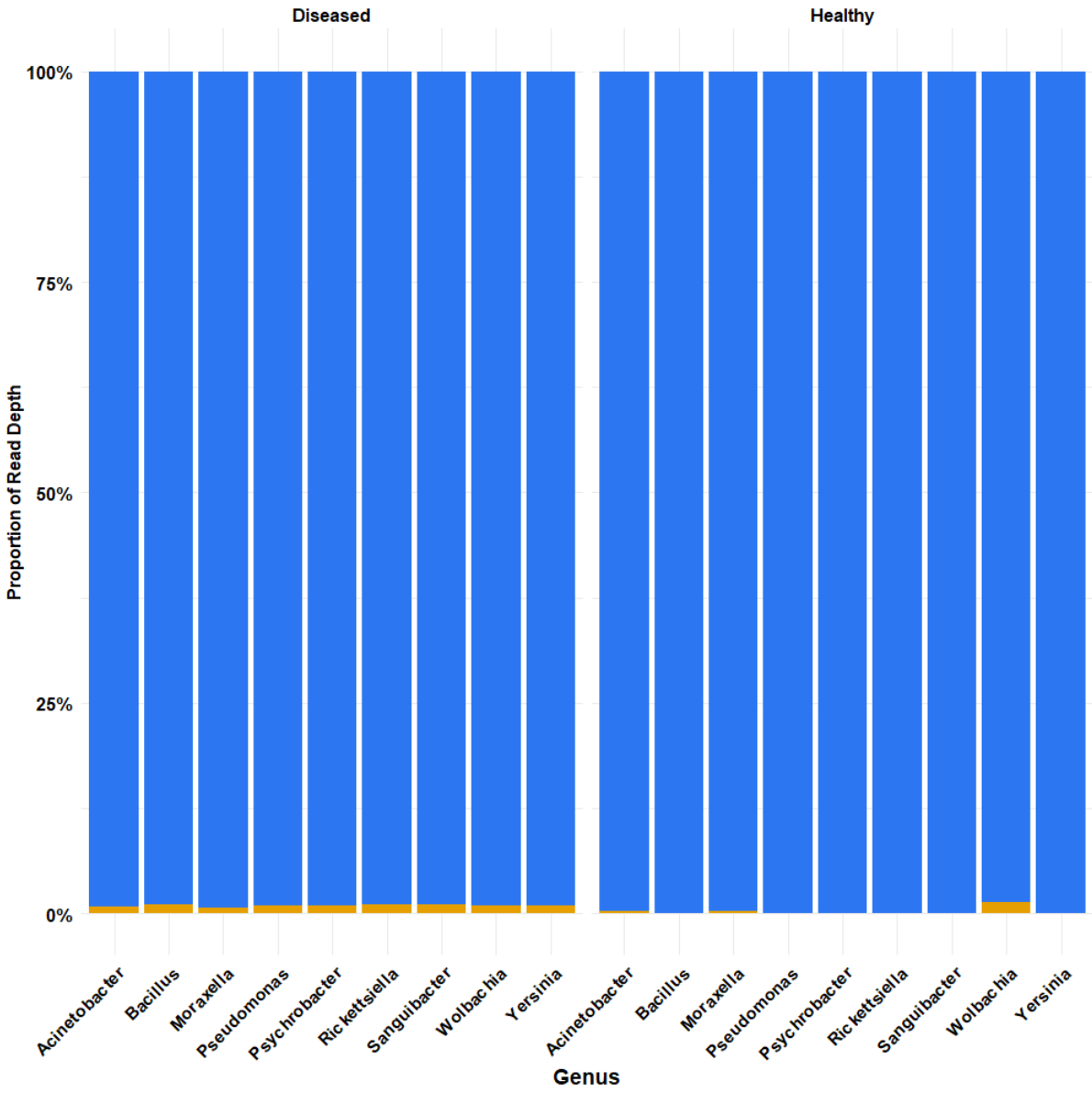
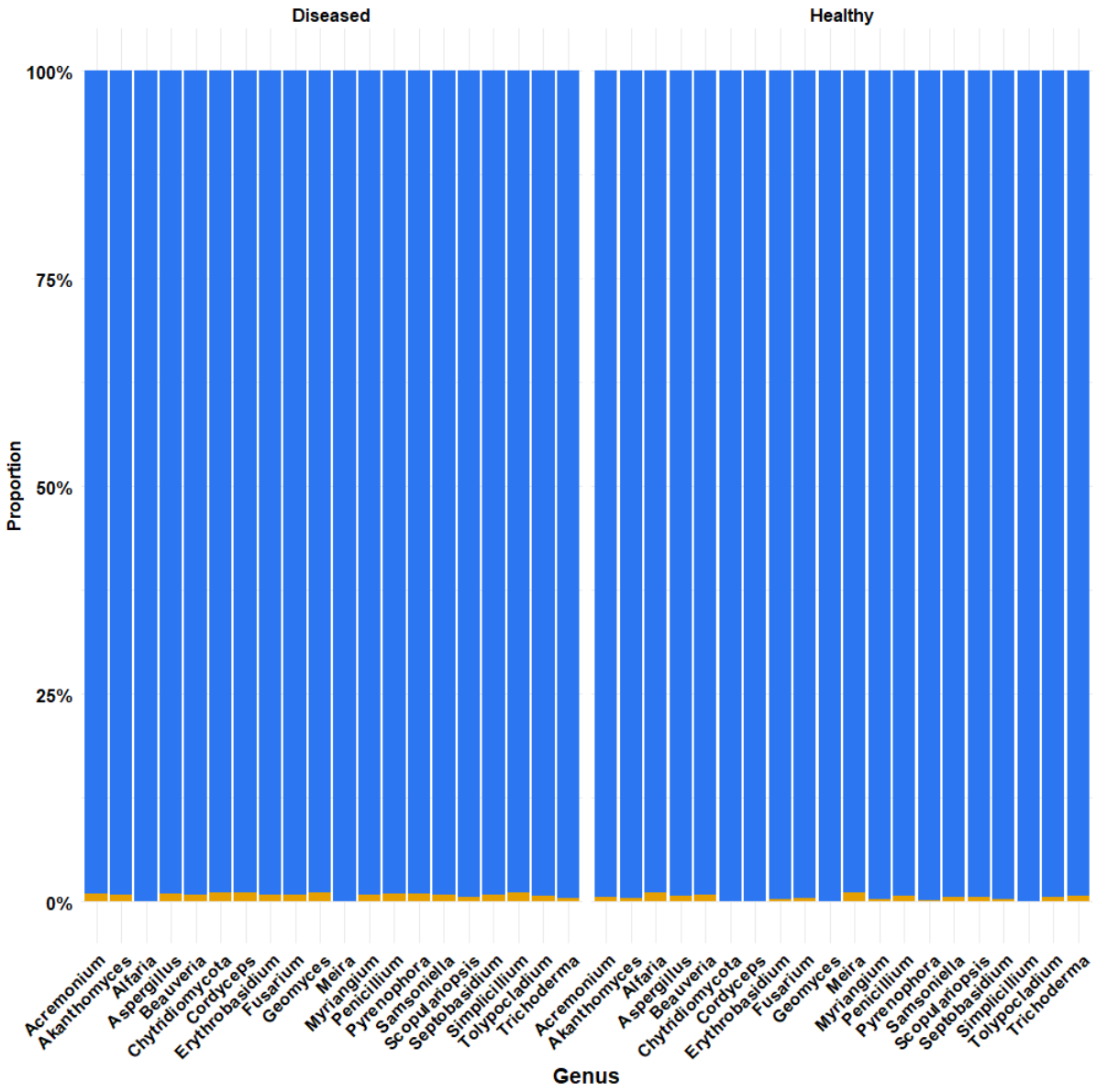


Figure 5.16: Stacked bar chart showing proportion of read depth made up by entomopathogenic genera in healthy and diseased bacterial samples.



2787

2788 Figure 5.17: Stacked bar chart showing proportion of read depth made up by entomopathogenic
2789 genera in healthy and diseased fungal samples.

Table 5.7: Results of Chi Square test which was used to test for differences in the proportion of read depth made up by entomopathogenic genera in healthy and diseased samples.

	X^2	df	p
Bacterial	1.8889	24	1
Fungal	5.8412	57	1

Read Depth

Two methods were used to investigate which factors were affecting disease status. Both normalised read depth of communities compared between disease states and proportion of entomopathogenic read counts in both healthy and diseases samples. While there were significant differences in normalised read counts for both fungal and combined communities there was no unified pattern to suggest this method was useful for identifying patterns in disease state. Proportion of entomopathogenic read counts in healthy and disease samples was tested for both bacterial and fungal communities but when formally tested there were no significant relationship present.

Chapter 6 General Discussion

Chapter Summary

Through the combined use of state-of-the-art molecular techniques, bioinformatics pipelines, and statistical analysis this thesis has provided both new molecular protocols designed to detect parasitism in OPM samples, and in-depth analysis of the ecology of biocontrol agents to better inform the management strategies of OPM in the UK, satisfying the overarching aim established in Chapter 1. This aim was linked across three data chapters to address each of the three aims set out in the introduction:

Chapter 3

I. *Develop a rapid, cheap, DNA-based field assay to confirm the presence of C. iliaca in OPM larvae.*

Following industry standard protocols, a successful LAMP assay is developed and validated to test for the presence of *Carcelia iliaca* in oak processionary moth tissue samples. In this chapter, the assay was specific enough to be able to differentiate between *C. iliaca,* other potential parasitoids, and host DNA. The performance of this LAMP assay was vital to the completion of this work however, it is also important in the wider context of monitoring and managing pest and invasive species.

Chapter 4

II. *Sample OPM larvae and record the distribution of C. iliaca in London to better understand the distribution of C. iliaca in the Greater London area.*

III. *Use generalised linear models (GLM) to determine the environmental covariates associated with C. iliaca, using the data gathered from objective two to better inform management approaches.*

The LAMP assay designed in Chapter 3 was successfully deployed to detect *C. iliaca* from samples collected from a range of sites across the Greater London area. This provided the first widespread distribution data of the parasitoid. This was then added to models alongside abiotic environmental data to determine which factors would be good predictors. Whilst the relationships between *C. iliaca* and environmental variables was too complex to capture in the statistical analysis provided here, the results of this chapter did open several avenues of future work which may shed light on this.

Chapter 5

IV. *Use DNA metabarcoding to better understand the fungal and bacterial communities associated with healthy and diseased OPM larvae to determine whether there are novel entomopathogens that could be used for OPM management.*

In this chapter both bacterial and fungal microbiomes of diseased and healthy OPM samples are explored to better understand which species are drivers of disease. Whilst some common entomopathogens were discovered in the samples further statistical analysis was unable to highlight a strong relationship between the presence of certain species, species richness, and species diversity and disease status. A better understanding of the underlying mechanisms underpinning this interaction need to be described to properly utilise entomopathogens as forms of biocontrol. An unexpected finding as part of this work is the association of an endosymbiont, which has shown to play roles in conferring resistance to numerous insecticides and parasites, with healthy samples which underpins the importance of having diverse management techniques to sustainably control pests and invasive species.

The key findings of this thesis raise several possible implications which include the methodological implications of the protocols and workflows that have been developed, the ecological implications of the primary research observations, and the policy and management implications of the key ecological findings for the understanding and management of invasive pest species through biocontrol.

Method Implications

Rapid diagnostics to better sample parasitoids

Parasitoids are amongst the most common species on our planet with around 10%-20% of insects employing this life strategy (van de Kamp *et al.*, 2018). However, due to difficulty in morphological identification they can be underrepresented in ecological studies and even ignored despite the overwhelming impact they can have on ecosystem structure and the provision of services. Molecular techniques have revolutionised the way we conduct ecology producing phenomenally large quantities of data and providing insights that previously were not accessible (Kitson *et al.*, 2019; Evans and Kitson, 2020). However, they are still not fully utilised for the sampling and monitoring of parasitoids with many studies opting for more traditional entomological sampling approaches that focus on bulk sample collection followed by individual specimen identification. Molecular approaches, such as next generation sequencing, have become much more common place when processing bulk samples with the benefit of removing long hours of identifying individual specimens. However, despite the benefits these approaches can still be expensive, particularly for routine work that needs to be completed regularly like monitoring. More direct diagnostic methods which target only one taxon of interest can often be much cheaper and faster to implement. Therefore, in instances such as monitoring a select group of species, this approach is justifiable with only specific questions needing to be answered.

Rapid diagnostic techniques like LAMP have swiftly been adopted for the diagnoses and monitoring of plant pathogens and for the identification of cryptic species on imported goods, with these tools now seen as essential for this work (Blacket *et al.*, 2020; Zou, Mason and Botella, 2020; Deliveyne *et al.*, 2023). A similar approach should be taken to monitor and study parasitoids. Firstly, on the basis that whilst costs are ever decreasing for next generation sequencing work, the cost can still prove to be a barrier to accessibility. Rapid diagnostics are designed to be inexpensive by nature, as the need to test widely renders expensive tests unfeasible, therefore providing a cost-effective method which provides accessibility. Similarly, rapid diagnostic tests tend to require a lower training level and level of equipment, again increasing accessibility.

Secondly, from a sampling perspective, rapid diagnostics provide two benefits. Further to the accessibility benefits of lower costs, there is also the benefit of accessibility to identification. This is pertinent to all molecular methods, which allow taxonomic novices to attain highly resolved taxonomic data comparable to that of experts without the need for extensive training. However, when combined with the quick access to these results compared to technologies like NGS, it allows for near instantaneous results for a much broader audience. The second benefit from a sampling perspective is the generation of abundance data which many sequencing technologies used for metabarcoding cannot provide, which can then be used to answer a range of ecological questions.

Here this work demonstrates the benefits of producing accessible, rapid diagnostic tests to monitor parasitoids of an invasive pest species. Multi-year parasitoid distribution data from across Greater London has been able to be generated for a fraction of the cost of other molecular techniques and the work has provided data for sites that would not typically be able to afford other methods. This data is now readily available for sites to incorporate into their decision making and with an accessible test they can also monitor parasitism rates going forward.

Policy and Management Implications

Despite the technical nature of many of this thesis chapters the core aim at the heart of this work has been to inform decision making around the management of OPM. In many respects this work only scratches the surface of the information needed for a fully comprehensive management framework however there are some key thoughts to be taken from this.

Importance of monitoring

Annual variation in insect populations is driven by a range of factors including biological interactions, environmental pressures, resource availability, and intergenerational effects (Boggs and Inouye, 2012; Sánchez-Bayo and Wyckhuys, 2019; Hu *et al.*, 2021; Wilson and Fox, 2021). This is true for both OPM and *C. iliaca* and untangling how important each of these factors contributes to fluctuations is important for two reasons:

I. Determining when OPM will have outbreak years so that effective allocation of management resources can take place.
II. Determining whether *C. iliaca* populations are stable enough in a given area to suppress the local OPM population.

A lack of resources is often credited as a barrier to effective long-term monitoring in ecology and as OPM continue to spread, the increased cost in both time and person power will likely contribute to lower efforts to monitor as robustly. However, in this work, the assay provided in chapter three could offer a solution to be able to monitor both populations at the same time. OPM populations are predicted to cycle, with a population steadily increasing to a peak before falling back to base levels over that period (Battisti *et al.*, 2015). As OPM is the primary resource for breeding *C. iliaca* it is hypothesised that this cycle is also followed by this species as resource availability peaks and wanes, a hypothesis that has some evidence based on the time series of parasitism presented in chapter four.

If parasitism is to be incorporated into management plans, then a regular assessment will need to be made to make sure that practises are data informed. Therefore, the deployment of the assay on a regular basis will not only provide a parasitism rate for a site but also an insight into how OPM populations are performing. Whilst an individual year may not provide much information, being able to infer the trend of OPM populations would allow for more focussed planning and strategic resource allocation when bad OPM years are predicted.

Potential resistance forming

The evolution of resistance to pesticides is a well-documented problem with widespread changes to management plans occurring to manage resistance and find more sustainable options. A factor that contributes to the formation of pesticide resistance is the lack of options available to land managers. Limited arsenals provide only one form of action, meaning it is easier for organisms to evolve some kind of resistance. This is highlighted particularly well with OPM where only *Bt* is currently used to control the species. However, previously there has been no suspicion that resistance to *Bt* has formed within OPM. This work, as part of an investigation into novel entomopathogens, has shown a link between healthy OPM samples and an endosymbiont that is known to convey resistance to pesticides in some species. Whilst the relationship between OPM, *Wolbachia* and health status of collected caterpillars currently remains just a link it is a fundamental question that needs to be answered with huge ramifications for policy and management if the effectiveness of *Bt* is at risk. With the UKs commitment in its 25 year environmental plan to reduce pesticides in the environment it also serves as a timely reminder that a review of the current available options for control and their long term sustainability should be a priority (HM Government, 2018).

Ecological Implications

Case study for future species

Oak processionary moth is not the first invasive forestry pest in the UK and will not be the last with species such as emerald ash borer (Dawson *et al.*, 2022; Forest Research, 2022c) likely to invade and establish within the next decade. However, what does make this species unique is that it comes during a time of transition for many management approaches across the UK and Europe as a whole, as management strategies seek to incorporate a wider range of nature-based solutions in favour of chemical pesticides. As OPM continues to spread and as more techniques are trailed in its management there are many lessons to be learned in how the countries future approach to invasive species will look. However, there are some immediate lessons from this body of work.

Firstly, it is clear that to incorporate nature-based solutions such as conservation biocontrol, an in depth understanding of agent life history and ecology is required (Miller, Polaszek and Evans, 2021). In many respects the work in this thesis raised more questions than answers around the dynamics of OPM and *C. iliaca* however, what has become apparent is that the relationship between the two species is complex and requires further study. If biocontrol is to take a prominent role in the tool kit of land managers and conservationists, then the resources must be made available to begin to understand the fundamental ecology that drives the interactions between important species.

Secondly, the traditional methods in which we have surveyed ecosystems for ecologically important information that relates to management strategies, like parasitism rates, may no longer be enough. Over the course of the 2010s and into the 2020s ecology has become a big data science employing powerful tools from other fields like next generation sequencing and machine learning which has advanced ecological research immeasurably. However, for applied ecologists or those who use applied ecology in their field, uptake of these technologies can still be slow, primarily due to cost or lack of trust. This work provides more evidence that cost effective, reliable tools can be developed from these technologies which will aid in the gathering of ecological data and lead to more evidence-based decision making.

Limitations

Chapter 3

For chapter 3 the first limitation is with the primer design where only a small number of sequences were available for a multiple sequence alignment to base primers on. This theoretically means that there may be individuals with single nucleotide polymorphisms (SNPs) at priming sites that are not detected. However, during testing on DNA extracted from parasitoids no individuals were found that did not amplify with the primer sets presented in this work. Ultimately with no evidence to suggest otherwise this remains a theoretical limitation and, based on the results so far, would largely have little consequence on results with appropriate sample sizes. This question could be clarified further with more sequencing of *C. iliaca* genomes from across the UK.

The second limitation of this chapter remains around the lack of "pure" *C. iliaca* DNA which was needed to test the specificity of the primers and rule out any non-specific amplification. Both larval and adult *C. iliaca* were used for DNA extractions and both tested using all sets of primers. However, whilst it Is almost certain that any extraction using larval *C. iliaca* would contain OPM DNA, due to the larvae feeding and living exclusively in OPM larvae, the issue stems from the uncertainty around how adult *C. iliaca* may retain OPM DNA after they have emerged. Another approach may have been to extract DNA from a host specific viewpoint and test to see whether parasitoid lacking hosts amplified. However, this approach may lend itself to confusion with failed parasitism attempts being detected due to LAMPs ability to detect extremely low quantities of DNA. Whilst this issue was resolved by introducing an amplification threshold it was not possible to completely rule out the possibility of cross reaction with host DNA occurring. Future work on host-parasitoid systems may look to avoid this issue by using synthetic DNA to create "pure" target controls.

Chapter 4

Limitations affect two aspects of this chapter. The first is the availability of sites for fieldwork where the majority of sites were distributed in the northwest and southwest and there is a clear lack of sites in the southeast meaning that spatial trends cannot reliably be inferred. Secondly, there are several sites that have much higher numbers of samples compared to others. Whilst these sites are not necessarily an issue the sites with lower numbers of samples

do represent a lower accuracy in terms of the final parasitism rate. There is little that can be done to mitigate post sampling apart from caution when interpreting results. However, pre-sampling survey design was as robust as possible with several back up sites used where sampling was not possible on primary sites. Finally, the resolution of the data used in the modelling must also be considered. Data that was collected as part of fieldwork (primarily habitat cover and grassland type) were quick snapshots of generic habitat types. It was not possible to do in depth vegetation surveys, the likes of which may be able to explain the presence of *C. iliaca* particularly with tachinids apparent favour of Apiaceae. Like wise much of the climate data comes from fairly large spatial scales which may not capture what is occurring at the local scale that OPM and parasitoids might operate at. Despite this, the data available was the best data possible and thus the models can only be interpreted with this in mind.

Chapter 5

The primary limitation with the work in this chapter lies with the samples used. The discovery of the diseased larval samples came during nest dissections of nests that had been collected during mid-summer. Any remaining healthy larvae had spun a pupal chamber and begun its pupation meaning the only samples available for comparison were pupae. There have been several studies documenting the change in microbiome of lepidopteran larvae over the course of their larval development and into pupae (Hammer, McMillan and Fierer, 2014; Gao *et al.*, 2019; Mereghetti *et al.*, 2019; Wang *et al.*, 2020). Whilst this has not been documented with OPM it would be wise to assume so when interpreting these results. To try and mitigate this impact, only known entomopathogenic genera were chosen for analysis as species of these genera are unlikely to be as heavily influenced by life stage if they are acting as infectious agents.

Knowledge Gaps

Understanding the OPM invasion pathway and OPM evolution

The initial pathway for OPMs arrival into the UK has already been well described however, there remain several questions around the UK population of OPM. Currently it is not understood whether this has been the only successful migration event of OPM to the UK. If this is the case what is the genetic diversity of this population? Is the population inherently

unstable due to a low gene pool? And does this population have genetic adaptations that have allowed it to be successful in the UK climate? If there has not been only one successful migration event, what is the origin of new influxes? With a resident population do channel crossing males have a chance to input into this gene pool? Or are more individuals being missed on improperly checked imports? Likewise, the same questions could be asked around parasitoids such as *C. iliaca* and *Pales processionea,* which are now able to establish in the UK due to OPM, including what will the final biocontrol dynamic look like and how will these new parasitoids affect native species?

The answers to these questions have wide reaching consequences from local level management to international trade strategies. At a local level a low genetic diversity in the UK OPM population may mean that it is less resistant to novel biocontrol strategies and also less likely to develop resistance to these strategies in the future meaning land managers can focus on one or two successful techniques. From a big picture perspective, if multiple invasions have occurred, or are currently ongoing, then it means that screening for OPM across the UK border is not successful. This would be in line with other invasive pest species as new establishments continue to occur, as well as continued establishments of novel pest species. This predominately occurs through global trade with the majority of forestry pests arriving on wood products or packaging. This is further compounded by limited biosecurity checks at ports which are constrained by a lack of resources, both financial and physical, lack of standardisation in monitoring practise and limited technical knowledge of staff. Addressing these issues should be a priority for any biosecurity policy reforms to ensure that OPM and other pests are more reliably intercepted and ensuring a truly bio secure border.

Also, understanding whether the UK OPM population has a genetic adaptation that has enabled its success is vital for understanding how much of a threat the UK population (and its source population) proposes to other areas. For instance, a population that is more resistant to increased rainfall and colder temperatures in its early instars may have no trouble expanding further north into territories that have never had OPM such as Sweden, Norway, and Finland. Population genomics would likely be a suitable approach to begin answering these questions and the knowledge gained may be vital to ensuring successful management of this pest where it has caused issues and where it may be a future pest.

Mechanisms underpinning *C. iliaca* parasitism of oak processionary moth

This work is the first descriptive work on *C. iliaca* populations in the UK. However, it does not go much beyond that. Many aspects of *C. iliaca* life history are missing including, adult resource use, self-regulatory mechanisms, dispersal capability, how adults detect host larvae, whether *C. iliaca* suffers from hyper parasitism, and whether *C. iliaca* is truly monophagous. With the work provided in this thesis land managers will be able to generate critical information regarding parasitism rates and *C. iliaca* site distribution which will allow them to understand the impact the parasitoid is having on its host. However, answering the above questions would allow land managers to go beyond this and be proactive about managing in a way that actively benefits *C. iliaca* and allows for planning beyond the current year of testing. This fundamental lack of knowledge around parasitoid life history and how it interacts with its host should be looked to as a guideline for what knowledge we need for future invasive pest species. This combined with horizon scanning could allow for science projects in collaboration, with the international pest management community to build a solid evidence-based database of effective biocontrol agents for important pest species. This would allow the UK at a country wide level to respond to incoming pest species rapidly, sustainably and with evidence-based tools, rather than through reactive approaches that inflict more damage than is necessary.

The role of the microbiome in the success of invasive species and their resistance to management

Recent advances in molecular methodologies have allowed for a deeper understanding into the relationship between insects and their microbiomes, particularly the bacterial symbionts that inhabit them. Many of these relationships are critical for the survival of insects, facilitating feeding and growth, breeding, and immune system responses to pathogens, parasites, and insecticides. These relationships are often good avenues for research as disrupting them can provide novel control methods. However, much of this work has been on model systems, such as *Drosophila,* and widespread agricultural pest species, such as aphids.

The benefits of translating this work to forestry pests such as OPM were shown in this work with an investigation into the microbiomes of healthy and diseased OPM samples. However, to truly be able to explore the relationships between OPM and individual species in the microbiome further work would be necessary. Further research in this system may be able to answer questions around insecticide resistance, as well as discover novel control methods

such as the application of Wolbachia to interfere with immune responses or larval growth. With the answer to these questions having implications for the way OPM is currently managed both in terms of sustainability of current methods but also allowing for a wider range of control options that may be species specific.

Conclusion

This thesis has demonstrated how the novel application of molecular techniques can be used to aid in the control of invasive pest species. Rapid, cost-effective diagnostics have been shown to be an incredibly useful tool in the monitoring of useful species such as biocontrol agents, providing unprecedented accessibility in terms of both cost and identification of species. Likewise, tagged nested metabarcoding has shown its effectiveness in being able to untangle interactions which previously have been difficult to identify. The initial application of these techniques in this work has already provided information that will be useful for local scale management and for informing policy decisions. However, with OPM in the UK there are still many questions that need to be answered regarding the ecology and life histories of the pest and biocontrol agents. Therefore, there is scope for further application of these techniques to answer these questions. The foundational knowledge that has already been provided by these techniques also shows that they will continue to play a role in the prevention and management of forestry pests throughout the 21st century.

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