SAVING AN ICONIC SPECIES FROM EXTINCTION IN THE UK: INTERACTIONS BETWEEN DIET, PARASITES AND ENVIRONMENTAL CHANGE

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy (PhD) in the School of Biosciences by

REBECCA ELIZABETH YOUNG

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Illustration by Rebecca Young

Acknowledgements

In loving memory of my grandmother Mary Hill

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Thesis summary

The Afro-Palearctic migrant European turtle dove (*Streptopelia turtur*) is one of the UK's fastest declining species. Breeding across Europe, and wintering in sub-Saharan Africa, there are several drivers of this species decline, including a dietary switch from predominantly wild to more cultivated seeds, and infection with the protozoan parasite *Trichomonas gallinae*.

This thesis uses molecular methods to investigate the diet of turtle doves, infection with *T. gallinae,* and how these factors interact, and GPS data to analyse home-range and habitat use in the wintering grounds. Studies of this species have primarily focussed on the western European breeding grounds, therefore this thesis includes birds from the wintering grounds, and the eastern and western European breeding grounds.

DNA metabarcoding of dietary items revealed variation between the genera consumed in the breeding and wintering grounds. Cultivated seeds were a prominent component of the diet in all sites, but a range of wild seeds were also detected, and habitat use indicated the importance of natural grasslands for foraging in the wintering grounds. Home-range of wintering turtle doves were considerably larger than previously described on the breeding grounds, potentially due to a combination of scarce food resources and a lack of territorial nesting behaviour. Home-ranges were smaller than previously described for turtle doves wintering in Senegal.

Prevalence of *T. gallinae* ranged from 50% to 83% between sampling populations. Ten haplotypes were detected, four of which have not previously been identified, and co-infection between multiple strains occurred in 17% of birds sampled. The frequency of co-occurrence of strain pairs was lower than would be expected if infections were random, suggesting a mechanism to reduce infection with multiple stains acting within the host. No correlation was detected between *T. gallinae* infection and the consumption of cultivated seeds, and high dietary overlap was observed between infected and uninfected individuals.

Chapter 1 – General Introduction

Photograph by Nicolas Viacrroze

1.1 Overview

Biodiversity is the diversity of plant and animal life present in a habitat, and generally, natural systems exhibiting higher levels of biodiversity are considered more healthy (Swingland 2001), while extensive damage to an environment results in the loss of flora and fauna. With up to 50% of higher taxonomic groups listed as 'critically endangered', the Earth is facing its greatest mass extinction in 65 million years (Cushman 2006; Smith *et al.* 2009). With this vastly elevated rate of species loss and impending extinction rates far exceeding expected background extinction rates, it has never been more important to consider the driving forces behind such declines. From a purely anthropocentric point of view, biodiversity is crucial to most aspect of our lives (WWF 2018). Ranging from food security to medicine, from economic activity to general well-being of individuals, nature and biodiversity are critical in our society (WWF 2018). The factors causing observed population declines are largely a result of human activity, and there are numerous recognised anthropogenic drivers of species loss, including climate change, habitat loss and introduction of invasive species, each of which are known to strongly contribute towards species loss (Lambin *et al.* 2001; Thomas *et al.* 2004;

Clavero and García-Berthou 2005). Understanding the ecological mechanisms underlying the effects of these changes on free-living species is crucial in combating species loss (Cardinale *et al.* 2012). However, the complexity of natural systems can make investigating population dynamics of a species inherently difficult, and it is important to consider multiple ecological contributors and their interactions.

Despite our reliance on the natural world, anthropogenic change in land use is considered to be the main driver of biodiversity decline globally (Sala *et al.* 2000; Chapin Iii *et al.* 2000). In the UK, a period of dramatic change in land use occurred between the 17th and 19th Century, with the British Agricultural Revolution and the advent of modern farming increasing crop and livestock production three to four fold (Pretty 1991). During the last 300 years, more than 50% of the global land surface has been altered, with in excess of 30% of the land surface being used for agriculture and over 25% of forests being cleared permanently (Hurtt *et al.* 2011). Since World War II, further developments in farming practices have resulted in an unprecedented rise in agricultural intensity, particularly in western Europe and North America (Benton *et al.* 2003; Marx *et al.* 2017). The amalgamation of fields by the removal of hedgerows and wild field margins; extensive use of chemical herbicides, pesticides and fertilizers; cultivating crops in monocultures and increased use of autumn sown crops have facilitated the worldwide doubling of agricultural food production between 1965 and 1999 (Benton *et al.* 2002). However, whilst these developing farming practices have improved the yield of crops for human consumption, they have contributed to widespread depletion in natural food resources, loss and degradation of habitats, and overall declines in farmland biodiversity (Brickle *et al.* 2000; Benton *et al.* 2002; Benton *et al.* 2003; Gillings *et al.* 2005). Feeding ecology is an important consideration in species' conservation management, as declines observed in numerous species across a range of habitats are believed to be caused, at least partially, by a loss of habitat providing appropriate food sources (Liu *et al.* 2018). This correlation between food availability and species decline makes diet analysis a key aspect of ecological investigation (Liu *et al.* 2018).

Another important factor to consider, which was overlooked for many years as a driving force in ecological declines, is disease (Tompkins and Jakob-Hoff 2011). Now considered among the top five causes for species extinction globally (Smith *et al.* 2006), disease was possibly disregarded in the past due to a lack of understanding and information surrounding this area of ecology (Smith *et al.* 2009). The International Union for Conservation of Nature (IUCN) Red List has records of species extinctions dating back to the 1500s. In their 2006 review, Smith *et al.* (2006) found that only 3.7% of the 833 known extinctions listed at the time were attributed, at least in part, to infectious disease. This may be an underestimate due to the difficulties of collecting retrospective evidence of disease causing extinction (McCallum and Dobson 1995): disease has been implicated in the extinction of numerous species of different genera, but such cases lack definitive evidence (McCallum 2008). For example, Avian Malaria and Avian Pox are thought to have led to the extinction of up to 13 Hawaiian land birds (Smith *et al.* 2006), but insufficient evidence exists to rule out other factors. Similarly, the fungal pathogen *Batrachomchytrium dendrobatidis* causes the disease chytridiomycosis in amphibians, and is believed to be responsible for dramatic declines and possible extinctions in several amphibious species, however again unequivocal evidence of this is lacking (Skerratt *et al.* 2007).

Impact of disease may also be underestimated as a result of incomplete information regarding disease in the wild: in 2006 only 39% of critically endangered and endangered artiodactyls, carnivores and primates had published records of pathogens from wild populations (Pedersen *et al.* 2007). It seems likely that of the 61% without records, disease will serve some level of threat to survival, particularly as outbreaks of infectious diseases have the potential to spread rapidly and diminish populations (Lawson *et al.* 2011).

The threat from disease may be greater following population decline, as transmission of disease can hinder population recovery, or worse, kill off the remaining population (Cunningham and Daszak 1998; Bunbury *et al.* 2007). For example, the Polynesian tree snail, *Partula turgida*, was endemic to the Society Island of Raiatea, French Polynesia, but became extinct in the wild following the introduction of the predatory snail *Euglandina rosea*

(Cunningham and Daszak 1998). This carnivorous snail was widely used as biological control to manage the population of giant African land snails (*Achatina fulica*) on several islands, including French Polynesian islands, Mauritius and Hawaii (Griffiths *et al.* 1993; Lowe *et al.* 2000; Gerlach 2001; Holland *et al.* 2012). However, predation of *P. turgida* by *E. rosea* resulted in the extinction of the former in the wild, reducing this species to a single captive population (Cunningham and Daszak 1998; Coote and Loeve 2003). Exacerbated by high population density, the rapid spread of a microsporidian parasite within the remaining captive population of *P. turgida* resulted in the extinction of the Polynesian tree snail in 1996 (Cunningham and Daszak 1998). This was the first, and to date only, unequivocal example of species extinction resulting from disease, exemplifying the impact disease can have, particularly on already diminished populations, and thus its importance from a conservation perspective. In recent years, as the recognition of these damaging effects of disease on wild populations has increased, so has the interest and research effort in this area.

1.2 European Turtle Dove

The European turtle dove, *Streptopelia turtur* (hereafter turtle dove) is an Afro-Palearctic (AP) migrant member of the Columbidae family: the UK's only long-distance migrant columbid (Jarry 1995). Their wide, western Palearctic breeding range begins at the Mediterranean, incorporating most of Europe, excluding the most northerly regions, and they spend winter in sub-Saharan Africa (Figure 1.1) (Browne and Aebischer 2005). Following three major migratory flyways: the western, central and eastern pathways (Figure 1.1) (Browne and Aebischer 2005; Marx *et al.* 2016), turtle doves undergo a 4000km journey to breeding grounds across Europe, where they arrive in late April (Browne and Aebischer 2003b). Based on ring recovery data from Czech, Hungarian, British, German and French birds, Marx *et al.* (2016) found that the vast majority of British (94%) and German (92%) birds, and a large proportion of French (62%) birds followed the western flyway. They identified more of an overlap in birds from the Czech Republic and Hungary using the central and eastern flyways (Figure 1.2), with 56% of Czech birds using the central flyway and 55% of Hungarian birds using the eastern flyway. This differential use of

migratory routes suggests a divide between populations from different regions across Europe.

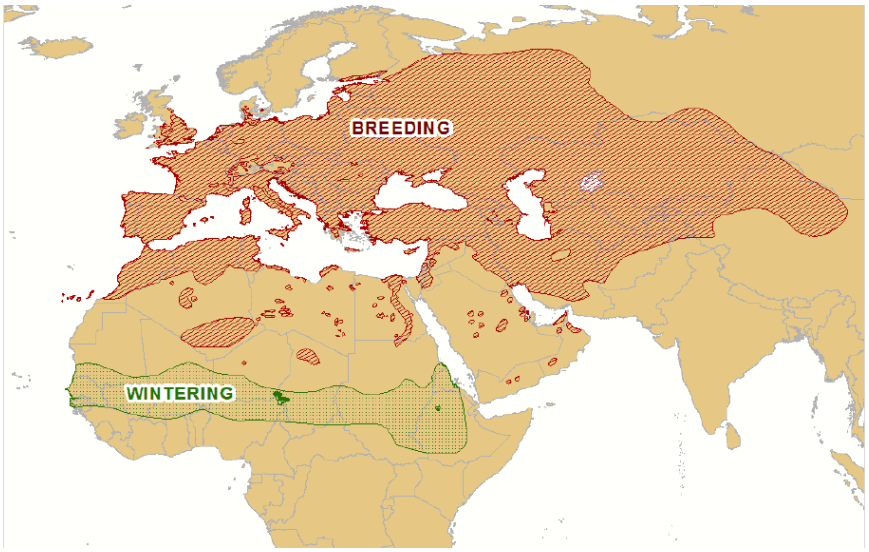

Figure 1.1: Map of the breeding and wintering range for the European Turtle dove (Streptopelia turtur). Breeding in red lines, wintering in green spots. (Figure from BirdLife International 2016, via Fisher et al. 2018).

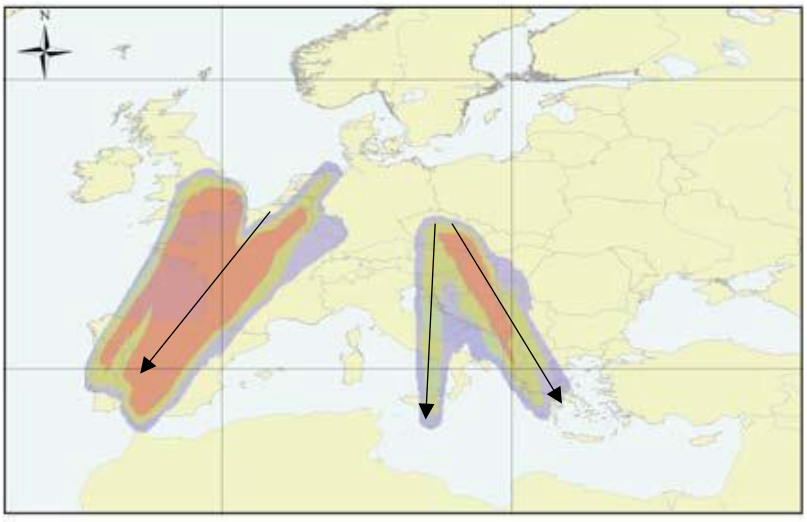

Figure 1.2: Flyway usage of turtle doves from five countries across Europe (UK, France, Germany, Czech Republic and Hungary) Figure is taken from Marx et al 2016 with adaptation of arrows added to clarify flyways. Line density kernels for 70% (red), 80% (yellow) and 90% (blue) of turtle doves.

In the UK, turtle doves are classified as a farmland species, nesting in overgrown, thorny bushes, hedgerows and scrub, with proximity to appropriate feeding sites, such as grassland areas, playing an important role in nesting site suitability (Browne and Aebischer 2004; Browne and Aebischer 2005; Dunn *et al.* 2017). Whilst in the UK they avoid nesting in dense wooded areas, it has been shown that scrub and woodland can support, on average, more territories for turtle doves than farmland, with woodland in Britain supporting densities of turtle dove territories up to 6.5 times greater than farmland (Browne and Aebischer 2005). This is consistent with the propensity of turtle doves to inhabit primarily forested habitats observed traditionally across Europe, including open woodland and forest borders (Dunn *et al.* 2017). On the wintering grounds, turtle doves may be found in a range of habitats, typically roosting in open scrub, forest and fruit orchards but also utilising agricultural landscapes and grasslands (Eraud *et al.* 2009; Hanane 2017).

Turtle doves generally begin breeding upon arrival in the breeding grounds in late April, with the peak in breeding activity falling in early June (Browne and Aebischer 2005). Typically clutches contain two eggs, but clutches of one are not uncommon, and larger clutches of three may occur, but are less common (Browne and Aebischer 2005). With a short incubation period of 14 days and a further 15 days before fledging, they can produce up to three broods per breeding season, and second or third breeding attempts commonly begin as late as August (Browne and Aebischer 2005). In recent decades, there has been a strong decline in the reproductive output of turtle doves. A 61% fall in the number of young successfully fledged per pair was observed between the 1960s and 2000s (Browne and Aebischer 2004), and a 19% reduction in the number of breeding attempts commencing in August was being observed between the 1960s and 1990s (Browne *et al.* 2005; Browne and Aebischer 2005). A shift in the commencing of the autumn migration has also been identified, which now occurs, on average, eight days earlier than it did during the 1960's, resulting in the breeding season being reduced by an average of 12 days (Browne and Aebischer 2005).

This reduction in breeding effort, along with several other environmental stressors, have led to a major population decline for turtle doves in recent

decades (Sanderson *et al.* 2006). Long-term declines have been observed across the breeding range: between 1970 and 2018, there was a 98% decline in abundance of turtle doves in the UK, and a similar trend is apparent across Europe, with an 85% fall observed between 1980 and 2015 (DEFRA 2019; Hayhow *et al.* 2017; PECBMS 2017). On shorter time scale, in just five years, between 2013 and 2018, UK turtle dove numbers dropped by 44% (DEFRA 2019). These dramatic declines resulted in the turtle dove being classified as 'Vulnerable' on the International Union for Conservation of Nature (IUCN) Red List in 2015 (Birdlife International 2019). Furthermore, a study by Stanbury *et al.* (2017) which utilised the same criteria as the IUCN Red List, with an extra stage allowing assessment at a regional level (IUCN, 2012), classified the turtle dove as critically endangered in the UK, having undergone declines of over 80% during the previous three generations (Stanbury *et al.* 2017). The evident vulnerability of this species has encouraged increased research into the relatively neglected ecology of turtle doves (Browne and Aebischer 2003a; Browne and Aebischer 2005).

Numerous factors have been implicated in the decline of turtle doves. Agricultural intensification, resulting in degradation of breeding habitat and change in food availability, in both breeding and wintering grounds, is considered a leading contributor (Chamberlain *et al.* 2000; Browne and Aebischer 2003a; Browne and Aebischer 2003b; Browne *et al.* 2004). Diminishing food resources has been linked to declines in a diverse range of species, across vertbrate and invertebrate taxa, and from several habitats, both terrestrial and marine. For example, the decline of favoured host plant species (i.e. food source) was positively correlated with the decline of bee species (Scheper *et al.* 2014), and extensive collection of edible cockles *(Cerastoderma edule)* in the Dutch Wadden Sea has led to a loss of shellfish prey for migrant Red Knots *(Calidris canutus islandica)*, and thus their disappearance from this area (van Gils *et al.* 2006).

The favoured food source for turtle doves is naturally occuring weed seeds, such as those found in meadows and field margins (Browne and Aebischer 2005). But as this type of land is increasingly being replaced with arable land, turtle doves have been forced to adapt, undergoing a dietary switch so their

diet consists more heavily of crop plants than it would have 50 years ago (Browne and Aebischer 2003a). Recreational hunting is also believed to impact on the population of turtle doves (Hirschfeld and Heyd 2005). Whilst hunting is permitted in several EU Member States, it is monitered in these countries, and EU regulations are in place to limit impact on wild populations. This includes restriction on the timing of hunting (i.e. outside spring-migration and breeding periods) and, in some but not all countries, a maximum number of birds that can be shot, to ensure that hunting "does not jeopardise conservation efforts" (Fisher *et al.* 2018). Recent hunting moratoriums have been agreed to help alleviate the strain on the species, with the latest being a ban on turtle dove hunting during 2021 across most regions of Spain, and a formal ban on turtle dove hunting in France (RSPB 2022). Despite these set limits, the pressure put on the species by hunting has been described as high and it is believed that this added pressure will accelerate population declines (Hirschfeld and Heyd 2005; Fisher *et al.* 2018).

Disease is also believed to be of importance: the protozoan parasite *Trichomonas gallinae*, which is known to cause adult and nestling mortality, occurs widely across the turtle dove range (Lennon *et al.* 2013; Stockdale *et al.* 2015; Marx *et al.* 2017). Two factors of particular interest for this thesis are the dietary switch, whereby the reduction in the availability of natural foods has resulted in a shift in the diet from primarily naturally occurring weed seeds, to a higher proportion of crop plants, and the spread of protozoan parasite *Trichomonas gallinae*, and how these factors may interact with each other.

1.3 The link between agricultural intensification, diet and declines in biodiversity

Anthropogenic changes in land use, including urbanization and agricultural intensification, are increasing worldwide (Lambin *et al.* 2001), resulting in a widespread removal of natural habitats. This habitat degradation results in a loss of nesting sites and reduction in food availability. Such factors have been attributed as causes of the decline of mammalian species, like the common dormouse (*Muscardinus avellanarius*) (Hester and Harrison 2007), invertebrates, such as bees (Goulson *et al.* 2015), herpetofauna, both reptile

and amphibian (Gibbons *et al.* 2000) and birds, including numerous UK and European farmland bird species (Donald *et al.* 2001; Benton *et al.* 2003).

As birds are considered strong indicators of wider farmland biodiversity (Donald *et al.* 2001), they have been the focus of a wide range of studies investigating the effects of agricultural intensification, with the heavy declines recognized over recent years being a cause for concern. Chamberlain *et al.* (2000) used data collected in the long-term Common Bird Census monitoring scheme to evaluate changes in the populations of 29 farmland birds in comparison with changes in farming intensity. Of the birds they examined, over half were in decline, nine severely so, including the turtle dove, bullfinch (*Pyrrhula pyrrhula*) and grey partridge (*Perdix perdix*) (based on the British Trust for Ornithology alert limit system, whereby severe decline was indicated by a decline of at least 50% over the 25 years preceding the report (Crick *et al.* 1998)). Another study found an estimated 10 million breeding pairs of 10 species of UK farmland birds were lost between the late 1970s and 1990s (Krebs *et al.* 1999). As well as population declines, species are reducing their range of habitation (Benton *et al.* 2003). Fuller *et al.* (1995) observed that 86% of 28 farmland bird species investigated in Britain between 1970 and 1990 reduced their range, and of the 18 they were able to assess, 83% had declined in abundance, almost half of these by an excess of 50%.

Various methods used in modern farming result in a loss of biodiversity, with intensively managed monocultures becoming widespread across the landscape. A Countryside Survey carried out in Britain between 1978 and 1990 assessed the numbers of plots of different community types lost or gained from both arable and pastoral land (Robinson and Sutherland 2002). The results showed a loss of mixed weed and broadleaf weed plots, and an increase in weed-free plots (Robinson and Sutherland 2002). Chamberlain *et al.* (2000) reviewed several other changes in land use in England and Wales that had occurred over the preceding 40 years. At the time of the publication, there was an increase in tilled land by almost 1 million hectares since the 1960s, and a change in dominant crop types. The amount of wheat, sugar beet and oilseed rape increased, and a decrease was observed in the area used to farm oats, barley, root vegetables and left as bare fallow (Chamberlain *et al.* 2000). Bare

fallow is an important habitat for a range of species. Home range analysis of the widely declining European hare (*Lepus europaeus*) identified selection of fallow land throughout most of the year, except winter (Smith *et al.* 2004), and fallow land has been identified as a requirement for the obligate grassland species the calandra lark (*Melanocorypha calandra*), which is highly sensitive to habitat fragmentation (Morgado *et al.* 2010). Despite its importance for biodiversity, the amount of fallow habitat in England and Wales has decreased from 20,000 hectares in the 1960s to around 5,000 hectares in the 1990s (Chamberlain *et al.* 2000).

A widely used practice in modern agriculture is the application of inorganic chemical pesticides, which was three times greater in the mid-1990s compared to the mid-1970s. Whilst these added chemicals improve crop yield by effectively minimising pests, they have had severe implications for biodiversity (Mahmood *et al.* 2016). The widespread application of pesticides can result in population declines of non-target species, through direct poisoning, bioaccumulation through the food chain and through removal of food resources (Isenring 2010). In the UK, declines were observed in local populations of wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*) and field voles (*Microtus agrestis*) as a result of direct poisoning of non-target species following the application of rodenticides to control rat populations (Brakes and Smith 2005). At higher trophic levels, secondary poisoning may occur as predators are exposed to pesticides when feeding on the carcasses of poisoned animals (Brakes and Smith 2005), and pesticide residues have detected in a range of higher predators, including the European mink (*Mustela lutrola*) (Fournier-Chambrillon *et al.* 2004), red foxes (*Vulpes vulpes*) and common buzzards (*Buteo buteo*) (Berny *et al.* 1997).

As well as the threat of direct poisoning, use of such chemicals in modern land management have severe implications for food availability, with chemical herbicides, insecticides and molluscicides reducing the availability of weed and invertebrate food respectively, in both agricultural land and managed grasslands (Chamberlain *et al.* 2000; Vickery *et al.* 2001). The corn bunting (*Emberiza calandra*), a farmland species which has been in steep decline in Britain since the mid-1970s, feed their chicks invertebrates (Brickle *et al.*

2000). Brickle *et al.* (2000) observed preferential foraging in more natural, grassy margins and un-intensified grassland: areas home to significantly higher numbers of the four most common chick-food invertebrates than intensively managed grasslands. The presence of such invertebrates was negatively correlated with pesticide application in cereal fields, indicating the negative impacts of chemical-driven farming practices on invertebrate prey availability and breeding success of wildlife (Brickle *et al.* 2000). The removal of weeds via use of chemical herbicides also depletes crucial food resources, both directly, for granivorous species such as the turtle dove, which rely on the seeds of wild plants (Dunn *et al.* 2015), and indirectly, through the removal of the host plants for invertebrate prey of species such as the grey partridge (GWCT, 2004 *in* Isenring 2010).

In addition, a substantial increase in autumn-sown crops since the mid-1970s has reduced the presence of winter stubble, with over 99% of wheat being sown in autumn by the mid-1980s (Chamberlain *et al.* 2000). Winter stubble is an important feeding habitat for wildlife, as weeds are allowed to grow amongst stubble prior to ploughing and planting of the next year's crop in spring, the loss of which diminishes food availability throughout winter and early in the breeding season (Eggers *et al.* 2011). Agricultural intensification has diminished quantity and quality of food available to the Asian subspecies of the great bustard (*Otis tarda*) over the winter period, when they feed predominantly on seed and vegetation (Alonso *et al.* 2010; Liu *et al.* 2018). The contraction of wintering range has largely been attributed to this change in land use and the resultant habitat loss (Alonso *et al.* 2010; Liu *et al.* 2018).

As described above, many changes in land management methods have impacted food availability, which is an important factor driving species declines, with wildlife feeding increasingly on anthropogenic sources in numerous different habitats (Wilson *et al.* 1999). Results are not always negative, with scavenging animals such as red foxes, Spotted Hyenas (*Crocuta crocuta*) and Black-headed Gulls (*Chroicocephalus ridibundus*) thriving in urban environments, as they take advantage of a plentiful and often nutritionally and calorically rich food supply (Contesse *et al.* 2004; Yirga *et al.* 2012; McCleery *et al.* 2014; Scott *et al.* 2015). However, this is not always the

case, and when novel foods are of poorer quality, exploitation of anthropogenic food and feeding sites, over natural resources, has detrimental effects on wild populations.

For example, the waste discarded by fisheries may be considered a beneficial, easy to access food resource extensively used by piscivorous birds as stocks of pelagic fish are diminished by the fishing industry (Grémillet *et al.* 2008). Cape gannets (*Morus capensis*) use such waste to supplement their diet in the absence of live food, but this resource is of poorer quality than live fish, and thus was observed not to support successful rearing and fledging of chicks (Grémillet *et al.* 2008). Lipid poor diets, associated with a lack of availability of oily fish, have also been associated with stunting the growth and development of young red-legged kittiwakes (*Rissa brevirostris*), and the decline of Stellar's sea lions (*Eumetopias jubatus*) (Rosen and Trites 2000; Kitaysky *et al.* 2005). There are also examples of ailments observed in wildlife as a result of nutritional deficiencies. 'Angel wing' is a deformity most commonly observed in waterfowl and believed to be a result of a diet consisting of foods rich in calories, but lacking the necessary vitamins and minerals, such as bread commonly fed to ducks (Flinchum 2006, *in* Murray *et al.* 2016). A balanced diet is also important for effective immune function, with protein deficient diets potentially resulting in poorer immune response, as demonstrated by free-living impala (*Aepyeros melampus*), where reduced protein intake was associated with elevated gastrointestinal parasite load (Ezenwa 2004).

Due to the clear anthropogenic influence on food availability and its effect on biodiversity, understanding the relationship between agriculture, food resources consumed and the effect on body condition and vulnerability to disease is an important area of research. The majority of studies of turtle dove diet to date have been conducted on the breeding grounds in western Europe where these modern farming practices have been widely implemented (Browne and Aebischer 2003a; Gutiérrez-Galán and Alonso 2016; Dunn *et al.* 2018; Mansouri *et al.* 2019). However, the intensity of agriculture varies geographically, for example between western and eastern European countries, with a greater extent of traditional, less intense farming methods used in eastern Europe (Donald *et al.* 2001). These areas of lower agricultural

intensity are poorly studied in comparison, and it would be highly beneficial to compare birds caught in more and less intensively farmed regions to assess the variation in food consumed between the two. Furthermore, due to the focus of dietary work on the breeding grounds, relatively little is known about the diet of turtle doves in the wintering grounds which, as birds spend over two thirds of their annual cycle along migratory routes or on the wintering grounds, may have important implications for survival (Lormée *et al.* 2016).

1.4 Disease as a conservation issue

Dietary change is one of several potential drivers implicated in the decline of the turtle dove, another of which is infection with the protozoan parasite, *Trichomnas galliane*. *Trichomonas galliane* is the causative agent of avian trichomonosis: an important disease affecting numerous bird species, including turtle doves (Stabler 1948). Affecting the upper alimentary tract, *T. gallinae* is commonly found in columbids, such as doves and pigeons, and can also occur in raptors and passerines (Stabler, 1948; Anderson *et al.* 2009; McBurney *et al.* 2015). There is evidence for infection with this parasite to cause mortality in both adult and nestling turtle doves (Stockdale *et al.* 2015), therefore, studying the dynamics of infection with this parasite is a valuable contribution to understanding turtle dove decline.

Despite its importance as a conservation issue, disease is still commonly overlooked as a contributor to species decline (Tompkins and Jakob-Hoff 2011). In their literature review assessing causes of extinction according to the IUCN Red List, Smith *et al.* (2006) found that less than 4% of known species extinctions since 1500 and less than 8% of the 2852 critically endangered plants and animals indicated disease as a contributing factor. They also identified no instances where infectious disease was listed as the sole driver of a species extinction, unlike forces such as habitat loss and overexploitation, which are relatively frequently listed as lone causes (Smith *et al.* 2006). A variety of factors will influence how much of an effect established infections will have on a population as a whole, and Smith *et al.* (2006) determined that disease is significantly less likely to affect species populations when acting independently from other stressors. This is supported by theoretical models of epidemiology, whereby certain conditions must be met for disease to drive a

species to extinction. Such conditions include the presence of a reservoir host, or the ability of infectious agents to survive in the abiotic environment, thus mitigating the necessity for the pathogen to maintain a population in the target host (De Castro and Bolker 2005). For example, several strains of avian poxvirus, are known to infect common UK avian species including the house sparrow (*Passer domesticus*), European starling (*Sturnus vulgaris*) and woodpigeon (*Columba palumbus*), and is estimated to be able to persist in a viable state for months in the environment (Lawson *et al.* 2018). Another condition is small population size, as was the case with the Polynesian Tree Snail (Cunningham and Daszak 1998). Whilst these are examples of such criteria being met and disease being implicated as causative agents in species declines and extinctions, such instances generally lack definitive evidence for disease causing extinction, with the only exception to date being the Polynesian Tree Snail.

However, there is strong evidence of declines and extinctions caused by a fungal pathogen *Batrachomchytrium dendrobatidis* (hereafter referred to as *Bd*), which emerged in Australia and America during the 1970's, and is known to use reservoir hosts and persist in the environment (Berger *et al.* 2016). *Bd* is a highly virulent and pathogenic skin fungus causing the disease chytridiomycosis, which was first described in 1998, and is believed to be responsible for globally observed declines and extinctions of numerous amphibious species (Berger *et al.* 2016). As *Bd* has a life-cycle stage which can survive in water, as well as being able to persist on a range of amphibious species, both larval and adult, at low intensities, allowing them to act as reservoir hosts (Skerratt *et al.* 2007), this pathogen meets both of these conditions. In their review, Skerratt *et al.* (2007) reported that nine amphibious species had become extinct since 1980, with a further 113 possible extinctions and 435 species experiencing dramatic declines. Whilst declines in 233 species were attributed to overexploitation and habitat loss (Stuart *et al.* 2004), there had been no conclusive cause attributed to the declines in the other 202 species, rather they are labelled "enigmatic" declines (Stuart *et al.* 2004). Skerratt *et al.* (2007) examined an array of literature from studies on the pathogenicity, virulence and spread of *Bd* among regions experiencing

dramatic population declines, and found strong evidence implicating chytridiomycosis as a major driver of these enigmatic declines. Whilst this evidence for disease causing declines and extinctions is not definitive, the lack of supportive evidence for alternative theories led Skerratt *et al.* (2007) to conclude that "The impact of chytridiomycosis on frogs is the most spectacular loss of vertebrate biodiversity due to disease in recorded history". Furthermore, Schloegel *et al.* (2006) determined, from extensive review of available evidence, that *Bd* was likely the driver behind the rapid population crash which lead to the extinction of the sharp-snouted day frog (*Taudactylus acutirostris*). If this is the case, this would be the first reported case of extinction of a free-ranging wildlife species caused by disease, with likely many more unknown cases (Schloegel *et al.* 2006).

Another condition stated to help disease drive a population to extinction is small population size (De Castro and Bolker 2005). Bighorn sheep (*Ovis canadensis*) live in a highly fragmented landscape, resulting in segregated, small populations with minimal gene flow between them (Singer *et al.* 2000). This is detrimental to the maintenance of genetic diversity, and thus retention of resistance alleles (Altizer and Pedersen 2008), making such populations more susceptible to extinction by spread of infectious disease. In New Mexico, a population of over 200 individuals was reduced to a single ewe in less than 20 years due to a combination of drought, predation and an epidemic of psoroptic scabies (Boyce and Weisenberger 2005). Whilst this example is on a relatively small scale, focusing on one sub-population of a larger, fragmented population, it effectively demonstrates the potential for disease to cause sudden and dramatic population crashes, particularly when other stressors are acting simultaneously on a population, thus highlighting the importance of research surrounding disease and its interaction with other factors.

Disease can have important implications for avian populations and, due to their free-flying nature, birds can act as long-range vectors, allowing disease outbreaks to rapidly spread (Reed *et al.* 2003). One example of this is the spread of *Mycoplasma gallisepticum* (MG) among North American house finches (*Haemorhous mexicanus*). The first MG incident in house finches was reported in Maryland in 1994, following a single host switch event, caused by

a mutation in MG, allowing transmission of the infection following contact between a house finch and infected poultry (Delaney *et al.* 2012; Staley *et al.* 2018). This novel infection caused severe conjunctivitis symptoms and resulted indirectly in mortality, as a result of impaired vision (Hochachka and Dhondt 2000; Bonneaud *et al.* 2011). Since this first incident, rapid epizootic spread of MG across eastern North America resulted in the population of house finches reducing by around 50% within this region by 1997 (Staley *et al.* 2018). This disease has continued to spread across the USA, occurring throughout the eastern United States, in Canada, and more recently in the western states of the USA (Fischer *et al.* 1997; Staley *et al.* 2018). Another disease that caused rapid declines since its introduction into a naïve population is West Nile virus, which was introduced into North America in 1999. Using a dataset comprised of a quarter-million birds, George *et al.* (2015) found that populations of 47% of the 49 species studied were negatively affected by West Nile virus, with over half of these populations failing to show signs of recovery since the introduction of the disease (George *et al.* 2015). In addition to the introduction of a novel parasite to a population, the introduction of a parasite vector can cause dramatic declines by facilitating the transmission of disease which previously would not have been possible. Avian malaria was the driver of wide scale losses in endemic bird populations in Hawaii following the introduction of the principal transmission vector, the mosquito *Culex quinquefasciatus* (Warner 1968; Lapointe 2008). Prior to this accidental introduction, endemic Hawaiian species were not infected with avian malaria, despite the presence of *Plasmodium* in the region, which was brought in by overwintering birds, because without the appropriate insect vector, there was no mode of transmission between the birds (Warner 1968).

These instances reflect the rapid spread of infections following the introduction of a novel parasite. Conversely, the co-evolution of parasites and hosts result in a diverse array of host adaptations that reduce the cost of infection, thus not all host-parasite interactions result in population declines (Ebert and Fields, 2020). This may be as a result of host resistance to parasites, reducing the impact of parasite infection through avoidance of infection or immune responses to rapidly clear infection, or the development of host tolerance to

parasites, whereby hosts are able to survive despite parasite infection by ameliorating the damage caused by infection (Best *et al.* 2008).

1.5 Trichomonas gallinae

As previously mentioned, an important avian parasite is *Trichomonas gallinae.* Trichomonadidae are a family of amitochondriate, flagellated protozoa, utilising a range of vertebrate and invertebrate hosts, with both commensal and parasitic relationships being observed (Honigberg 1963; Kleina *et al.* 2004). Within the parasitic species there are a few with significance to humans, both medical and veterinary, the best understood of which is *Trichomonas vaginalis*, which causes the human infection trichomoniasis (Graves and Gardner 1993). Infecting the genitourinary system, *T. vaginalis* is the most commonly occurring sexually transmitted parasite in the world, and is considered to have implications in elevating the transmission of human immunodeficiency virus (HIV) (Mayta *et al.* 2000). Another species of note is *Trichomonas foetus,* again a sexually transmitted parasite affecting the genitourinary system of cattle; this parasite is important from a veterinary perspective (Yule *et al.* 1989). *Trichomonas foetus* can cause infertility and abortion in infected cows, resulting in major losses within the farming industry (Yule *et al.* 1989). *Trichomnas galliane* was first recognised as an emerging infectious disease following an outbreak of trichomonosis among greenfinches (*Chloris chloris*) and chaffinches (*Fringilla coelebs*) in the UK in 2005 (Lawson *et al.* 2006).

There are a range of symptoms associated with trichomonosis with varying severity. As a disease that primarily affects the throat and gullet, some common visible symptoms associated with trichomonosis include drooling, regurgitation of food and difficulty swallowing (McBurney *et al.* 2015). In more severe cases, individuals may develop necrotic legions in the oropharynx and crop (Stabler 1954). This can lead to death via starvation or asphyxiation, if lesions grow so large that they block feeding or prevent normal breathing, respectively (Stabler 1954). Less commonly, trichomonosis may manifest itself in the liver tissue, air sacs and parts of the cranium (Stabler 1954; Narcisi *et al.* 1991). The variation in severity of symptoms is thought to be a result of difference in susceptibility between host birds, as well as genetic diversity of

T. gallinae, with at least 23 strains of *T. gallinae* having been identified as infecting avian species and reported on NCBI GenBank (Clark *et al.* 2016).

The aforementioned finch epidemic began in Western England and Wales and spread across Eastern England, into Ireland and mainland Europe (McBurney *et al.* 2015). In 2007, geographical regions with the highest incidence rates (West England and Wales) suffered 35% and 21% declines in breeding populations of greenfinches and chaffinches, respectively (Robinson *et al.* 2010). This decline has continued, particularly in greenfinches, of which the British race (*C. chloris harrisoni*) is now included on the red list of Birds of Conservation Concern (Eaton *et al.* 2015). In the UK, the breeding population of greenfinches was estimated at 4.3 million just before the trichomonosis outbreak in 2006, but in 2016, was recorded at approximately 1.5 million individuals (Lawson *et al.* 2018). This decline reflects a mean loss of around 280 000 individuals per year: the highest recorded impact of infectious disease on a European wild bird population (Lawson *et al.* 2018). This epidemic continued to spread, reaching Southern Fennoscandia in 2007, Germany by 2009 and expanding eastwards into continental Europe by 2012 when it was found in Austria and Slovenia (McBurney *et al.* 2015). Based on ringing data, chaffinch migration was believed to be the most likely cause of the wide reach of this epidemic, emphasising how easily disease can be spread within free-living populations (McBurney *et al.* 2015). The sudden and extensive spread of trichomonosis in the novel finch host indicates a low host specificity of *T. gallinae,* a concerning factor regarding this disease from a conservation perspective (McBurney *et al.* 2015).

The worldwide spread of trichomonosis has become increasingly recognised as a concern for conservation and management of avian population declines (Bunbury *et al.* 2007). The generality of hosts increases the risk of *Trichomonas* in wild populations, as *T. gallinae* has been found in numerous avian orders, including columbiformes, passeriformes, falconiformes and psittaciformes (McKeon *et al.* 1997; Sansano-Maestre *et al.* 2009; Lawson *et al.* 2012). In columbids, it is suggested that *Trichomonas* may have contributed to the rapid decline and subsequent extinction of the passenger pigeon, *Ectopistes migratorius,* after the introduction of the domestic pigeon to North

America (Bucher 1992; Stabler 1954). It is also considered that trichomonosis is a limiting factor in the recovery of the endangered Mauritian pink pigeon, *Nesoenas mayeri* (Bunbury *et al.* 2007).

One important proposed mechanism of the rapid spread of *T. gallinae* is horizontal transmission, both intraspecific and interspecific, between birds congregating in high densities to feed, particularly at artificial feeding sites (Robinson *et al.* 2010; Gerhold *et al.* 2013; McBurney *et al.* 2015). For example, the sudden and extensive spread of the parasite among finches was suggested to be a result of pathogen spillover, possibly facilitated by congregation of birds at feeding sites, such as garden bird feeders (McBurney *et al.* 2015; Stockdale *et al.* 2015). Pathogen spillover refers to an event occurring when a reservoir host, exhibiting a high pathogen prevalence, comes into contact with a novel host population not previously exposed to that pathogen, allowing infection transmission to the new host (Daszak *et al.* 2000). This is a cause for concern from a conservation standpoint, as parasite exposure can result in high levels of mortality, particularly in naïve populations (Altizer and Pedersen 2008). It is suggested that this is the cause for local extinction of the African wild dog (*Lycaon pictus*) (Daszak *et al.* 2000). Following wide scale declines since the 1960's, the African wild dog is now considered endangered, with a fragmented population of fewer than 5000 individuals. In 1991, the extinction of the wild dog population in the Serengeti was suggested to be linked to the concurrent presence of canine distemper in sympatric domestic dogs with pathogen spillover potentially causing the loss of this population (Macdonald 1992; Ginsberg *et al.* 1995).

Spillover may also occur as a result of the introduction of a novel species that, in turn, brings novel pathogens with it. For example, the introduction of non-native North American crayfish (*Pacifastacus leniusculus)* into Europe, brought with it crayfish plague: a disease to which North American hosts are resistant (Holdich *et al.* 2009). As crayfish plague does not naturally occur in Europe, native species are particularly susceptible, resulting in the local extinction of native European crayfish (*Austropotamobius pallipes*), following the introduction of North American crayfish (Holdich and Reeve 1991). Similarly, the arrival of both invasive birds carrying avian malaria (*Plasmodium spp.*),

and a suitable introduced mosquito vector (*Culex quinqufasciatus*) in Hawaii facilitated the infection of native bird species with novel avian malaria. Whilst no definitive evidence is available, there is considerable circumstantial evidence implicating introduced avian malaria in the widespread decline and extinction of endemic avian species, as discussed earlier regarding extinctions due to disease (Warner 1968). Whilst these are extreme examples, resulting in local extinctions, it is the same mechanism as described previously for the introduction of rock doves around the world, facilitating the global spread of *T. gallinae*. These examples also demonstrate how much more detrimental the effects of disease can be on wildlife populations when acting in conjunction with other factors, in these instances, population fragmentation and introduction of alien species.

A common factor observed in all geographic areas where trichomonosis is emerging is mortality where birds gather at feeding and watering sites (Robinson *et al.* 2010; McBurney *et al.* 2015). With the reduced availability of natural food sources, birds are increasingly taking advantage of opportunistic feeding on man-made sites and garden bird feeders (Browne and Aebischer 2003a; Robb *et al.* 2008a; Dunn *et al.* 2018; Lawson *et al.* 2018). Water sources are also expected to be a common location for disease transmission to occur, particularly for birds inhabiting or wintering in regions with hot, dry climates, such as sub-Saharan Africa, as water is scarcer in such regions (Harmon *et al.* 1987; Amin *et al.* 2014). Consequently, high densities of birds congregate at watering holes, increase both the likelihood of parasites being deposited in pools by infected hosts, and infective agents being picked up by uninfected hosts (Bunbury *et al.* 2007). Typically, when in Africa, turtle doves go to drink at first light or sunset, when large congregations of birds are observed (Browne and Aebischer 2005). This, along with exploitation of anthropogenic food sources throughout the year, may contribute to elevated risk of disease transmission in turtle doves

Few studies into *Trichomonas* infection have been carried out on free-living turtle dove populations. It was previously considered that turtle doves may be at lower risk of *T. gallinae* infection than other UK columbids, due to reduced exposure (Lennon *et al.* 2013). This was a result of their lower tendency to

feed in gardens and being out of the country during the winter months, when finch trichomonosis peaks (September-February) (Robinson *et al.* 2010; Lennon *et al.* 2013). There appeared to be support for this idea as, prior to 2013, there were no reported cases of *T. gallinae* in turtle doves within the UK. However, Lennon et al. (2013) found the first recorded occurrences of *T. gallinae* in UK turtle doves, detecting 86% infection across a wide geographical area. This is supported by work carried out by Stockdale et al. (2015), who tested turtle doves in the UK and found an infection rate of 100% in both adults and nestlings. Further research conducted in Europe supports this, with a 93% prevalence of *T. gallinae* in swab samples from turtle doves captured across Spain, Malta, Italy and Germany (Marx *et al.* 2017) and a 93-100% prevalence of *T. gallinae* in turtle doves sampled in France and the UK (Thomas 2017). There has been little research into *T. gallinae* infection on the wintering grounds, but one study identified 90-100% *T. gallinae* prevalence in turtle doves sampled in Burkina Faso and Senegal (Thomas 2017). These studies demonstrate that infection with *T. gallinae* is a more widespread concern in the UK and Europe than previously thought. Whilst *T. gallinae* infections in turtle doves are commonly asymptomatic (Stockdale *et al.* 2015; Dunn *et al.* 2016), having limited effect on the turtle dove host, trichomonosis has been identified as the causative agent in mortality in both adults and nestlings, thus posing a threat to turtle dove populations (Stockdale *et al.* 2015). This potential for both asymptomatic infection and mortality supports the need for monitoring *T. gallinae* infection in turtle doves, with appropriate techniques to detect infection when individuals do not exhibit symptoms.

1.6 DNA analysis of diet and disease

Understanding the role of diet and disease in species decline is reliant on adequate methodology to assess the level of change and impact of such change on population dynamics. This can be challenging as, whilst there is an array of traditionally used methods for investigating diet and disease, they all come with their own merits and drawbacks. For example, behavioural observation (Robinson and Holmes 1982), analysis of stable isotopes (Cherel *et al.* 2007), identification of hard remains in faecal matter (Deagle *et al.* 2009) and gut contents analysis (Young *et al.* 1998) have all been used to investigate

diet. Whilst information obtained from these methods can be useful, particularly if multiple methods are used in conjunction, they do not provide a universally adequate method of diet analysis (Deagle *et al.* 2009). Similarly, traditional methods of wildlife disease monitoring are commonly reliant on individuals expressing visible symptoms, and post-mortem analysis to identify infection may be opportunistic, whereby data on infection is only collected as an aside from samples collected for other purposes (Spalding and Forrester 1991; Lennon *et al.* 2013). These methods are likely to lead to an underestimation of infection in free-living populations for several reasons, which will be discussed later. However, as technology advances, so does our ability to unravel intricate ecological processes and gain a greater insight into interactions in nature. DNA analysis has been applied to a wide range of ecological areas, including trends in population dynamics, breeding ecology, diet and disease (Hadrys *et al.* 1992; Yoccoz 2012). I will discuss the drawbacks of traditional methods, as well as how developing methodology utilising modern DNA based technology can mitigate these problems.

1.6.1 Diet

For traditional methods of diet analysis, observational studies require a lot of time and, whilst they can provide a snapshot of information, these findings may not be spatiotemporally repeated. Furthermore, there are logistical restrictions of this method, for example in nocturnal animals feeding at night, or small invertebrates feeding within dense vegetation (Symondson 2002). Identification of hard parts in faeces is not suitable for all food types, and will only yield results for food items that cannot be fully digested. Furthermore, some predators avoid ingestion of hard parts, such as bones, and many eat foods that are easily digested and leave no discernible remains, for example, soft-bodied prey, or easily digestible plants, providing further drawbacks to the use of this method (Symondson 2002; Deagle *et al.* 2009). Similarly, with stomach contents analysis, digestion of food renders results less reliable. Variation in digestion of different foods means that those that are easier to digest will remain in the gut for less time or rapidly become unrecognisable (Robeson *et al.* 2017). This gives rise to possible bias towards more recently consumed food items or those that are harder to digest in this kind of analysis,

and can result in certain prey items being frequently underrepresented (Reynolds and Aebischer 1991; Bowen 2000). Furthermore, gut contents analysis is done on matter extracted from deceased animals, which raises its own ethical considerations if wild animals are culled purely for the purpose of studying their diet, encouraging researchers to move towards less destructive methods (Trites and Joy 2005). This aspect is particularly important to consider with regards any vulnerable or endangered species, as it severely restricts the scope of this method for analysis of diet.

By combining DNA-based identification of prey/plant remains in faecal samples, using metabarcoding, and high-throughput sequencing (HTS) techniques, multiple specimens of varying taxa can be identified from one single sample via use of an HTS platform (Clarke *et al.* 2014). This approach has the capability to deliver new levels of insight into ecological networks (Clarke *et al.* 2014). Unlike traditional methods, such as Sanger sequencing, HTS allows the sequencing of multiple molecules at once, thus allowing the simultaneous identification of multiple organisms from a single sample, as opposed to previous sequencing methods, which sequenced only single organisms at a time (Taberlet *et al.* 2012). By adopting this approach, it is possible to discern individual species making up whole communities, based on the identification of a specific genetic region, or 'barcode' (Hebert *et al.* 2003; Cristescu 2014). The barcoding region is a standardized portion of the genome, which is conserved across organisms, ideally exhibiting minimal intraspecific variation, but with some variation between species (Clarke *et al.* 2014). Due to the wide availability of sequence data for such conserved regions, it is possible to use primers designed to bind with their corresponding flanking primer-binding sites to amplify these loci without the prior knowledge of the organism in question (Symondson 2002; Clarke *et al.* 2014). Using various different barcode loci, metabarcoding has been applied to fungi, plants and animals in order to investigate community ecology (Taberlet *et al.* 2006; Epp *et al.* 2012; Shehzad *et al.* 2012). The nature of metabarcoding, allowing simultaneous identification of unknown organisms comprising whole communities, makes it an ideal tool for diet analysis, and it is becoming an increasingly popular alternative in this field (Pompanon *et al.* 2012).

Metabarcoding has been used in an array of study systems, including invertebrates, birds, reptiles and mammals (Pompanon *et al.* 2012; Cuff *et al.* 2021; Hacker *et al.* 2021; Shutt *et al.* 2021). This method can identify food sources based on analysis of non-invasively collected faecal samples, as such samples will contain the remains of food that have been eaten by a host (Symondson 2002). Whilst, for the most part, food will have been sufficiently digested that excretion will not contain visibly recognisable remains of dietary components, samples will contain DNA from semi-digested food (Murray *et al.* 2011). Although DNA is degraded during digestion, the use of PCR to amplify intact DNA produces sufficient amounts of DNA to facilitate diet analysis using this method (Symondson 2002). Unlike species specific amplification, metabarcoding uses group-specific primers which will amplify a relatively conserved barcode region found across the target taxa (Deagle *et al.* 2009). For example, the CO1 region is used almost universally for animals, and combinations of plastid *rbcL, matK* and *trnH-psbA* are commonly used for plant identification (Kress *et al.* 2015). However, with the constant development of molecular methods, use of different barcoding regions such as 16S and ITS2 are becoming increasingly common (Baker *et al.* 2016; Moorhouse-Gann *et al.* 2018). By running resulting amplicons through an HTS platform, it is then possible to identify the source of DNA present in order to detect items that occur in the diet of the host (Murray *et al.* 2011). This molecular methodology will be used for the diet analysis component of this project, by using recently developed primers to target the ITS2 region to identify plants present in the diet of turtle doves (Moorhouse-Gann *et al.* 2018).

Despite its many benefits, there are drawbacks, which should be considered when using metabarcoding for dietary analysis. One caveat of traditional dietary analysis that still applies to some extent is the different rates of digestion among taxa (Arai *et al.* 2011). Whilst this method is more effective at detecting certain prey than hard parts analysis, it is still the case the rate at which DNA will be degraded in the gut is not uniform across taxa, therefore making this method biased towards taxa where DNA degrades more slowly in the gut (Lamb *et al.* 2019). In addition to this, there is a wide range of causes for biases, occurring both during PCR amplification and sequencing, including

particularly abundant or rare prey items, variation in sequence length and sequencing artefacts (Brooks *et al.* 2015; Moorhouse-Gann *et al.* 2018; Jusino *et al.* 2019). Thus, there is debate as to whether it is appropriate to infer quantitative results of prey consumption from metabarcoding data. Whilst the relative read abundance of sequencing outputs may be used to describe the abundance of items in the diet (Deagle *et al.* 2019), primer biases resulting in variation of sequence amplification is commonplace in PCR-based metabarcoding, thus it is argued that presence-absence metrics are more suitable (Elbrecht and Leese 2015; Lamb *et al.* 2019).

Another potential cause of bias is amplification of host DNA, which can be problematic when using broad coverage primers, as shed epithelial cells from the gut will be present in faecal samples, resulting in high levels of host DNA potentially being detected, as well as desired dietary items (Pompanon *et al.* 2012). If the host species falls within the taxonomic coverage of the barcode used to encompass target diet items, host DNA will account for a relatively high proportion of the sequencing reads produced, resulting in a lower proportion of sequences being assigned to dietary items, and potentially preventing their detection (Pompanon *et al.* 2012). In addition to sequencing of predator DNA potentially swamping dietary item DNA, the presence of host DNA in faecal samples means metabarcoding is unable to detect cannibalism, which may be a behaviour of interest in dietary studies (Tercel *et al.* 2021). Whilst the issue of disproportionately high levels of amplification of host DNA can be mitigated by designing 'blocking' primers, which include an intentional oligonucleotide mismatch against host DNA (Vestheim *et al.* 2011), this can introduce biases in DNA amplification (Piñol *et al.* 2014). Furthermore, it cannot solve the problem of distinguishing between detection of host DNA and cannibalism (Pompanon *et al.* 2012; Berry *et al.* 2015). Similar to the difficulty of detecting cannibalism, metabarcoding is also unable to distinguish accidental consumption of prey and secondary predation. Secondary predation occurs when a primary predator is consumed by a secondary predator, and the gut contents of the primary predator is also detected in diet analysis (Symondson 2002). Using metabarcoding, it cannot be differentiated whether a dietary item has been consumed by the host, or by a prey item which

the host has then eaten, which may lead to incorrect inferences about the diet (Tercel *et al.* 2021).

Depending on the study system, these described problems can have lesser or greater significance, and many issues can be mitigated through the use of appropriate primers, to minimise the amplification of host DNA, bioinformatics processing, to ensure effective cleaning of data to remove false negatives and false positives, and statistical analysis, using tests to address factors such as secondary predation. Even with mitigation, these factors should be considered when interpreting results of dietary metabarcoding. As turtle dove diet is purely granivorous, plant specific primers will be used to assess the diet, which do not amplify the host DNA. In addition, as turtle doves do not eat animals, as they will not be consuming dietary items which have themselves eaten other prey, thus negating the issue of secondary predation.

1.6.2 Parasite infection

Similarly when considering disease monitoring, previously used methods, such as observational studies reliant on individuals exhibiting visible symptoms and post-mortem examination, can be problematic. Numerous factors contribute to why they are likely to lead to an underestimation of infection in free-living populations, thus leading to the poor understanding of the contribution of parasite infection to species decline. Firstly, widespread sub-clinical infection, whereby hosts are infected with parasite but this has not resulted in any clinical outcome, means that relying on observing individuals expressing visible symptoms will result in those asymptomatically infected being mistakenly identified as uninfected individuals (Sansano-Maestre *et al.* 2009; Stockdale *et al.* 2015). Observational monitoring of infection via visible symptoms is also difficult for species inhabiting remote areas, as well as nocturnal and more secretive species (Morner *et al.* 2002). As with sub-clinical infection, individuals with subtle, or short-lived symptoms, as may be the case for those recovering from a disease, or those that have conferred immunity as a result of prior infection, as well as individuals infected with less virulent strains, also risk being incorrectly assessed as clear of infection (Lennon *et al.* 2013). Equally, any assessment of prevalence relying on morbidity will overlook subclinical infections, or infections from which individuals recover (Lennon *et*

al. 2013). Another problem with relying on post-mortem analysis is that it requires the acquisition of carcasses: a factor which may be problematic for free-living species due to carcasses being scavenged by other species, or rapidly decomposing, resulting in an underestimation of the number of individuals succumbing to a disease (Peterson *et al.* 2001; Bunbury *et al.* 2008b; Prosser *et al.* 2008). Finally, particularly in migrant species, such as the turtle dove, seasonal movements of individuals may mask population declines caused by parasite infection (George *et al.* 2015).

As with many other parasites, *T. gallinae* infection can occur without the presence of clinical signs, but with individuals still able to transmit parasites (Bunbury *et al.* 2008a; Stockdale *et al.* 2015), thus identification of individuals with visible clinical signs is not sufficient for infection monitoring. To counter this, microscopic identification of infective agents has, for a long time, been used to detect infection. This may be through the use of techniques such as wet-mount microscopy, as is the case for *T. gallinae* (Bunbury *et al.* 2005), or inspection of fixed and stained blood smears for identification of several blood parasites, such as *Plasmodium* and *Haemoproteus* (Valkiūnas *et al.* 2008), both of which are used to investigate diseases in birds. Electron microscopy, has been used for decades to identify viruses from cultured cells in order to determine the etiological agent responsible for several outbreaks of unknown infectious diseases (Goldsmith *et al.* 2013). Whilst this method increases the chances of detection, it is still not sensitive enough to detect all infections. In addition, it is time consuming, requires a high level of taxonomic expertise and is often unable to differentiate species in early life stages (McManus and Bowles 1996).

The use of genetic tools has dramatically enhanced the ability of researchers to both detect parasites and to differentiate between species (Harris *et al.* 2020). By isolation of DNA and amplification via polymerase chain reaction (PCR), it is possible to identify positive infections more reliably and efficiently than using traditional microscopy methods (Valkiūnas *et al.* 2008). Richard *et al.* (2002) directly compared PCR assays and microscopic examination of blood smears for the detection of avian haemosporidians, concluding that use of PCR was more efficient and reliable. Durrant *et al.* (2006) reported a 10-fold

increase in number of positive haematozoa infections identified using PCR compared to microscopy. Furthermore, the use of genetic methods facilitates more fine-scale parasite differentiation, and is now widely adopted to identify different haplotypes within the same species (Mucheka *et al.* 2015; Quillfeldt *et al.* 2018; Chougar *et al.* 2019; Kılınç and Simsek 2019; Hidalgo *et al.* 2020).

Whilst the majority of parasitological studies use traditional Sanger sequencing (Harris *et al.* 2020), this method is limited in detection of multiple haplotypes of parasite species. As Sanger sequencing only detects the dominant amplicon sequence, if multiple species or strains are present within one sample, this method does not have the capacity to identify them separately (Huggins *et al.* 2019). Whilst clonal culturing is a viable method used to identify multiple strains within one host, it is both time consuming and costly, making it less appropriate for use in large scale monitoring activities (Grabensteiner *et al.* 2010). By sequencing a standardized barcoding region of the genome, the use of high-throughput sequencing (HTS) and metabarcoding makes it possible to ascertain the presence of multiple species or haplotypes, and by its nature of highly paralleled processing, it is more efficient than previous methods (Taberlet *et al.* 2012; Clarke *et al.* 2014).

Field studies identifying the presence of *T. gallinae* in wild populations have benefitted from the development of methods of identification of infection thanks to its close relationship to the anthropogenically infective *Trichomonas vaginalis*. Due to the widespread occurrence of *T. vaginalis* as a sexually transmitted disease in humans, more sensitive methods for detection of this closely related parasite have been developed, such as simple culturing methods using InPouch culture kits (Mayta *et al.* 2000). When comparing detection of *T. gallinae* from wild pink pigeons using wet-mount microscopy and InPouch culturing, 60% of birds sampled tested positive for *T. gallinae* when samples were cultured, but fewer than half of these positive infections were detected by the wet-mount method (Bunbury *et al.* 2005), demonstrating one of the drawbacks of a lack of sensitivity of traditional methods. Since this study by Bunbury *et al.* (2005), which studied the contents of InPouch kits, containing cultured parasites, microscopically, methods have further developed, with DNA isolated from parasites grown in culture being amplified

by PCR to screen for positive *T. gallinae* infection (Gaspar da Silva *et al.* 2007; Amin *et al.* 2014; Lennon *et al.* 2013; Marx *et al.* 2017). It has been shown that, in order to obtain sufficient DNA to reliably detect sub-clinical *Trichomonas* infections using PCR, this culture step is necessary and swabs must be taken from the oral cavity, oesophagus and crop, then incubated in culture medium to allow the parasite to propagate prior to DNA extraction and amplification (Cover *et al.* 1994; Dunn *et al.* 2016). *Trichomonas* DNA can then be amplified to identify positive infections, even from individuals showing no clinical symptoms, and subsequently used for high-resolution genetic differentiation between strains by sequencing of PCR products.

By using DNA analysis and HTS, I aim to learn valuable, and previously inaccessible information about a range of ecological areas, such as diet and disease, allowing me to discern patterns and interactions between them, and aiding in untangling intricate ecological networks. I hypothesise that there will be significant variation in the diet of turtle doves in the different regions and that the prevalence of *T. gallinae w*ill vary between regions, with the highest *T. gallinae* strain diversity being expected in the wintering grounds, migrating individuals from across Europe come together. By looking at turtle dove diet in conjunction with *T. gallinae* infection, this project will investigate possible interactions between food choice and parasite infection, with the hypothesis that the presence of *T. galliae* will be associated with a higher proportion of cultivated food in the diet, as a result of increased inter and intra-specific contact at sites with food provisioning. By assessing both diet and *T. gallinae* infection from individual birds, I aim to provide a basis for comprehensive land management plans to protect this iconic species.

Chapter 2 - Analysis of the diet of turtle doves across European breeding and African wintering grounds

Photograph by Rebecca Young

Contribution of collaborators involved in this chapter was mainly support in collection of samples from the field. Samples used in this project were collected from Senegal in three years prior to my PhD commencing by the RSPB and Nature-Communautés-Développement. The main team involved in sample collection was Chris Orsman (RSPB), Moussa Ka (NCD) and Mamadou Diallo (NCD). I worked with Chris Orsman, Moussa Ka and Mamadou Diallo and master's student Coen van Tuijl to collect samples from Senegal in 2017. I was supported in sample collection in France by Hervé Lormée and Cyril Eraud of the Office Français de la Biodiversité, Unité Avifaune Migratrice and in Hungary by Dr Orsolya Kiss, of the University of Szeged. During sample collection, all birds were handled under the licences of the aforementioned collaborators. The custom pipeline used for bioinformatic analysis used in this work was designed by Lorna Drake and Jordan Cuff (modified from that provided by Helen Hipperson and the NBAF team at the University of Sheffield).

2.1 Abstract

Agricultural intensification is a major driver in species declines, with changes in land use resulting in widespread alteration of resource availability. An increase in anthropogenic food resources, alongside decreasing natural resources, has resulted in species undergoing dietary changes that can have important ecological consequences, particularly for declining species. Here I use high throughput sequencing to analyse the diet of the migrant European turtle dove (*Streptopelia turtur*), a species that has experienced significant population decline throughout its European range. I analyse the diet of this species on both breeding and wintering grounds to gain an understanding of resource use throughout the annual cycle, and compare areas of more and less intensive agriculture in western and eastern Europe, respectively. I examine associations with body condition, spatiotemporal variation and the source of food (wild or cultivated). I identified 121 taxonomic units in the diet, with significant variation across sampling seasons, and very little overlap between the breeding and wintering seasons, as well as high levels of cultivated food resources in the diet of turtle doves in both breeding and wintering grounds. Body condition of birds on the wintering grounds increased as the season progressed, as they approached migration, concurrent with an increased consumption of wild seeds, indicating the importance of habitats rich in wild seeds and the need to consider food availability on the wintering grounds in turtle dove conservation strategies.

2.2 Introduction

Diet is an important aspect of an animal's ecology, with an appropriate diet key to ensuring they receive the required nutrients to maintain a healthy body condition for their own survival, breeding success and withstanding the energetic cost of migration (Martin 1987; Newton 2004a). However, there are increasing instances of wildlife undergoing a dietary switch and consuming novel food resources that they would not naturally eat. There are two potential drivers of this switch, both driven by anthropogenic influences: an increased availability of novel food resources, or a decrease in natural food resource availability (Dunn *et al.* 2018). Some common factors resulting in an increase

in novel food resources are supplementary feeding, human food waste and an increase in farmed crops (Browne and Aebischer 2003; Robb *et al.* 2008a; Newsome and Van Eeden 2017). Farmed crops not only increase the availability of these novel foods in the environment, but also, depending on farming practices, often remove many natural food resources from the environment. The removal of natural flora and invertebrates heavily impacts farmland biodiversity, affecting species feeding on a range of organisms, resulting in widespread species declines (Hester and Harrison 2007; Donald *et al.* 2001; Benton *et al.* 2003).

Some species do benefit from an abundance of food resources, such as white storks (*Ciconia ciconia*), which commonly take advantage of an abundance of food at landfill sites across Europe (Gilbert *et al.* 2016). However, species' declines are often associated with dietary switches and nutritional stress. In many instances, the alternative food resources will be of poorer quality than optimal natural resources (Will *et al.* 2015; Moorhouse-Gann *et al.* 2020), resulting in poorer body condition (Rosen and Trites 2000), and subsequently diminished survival rate and reproductive success (Litzow *et al.* 2002; Wanless *et al.* 2005). Furthermore, when feeding on lower quality food resources, the resultant reduction in body condition may compromise immune function, resulting in a higher rate of disease among wildlife (Murray *et al.* 2016).

Agricultural intensification is a leading cause of dietary shifts, with changing farming practices dramatically increasing crop yields, but depleting natural food resources, both on avian breeding and wintering grounds (Chamberlain *et al.* 2000; Brink and Eva 2009; Binswanger-Mkhize and Savastano 2017; Zwarts *et al.* 2018). There are a number of practices which reduce food availability in farmed habitats. The removal of traditional hedgerows and weedy habitats in favour of cultivated crops, and the extensive use of herbicides reduces wild plant cover and seed abundance (Marshall *et al.* 2003; Browne *et al.* 2005). Additionally, the shift to autumn-sown cereals since the 1970s has decreased the fallow period between seasons, limiting weed seed availability early in the breeding season (Gillings *et al.* 2005; Powolny *et al.* 2018). Browne and Aebischer (2003a) observed that areas of farmland with a high abundance of weeds within their study sites were key feeding grounds

early in the breeding season, frequented by large numbers of turtle doves. However, these sites were abandoned as feeding sites following the application of herbicides by farmers in early August in preparation for autumn seed sowing. Intensity of agriculture varies geographically, for example between western and eastern European countries, with a greater extent of traditional, less intense farming methods used in eastern Europe than in western Europe (Donald *et al.* 2001). This has been suggested as a cause for less severe species declines being observed in eastern Europe than western Europe (Báldi and Faragó 2007).

2.2.1 The European turtle dove and their change in diet

The turtle dove is one of the UK's most rapidly declining species, with long-term declines observed across the breeding range (Burns *et al.* 2020). Between 1970 and 2018, there was a 98% decline in abundance of Turtle doves in the UK, and over the five year period between 2012 and 2017, an annual decrease of 13.3% was observed (DEFRA 2019; Burns *et al.* 2020). A similar trend is apparent across Europe, with an 85% decline observed between 1980 and 2015 (PECBMS 2017). One factor which has been considered an important contributor to this population trend is agricultural intensification (Browne and Aebischer 2003a). As the only obligate granivorous Afro-Palearctic migrants in Europe, the turtle dove is ecologically unique, and particularly threatened by agricultural intensification, as some of the most significant farmland bird declines have been attributed to seed-based diets (Dunn *et al.* 2015; Robinson and Sutherland 1999).

There is much evidence that agricultural intensification, and the loss of biodiversity associated with it, has been a driver in the decline of farmland bird species throughout much of Europe (Chamberlain *et al.* 2000; Donald *et al.* 2001; Benton *et al.* 2002). In the UK, turtle doves are classified as a farmland species (Benton *et al.* 2003; Browne *et al.* 2004). Outside the UK, turtle doves are not necessarily considered farmland species, inhabiting primarily forest habitat in Continental Europe (Dunn *et al.* 2017), and utilising a range of habitats including forests, fruit orchards and hedgerows for nesting and cultivated areas and grassland for foraging in the wintering grounds (Eraud *et al.* 2009; Hanane 2017). However, much of the surrounding environment has

been subject to increased agricultural impact in these regions, therefore it is important to investigate the impacts of such changes on the diet of birds not necessarily nesting within farmland.

In the first wide scale turtle dove study since the 1960s, Browne and Aebischer (2003a) observed a rapid shift in diet composition, using microscopic analysis of faecal samples collected at two sites in East-Anglia (UK), during the 1998-2000 breeding seasons. Turtle dove diet previously largely comprised the seeds of non-cultivated arable plants (Murton *et al.* 1964). In the 1960s, weed seeds constituted over 95% of their diet, with common fumitory (*Fumaria officinalis*) and common chickweed (*Stellaria media*) being of particular importance (Murton *et al.* 1964). However, in a more recent study, Browne and Aebischer (2003a) recorded a significantly lower proportion of the diet (just 40%) being made up of seeds from natural weed flora. They observed that the main dietary component had been replaced in recent decades by cultivated seeds, principally wheat and rape, which would previously have amounted to just 5% of the average seed intake of turtle doves (Browne and Aebischer 2003a). These findings are supported by the use of more recently developed diet analysis tools. Dunn *et al.* (2018) investigated the diet of four UK columbid species (turtle dove, collared dove (*Streptopelia decaocto*), stock dove (*Columba oenas*) and woodpigeon (*Columba palumbus*)) through the use of High Throughput Sequencing (HTS) to identify dietary items consumed from DNA fragments extracted from faecal samples, allowing a more in depth study into the composition of columbid diet. Consistent with previous findings, the proportion of naturally occurring seeds appearing in the diet of turtle doves was lower than in the 1960s, with common chickweed appearing in the diet of only 26% of individuals. Along with a high prevalence of cultivated crops, this study was also the first to identify seeds such as niger (*Guizotia abyssinica*), sorghum (*Sorghum bicolor*) and hemp (*Cannabis sativa*), common components in garden bird food, in turtle dove diet in the UK (Murton *et al.* 1964; Browne and Aebischer 2003a; Dunn *et al.* 2018).

2.2.2 Supplementary feeding

Supplementary feeding is a common practice across the world for recreational purposes, (e.g. garden birdfeeders) (Robb *et al.* 2008a), game management

(Oro *et al.* 2013), tourism (Orams 2002) and conservation (Ewen *et al.* 2015). Whilst there are advantages to supplementary feeding, both for wildlife, and humans, it is important that appropriate food is provided (Murray *et al.* 2016). Supplementary feeding can benefit species when natural food resources are scarce, by reducing nutritional stress (Robb *et al.* 2008a; Wilcoxen *et al.* 2015), however, potential physiological and ecological effects of food provisioning should be considered.

In their comprehensive literature review, Murray *et al.* (2016) found that nearly half of the studies reported negative effects of food provisioning on the health of wildlife, such as deficiencies in protein and other micronutrients (Murray *et al.* 2016). As well as implications for individual bird fitness, food resources may impact breeding success, as demonstrated in study of greenfinches (*Chloris chloris*) and hawfinches (*Coccothraustes coccothraustes*), which indicated that a diet consisting heavily of sunflower seeds may be negatively impacting sperm quality (Støstad *et al.* 2019). These examples highlight the importance of using appropriate resources where food is provided for conservation purposes.

Providing supplemental resources also has implications for a range of ecological factors. For example, anthropogenic food subsidiaries can improve fitness in opportunistic species, facilitating population increases and potentially altering competition or predator-prey interactions, thus helping to shape communities and ecosystems (Oro *et al.* 2013). Congregation of birds at feeding sites can result in increased disease transmission, with increased prevalence of infectious agents including *Trichomonas gallinae*, *Salmonella typhimurium*, *Escherichia coli* and *Mycoplasma gallisepticum* all being associated with supplemental feeding (Daszak *et al.* 2000; Lennon *et al.* 2013; Moyers *et al.* 2018). When considering avian breeding ecology, the development and laying of eggs is an energetically demanding process, so breeding adults may benefit from access to supplementary food resources (Robb *et al.* 2008a). Several studies showed supplemental feeding resulted in earlier laying dates (reviewed in Robb *et al*. 2008a). Whilst chicks from early broods may have increased survival rates (Barba *et al.* 1995), earlier breeding attempts risk rearing nestlings out of synchrony with crucial natural resource

availability (Schoech and Bowman 2001). This illustrates one of many complex ecological interactions which must be considered when formulating species management plans.

When considering the health of individual birds, in studies of turtle doves, a higher proportion of cultivated seeds in adult turtle doves' diet was associated with better body condition, but the opposite was true for nestlings, which were in better condition when fed a higher proportion of wild seed species (Dunn *et al.* 2018). Furthermore, fledglings that were in better body condition were more likely to survive 30 days post-fledging, and body condition was positively correlated with the availability of weed seed-rich habitat (Dunn *et al.* 2017). Together, these studies indicate that, whilst anthropogenic food resources may be beneficial to adult birds early in the breeding season, the availability of wild food resources is important in increasing the likelihood of successfully fledging chicks. Far less is known about the diet of turtle doves in the wintering grounds, but due to differing nutritional needs throughout the annual cycle, and potential implications for survival and subsequent breeding, it is important to consider feeding ecology across the entire flyway of migratory species (Newton 2004a; Eraud *et al.* 2009; Atkinson *et al.* 2014).

In the wintering grounds, it is crucial that birds are able to gain enough fat resources to prepare them for their migration back to Europe. During the breeding season, it is key that adults have sufficient nutritional resources early in the breeding season to prepare them for reproduction, and that there are wild seed resources available to feed chicks and fledglings. Through a stronger understanding of feeding behaviour across their range, land-management and supplementary feeding plans can be better informed to ensure turtle doves have access to appropriate nutritional resources throughout the year, with the aim of increasing productivity, and thus, survival. Unlike other dietary studies that focus solely on the breeding grounds (Browne and Aebischer 2003a; Rocha and Quillfeldt 2015; Gutiérrez-Galán and Alonso 2016; Dunn *et al.* 2018; Mansouri *et al.* 2019), this study includes sites in both the breeding and wintering grounds. I use HTS to obtain insight into the use of feeding resources by turtle doves across a range of natural environments, testing the following hypotheses: i) In the breeding grounds, turtle doves will feed on a higher

proportion of wild seeds in Hungary, which has less intensive agriculture than France; ii) Birds feeding on a higher proportion of cultivated seeds will exhibit a higher body condition than those feeding on more wild seeds.

2.3 Methods

2.3.1 Sample collection

Faecal samples were collected in three countries, across both wintering and breeding grounds (Figure 2.1). A wintering population of turtle doves was sampled at the Beer Sheba Project (Beer Sheba Project, 2002), an agricultural resource centre encompassing a 100 hectares of naturally regenerated *Acacia seyal* woodland, small allotments growing fruit and vegetables, and a small reservoir, near Sandiara (14°22'N, 16°48'W), Senegal, between November and March over four winters (2014/2015 to 2017/2018). The surrounding environment consists largely of agricultural land, with major crops being sorghum, millet and peanut, with sporadic areas of grass and shrubland. Several small settlements are also present and low densities of livestock are allowed to graze freely on pasture. Water availability varied across season, with seasonal shallow depressions of water being available earlier in the season, which dried up as the dry season progressed. A larger reservoir was present throughout the wintering season approximately 6km from Beer Sheba. Mist nets were used to catch birds when they returned to the site to roost in the evening. Exact time varied day to day, but was around 17:00-18:00, so that birds were only handled during the cooler time of day to minimise stress.

Birds were caught at two different European breeding locations, in western Europe (France) and eastern Europe (Hungary) in order to sample geographically distinct populations using different migratory flyways (Marx *et al.* 2016). In Europe, birds were caught early in the breeding season, from sunrise, throughout the day. In France, samples were collected from 15th-30th May 2017 at three sites close to the French Atlantic coast. The sites consisted of two mainland sites, comprising large areas of deciduous forest: Chizé Forest (46°07'N, 0°25'W) and Aulnay Forest (46°02'N, 0°14'W), and a more open and varied habitat on Ile d'Oléron (45°93'N, 01°28'W), an island just off the west

coast. The larger of the two forests, covering around 3435 hectares, Chizé Forest is a mixture of mature and deciduous woodland, with areas of scrub. Aulnay forest covers around 2870 hectares, and is less mature, as much of the forest is regenerated following a storm in 1999, which destroyed much of the native forest, including its beech grove. The forest is largely deciduous, primarily *Quercus* sp. with areas of *Fagus sylvatica*. Situated within 15km of each other, both Chizé and Aulnay are surrounded by areas of arable farmland and small towns. Ile d'Oléron is a 174km^2 island just off the west coast. A more open and varied habitat, with small woodland areas, marshes, arable land and villages. In recent years Ile d'Oléron has had increasing areas of land that were previously farmed left to rewild due to high levels of wild boars (*Sus scrofa*) and rabbits (*Oryctolagus cuniculus*) interfering with crops (Hervé Lormée*, personal communication*).

In Hungary, samples were collected over a three-week period from 20th May-7th June 2018, from Balotaszállás-Öttömos (46°16'N, 19°35'W). This area covers 4000 hectares, consisting mainly of black pine (*Pinus nigra*) and black locust (*Robinia pseudoacacia*) plantations, interspersed with native poplar-juniper sand dune and open steppe oak forest, and steppe. The surrounding area consists of largely of agricultural fields (arable and pasture) and villages, areas of natural grassland and small woodlands. Whilst there was variation in the surrounding environment, all sites were located within a landscape containing a mix of agricultural land and small towns or villages, often interspersed with natural grassland.

In France, potter traps were baited with bird-seed mix (species composition identified by DNA sequencing; see Appendix 1, 1.1). On Ile d'Oléron, bait was just sunflower seed (*Helianthus* sp.), wheat (*Triticum* sp.) and maize (*Zea mays*). In mainland French sites (Chizé and Aulnay), the mix consisted of sunflower seed (*Helianthus* sp.), wheat (*Triticum* sp.), sorghum (*Sorghum bicolor*), maize, proso millet (*Panicum miliaceum*) and canary grass (*Phalaris canariensis).* In Hungary, birds were caught using mist nets, and the site was baited with ground maize. Whilst turtle doves and other birds were observed feeding on the bait, it was primarily intended to feed the wild boar. Maize was

put out every day for a week from 19th – 26th May. After this date, maize was used to bait the site every other day.

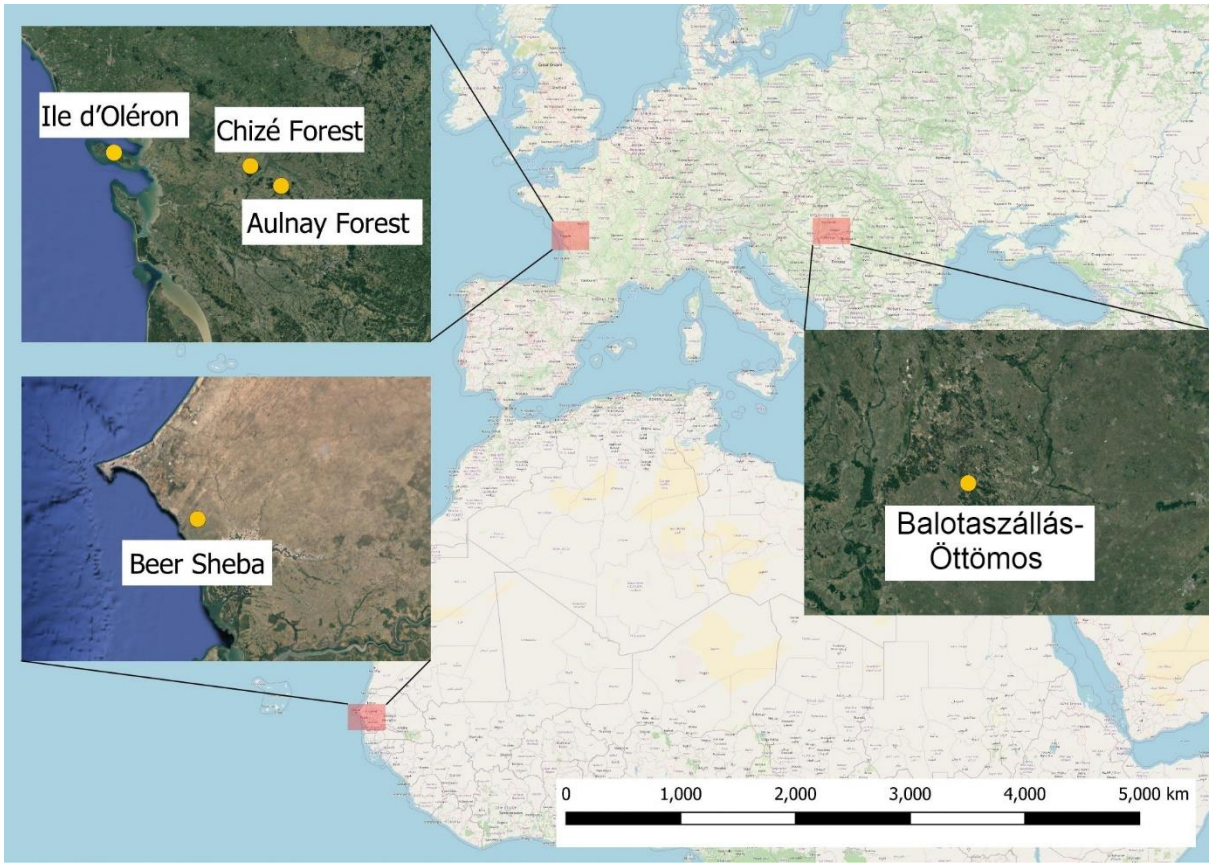

Figure 2.1: Countries sampled across the European turtle doves' breeding and wintering range

Faecal samples were collected from sterile bird bags, within which birds were temporarily held after capture, using a sterile toothpick, and transferred to a 2ml Eppendorf tube (Senegal) or a sterile plastic ziplock bag (Europe). When extracting faecal samples from bags, the white part of the sample, consisting of urea, was avoided as this contains PCR inhibitors (Segelbacher and Steinbruck, 2001) and could reduce efficiency of subsequent lab analysis. Faecal samples were dried in order to preserve plant DNA in faeces (Kowalczyk *et al.* 2011; Rayé *et al.* 2011). In the first three sampling seasons in Senegal (2014/15-2016/17), faecal samples were air dried by opening Eppendorfs leaving for 10-16 hours, covered with mesh fabric, to allow water to be released but prevent invertebrates or debris getting into the sample (Chris Orsman, *personal communication*). In Senegal 2017/18 and all

European sampling, faecal samples were dried using self-indicating silica gel to dry samples more efficiently. Samples were frozen as soon as possible, but this ranged from two weeks to three months due to field conditions and time between collection and import into the UK.

Each bird was fitted with a standard metal ornithological ring, and a blood sample taken from the brachial vein. A range of morphometric measurements were taken, including weight and wing length. In Senegal, birds were aged according to EURING age code (Baker *et al.* 1993) based on the moult plumage, which were subsequently divided into the age classes adult, juvenile or unknown for analysis (see Appendix 1, 1.2 for method of differentiation). All European birds were classified as adult as samples were collected at the beginning of the breeding season, thus all individuals were at least one year old. Sex information was not included as it is very difficult to accurately sex turtle doves visually based on sexual dimorphism, particularly during winter. Sexing was attempted using PCR on DNA extracted from both faecal and blood samples, but results were too inconsistent to reliably differentiate sexes. For full description of sexing PCR trials, see Appendix 1, 1.3). All bird handling was completed by licensed ringers.

2.3.2 DNA extraction and PCR amplification

2.3.2.1 Faecal samples for dietary analysis

DNA was extracted using either a QIAmp DNA Stool Mini Kit, or QIAmp Fast Stool Mini Kit (Qiagen, Hilden, Germany). The extraction protocol is largely the same between the two kits, with the only change being a replacement of the InhibitEX tablets with an InhibitEX buffer in the new kit. Extractions were conducted following manufacturers' protocols, including all recommended steps, with the following modifications, based on Dunn *et al.* (2016). (i) a sterile pestle was used to homogenise faecal samples in buffer ASL; (ii) samples were incubated at 70°C for 30 minutes; (iii) drying step was increased to 3 minutes centrifugation; (iv) final elution in 100µl buffer AE. Two extraction negatives, containing no faecal sample, were included in every extraction batch to detect potential contamination of buffers.

PCR amplification of the second internal transcribed spacer of nuclear ribosomal DNA (hereafter ITS2) region was carried out using the UniPlant primer pair (Moorhouse-Gann *et al.* 2018, Table S1.2) specifically designed for short amplicons (187-387bp) to maximise amplification of degraded plant DNA from faecal samples. Both forward and reverse primers were labelled with MID-tags (Multiple Identifiers) to provide a unique combination of tags for each DNA sample. PCRs were carried out in 25µl reaction volumes, consisting of 12.5µl Multiplex PCR Master Mix (Qiagen), 2.5µl nuclease free water, 2.5µl each of 0.01M UniPlantF and UniPlantR MID-tagged primers (Eurofins, Ebersberg, Germany), and 5µl template DNA. Within each 96-well plate, 11 extraction negatives, one PCR negative, two unused MID-tag combinations and two PCR positives (DNA extracted from plants endemic to Mauritius, thus not occurring within my study system) were included as controls (Taberlet *et al.* 2018).

Negatives were arranged within the plate such that every MID-tag had a negative control, facilitating identification of any contamination in primers. Two wells per plate were left empty to ensure that certain MID-tag combinations were not used at all during PCR amplification to allow quantification of tag jumping during the sequencing process. Replicate samples were also incorporated for quality control purposes, with a total of 16 samples to assess the consistency of sequencing.

PCR amplification was carried out using SimpliAmp^{TM} Thermal Cycler (ThermoFisher Scientific, Waltham, USA), using the following thermocycling protocol: 95°C (15 minutes); 40 cycles of 95°C (30 seconds), 58°C (90 seconds), 72°C (90 seconds); 72°C (10 minutes). Concentration of amplified DNA was determined by testing 5µl of PCR product using a DNA fast analysis cartridge with QIAxcel (Qiagen). Each plate of MID-tagged PCR product was combined into a single pool of uniquely identifiable samples, with approximately equal concentration per sample. Concentrations from the QIAxcel were used to calculate the volume of each PCR product to be added to the final pool, with plates being pooled individually due to unavoidable variation between QIAxcel runs. The maximum DNA concentration, C_{max} was determined for each plate, and divided by the concentration of each sample to

produce the volume to be added to the pool. The volume of negative samples to add to the pools was the average of all sample volumes added to the pool.

2.3.3 Cleaning and Illumina library preparation for HTS

2.3.3.1 Cleaning

Following pooling of samples there were five pools for ITS. The quality of the pooled samples and size of amplicons was checked via TapeStation2200 (Agilent, Santa Clara, USA). Pools were cleaned using SPRIselect beads (Beckman Coulter, Brea, USA) at a ratio of 1:1.1, to retain fragments of the desired size range (207-400bp). SPRI bead solution was vortexed for five minutes to ensure beads were fully suspended in solution then added to pooled samples and incubated at room temperature for five minutes to allow DNA to bind to beads. Tubes were placed on a magnetic rack, attracting the beads to the magnet and allowing removal of the supernatant without disturbing the beads. The supernatant, containing amplicons outside the desired size range, was discarded. Tubes were kept on the magnetic rack and rinsed twice with 85% ethanol. Following the removal of ethanol from the second rinsing step, beads were removed from the magnetic rack and left to air dry for approximately five minutes. Beads were then eluted in nuclease free water, with the volume of nuclease free water added determined by the number of samples included in the pool. Samples were incubated at room temperature for five minutes, then returned to the magnetic racks. The supernatant, now containing DNA previously bound to the beads, was removed and aliquotted into a new tube.

Once cleaned, the concentration of DNA was measured for each pool using a Qubit dsDNA High-sensitivity Assay (ThermoFisher Scientific) and pools were combined into a single equimolar pool.

The library preparation for Illumina sequencing was carried out using NEXTflex Rapid DNA-seq Kit (Bioo Scientific, Austin, USA), following the manufacturer's protocols.

2.3.3.2 Step 1: End-repair and Adenylation

To calculate the volume of pooled sample required to produce an input sample containing 100ng DNA, 100 was divided by the result from the final Qubit of the clean-up stage (21.1), giving the amount of sample to be added to a PCR tube. This was then diluted in a volume of nuclease free water to produce 32µl of diluted sample. 15µl NEXTflex End-Repair & Adenylation Buffer Mix and 3µl of NEXTflex End-Repair & Adenylation Enzyme Mix were then added, resulting in a total volume of 50µl. The mixture was then incubated in a thermocycler for 20 minutes at 22°C, then 20 minutes at 72°C.

2.3.3.3 Step 2: Adaptor ligation

Adaptors were first diluted to the desired adaptor concentration for the given input DNA concentration (3µM for 100ng input DNA). 2.5µl diluted NEXTflex Barcodes and 47.5µl NEXTflex Ligase Enzyme were added to the 50µl product produced in step 1, and mixed well prior to incubating for 15 minutes at 22°C in a thermocycler.

Samples were then cleaned using a similar method to SPRI bead clean (see section 2.3.3.1 Cleaning). 80µl AMPure XP beads were added to the sample and mixed thoroughly until homogenized. The volume added at this stage was adjusted from the standard protocol to allow for the removal of smaller, undesired amplicons. All subsequent steps were carried out as for the SPRI bead clean up, but the ethanol rinse was performed using 80% ethanol, and the rinsed beads were resuspended in 52µl resuspension buffer to elute the DNA, and 50µl transferred to a new tube for the next steps. This clean was repeated with three minor adjustments: adding 40µl AMPure Beads to start with, eluting in 22µl resuspension and retaining 20µl sample for the next stages.

2.3.3.4 Step 3: PCR amplification

A 50µl volume of PCR mix was made up of 20µl adapter ligated DNA (from previous step), 16µl nuclease free water, 2µl NETXflex primer mix, 12µl NEXTflex PCR master mix. The mix was placed in a thermocycler for the following cycles: 2 minutes at 98°C, 6 cycled of 30 seconds at 98°C, 30 seconds at 65°C, 1 minute at 72°C and a final step of 4 minutes at 72°C.

The PCR product was cleaned once more using AMPure beads, following the protocol as above, adding 40µl AMPure beads and with a final elution volume of 21µl resuspension buffer and 20µl of DNA being transferred to a new tube.

2.3.3.5 Step 4: Sequencing

Following adaptor ligation and cleaning steps, the final concentration of DNA (12.2ng/µl) and average library size (499bp) were determined via the Qubit and TapeStation, respectively. The concentration obtained was used to calculate the volume of sample to be sequenced, to achieve the optimum concentration for sequencing using an Illumina platform of 4nM. The library was sequenced using a Nano chip, with 250bp paired end reads on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

2.3.4 Bioinformatic analysis

Bioinformatic analyses were carried out using a custom pipeline designed for analysis of diet metabarcoding data from Illumina MiSeq (Drake *et al.* 2021). Data were first checked for truncation in the MID-tags, using the 'grep' function to extract information of the number of reads with and without the full 10 base pair MID-tag before the primer. This was then used to calculate the rate of truncation (Table S1.3). FastP (Chen *et al.* 2018) was then used to trim, align and check the quality of reads, discarding low quality reads, based on a quality threshold (Q) of 33. A minimum sequence length (170bp) was set, based on the expected amplicon length to filter out short, poor quality reads. Using Mothur v1.39.5 (Schloss *et al.* 2009), sequences were demultiplexed and assigned their sample ID. Following the assignment of an identifier, MID-tag and primer were removed, so resultant sequences consisted of only the barcode.

Following demultiplexing, samples were dereplicated and error-corrected via Unoise3, in Usearch 11, to remove sequencing and PCR errors and chimeras. Dereplication was performed using the fastx_uniques command. Denoising of data, performed by means of a UNOISE algorithm specifically designed for use on Illumina sequences, recovers correct biological sequences from reads obtained, referred to as zero-radius operational taxonomic units (hereafter zOTUs). Reads were then clustered based on zOTUs, also in Unoise3.

Clustering based on zOTUs uses a 100% clustering threshold, as opposed to the standard 97%, assuring high resolution and preventing variants being incorrectly collapsed into clusters (Edgar 2016; Edgar 2020). BLASTn v2.7.1 (Camacho *et al.* 2009) was then used to assign sequences to their closest matching taxa from GenBank, with the minimum percent identity set to 95% in order to remove poor matches. BLAST results were filtered using the *dplyr* package in R to retain only the top bit score for each zOTU, calculated by combining the percent ID and e-value to indicate how good the alignment is. These BLAST outputs were analysed using MEGAN v6.15.2 (Huson *et al.* 2016) to assign taxonomic names to each zOTU. In instances where there was more than one top hit, zOTUs would be assigned to the highest common taxonomic rank.

Following taxonomic assignment, results were manually checked to identify zOTUs not assigned to species level, generally resulting from variations in nomenclature or erroneous sequences on GenBank. Where possible, sequences were assigned to species level, however, where there were multiple top hits on GenBank, they were assigned to the highest taxonomic rank in common. The geographic range of all taxa identified was checked using Kew Science 'plants of the world online' (POWO 2019). Fifteen species assignments were identified which did not occur within the sampling range of the present study, thus were assigned the most appropriate taxonomic level occurring within the study region. The final step was aggregation, whereby all zOTUs that had been assigned to the same taxon were aggregated in R using the 'aggregate' function.

2.3.5 Cleaning the data set

There were two stages to the cleaning of data to remove artefacts from the final data set, which cause false positives. The two stages of filtering complement each other to provide a more comprehensive method of data cleaning (Drake *et al.* 2021)

The first stage of cleaning was to remove contamination. Contamination can occur at two stages: in the laboratory exogenous contaminants may be introduced into the samples during extraction or PCR, or internal

contamination, such as tag jumping, during library preparation or sequencing (Taberlet *et al.* 2018). In order to remove potential false positives occurring as a result of exogenous contamination, the highest read count in negative controls was identified for each taxon. The same was done for unused MID-tag combinations in order to account for tag-jumping. Theoretically, both negative controls and unused MID-tag combinations should contain no DNA, thus any reads occurring within these samples are a result of contamination (Drake *et al.* 2021). The highest read count (out of either negative control of unused combinations) was designated as the maximum contamination per taxon, and any read count below this maximum contamination threshold was considered exogenous contamination and read count was replaced with zero. By removing read counts below the threshold, as opposed to subtracting the maximum contamination across the board, the overall read count of samples exceeding the contamination threshold was not affected.

The second stage is intended to remove internal contaminants: artefacts created during PCR and sequencing; and is carried out on a per sample basis. Artefacts generally occur in far lower proportions than genuine sequences, thus are removed by filtering out low abundance OTUs. Despite every effort being made during the pooling process to make samples equimolar, thus limiting variation in read depth among samples, this remains an inevitable occurrence. This variation in read count between samples can have strong implications for the efficacy of data filtering if a single threshold is set based on raw read count, such as removal of samples with fewer than 10 reads, a commonly cited filtering method (Drake *et al.* 2021). With this method, artefacts are more likely to pass filtering thresholds in samples with higher read counts, as artefacts tend to occur in greater numbers at higher sequencing depths (De Barba *et al.* 2014; Drake *et al.* 2021). In order to overcome this, thresholds were set based on frequency of occurrence of taxa within samples. Following this proportional approach accounted for variation in read depth between samples (in this case, the read depth per sample range from 4,276-172,171) facilitating a standardised clean up across all samples.

It is important to carefully consider the threshold for removal of reads from the data, as if threshold is set too low, it risks a high number of false positives, and

conversely, if thresholds are too conservative, there is a risk that genuine reads will be lost, resulting in false negatives. In order to designate a filtering threshold, the positive controls were used, each of which contained one of three known plant species, endemic to Mauritius, which would therefore not occur in samples from this study. One positive control (*C. mappia)* was not correctly assigned, but the species occurring in high read counts in this control (*Chromolena ordorata*) had a read count of 27,565 in the positive control, and a maximum read count of 418 across all faecal samples and negative controls, giving confidence that this was in fact DNA from the positive control. Sample based filtering thresholds were assigned by looking at how often non-target DNA occurred within the positive control. Different sampling thresholds were tested to assess the efficiency of removal of false positives from positive controls (for full details, see Appendix 1, 1.5.1). In order to balance false positives and false negatives, a slightly more conservative threshold of 4% was decided upon, which removed most false positives from positive controls, without running the risk of removing too many genuine reads. For a full description of how this threshold was selected, see supplementary information 1.5.1, Table S1.4). This 4% threshold was applied across the dataset in a sample filtering basis, and read counts for any taxa failing to exceed 4% of total reads of a sample were replaces with zero.

These two filtering methods were combined and only read counts exceeding both the maximum contamination threshold and the 4% per sample threshold were retained for further analysis. Following data filtering, fungal and bacterial taxa were removed, as were any taxa which were not identified to at least family level.

2.3.6 Statistical analysis

All statistical analyses were carried out in R, version 4.0.1 – “See Things Now” (R Core Team 2020). Due to differences in the amplification efficiency of DNA from different plant species, presence or absence of each taxonomic unit within each sampling unit, rather than read count, was used in all statistical analysis (frequency of occurrence) (Pompanon *et al.* 2012; Deagle *et al.* 2013; Lamb *et al.* 2018).

As sample size varied across countries, with a relatively small number of birds being caught in Hungary, sampling completeness was assessed using nonparametric Chao estimators using the package *vegan* (Oksanen *et al.* 2020). The 'specpool' function was used for to extrapolate the species richness in each country sampled, using incidence-based estimates, using the default Chao equation. The extrapolated species richness was used to estimate the proportion of total taxonomic units of the diet likely to have been detected in each sampling site.

2.3.6.1 Dietary variation in turtle doves

Two GLMs with Poisson errors and logarithmic link functions were fitted to assess differences in dietary species richness (number of taxa found within a faecal sample) between: i) sampling seasons and ii) years. The latter just used data for Senegal, as this was the only site to be sampled more than once (four consecutive winters). For all GLMs, significant pairwise differences were determined using Tukey's post-hoc test, using the *multcomp* package in R (Hothorn *et al.* 2008). The majority of analysis carried out include the supplementary seed provided to birds in bait as this was considered an important aspect of the diet. However, some additional analyses were conducted to assess the effect of removing bait seed from analysis.

Multivariate Generalised Linear Models (MGLMs) were performed using the *mvabund* package (Wang *et al.* 2012) to investigate variation in the diet of turtle doves. Multivariate data are commonly used in ecology to assess the effect of environmental variables on communities, and is also applicable to the study of diet (Wang *et al.* 2012; Hardy *et al.* 2017). Though widely used in ecology, a distance-based method of analysis makes implicit assumptions about the mean-variance relationship of data, which are often not consistent with true data (Warton *et al.* 2012). This can have major consequences for ecological inferences made from analyses, therefore there has been a move towards model-based approaches to analysis in recent years (Wang *et al.* 2012). The *mvabund* package uses this model-based approach to provide increased statistical power to detect differences between communities (Wang *et al.* 2012).

As not all dietary items were differentiated to species, genus level was used for this analysis, including 85 different genera. Some sites had unique attributes, such as Senegal being sampled in multiple years and France having multiple sampling sites and a high consumption of bait, which facilitated different analyses, so several MGLMs were fitted using subsets of data to address different questions. The different models generated are summarised in Table 2.1. All models including birds captured in France were run twice, once using the full dietary data set and one with the bait seeds omitted from the genera consumed data, in order to assess the effect of bait seed on analysis. In all instances, the 'manyglm' function was used to create an MGLM using the binomial family and complementary log-log link function. An 'mvabund' object was created using the presence-absence matrix of dietary items, which was the dependant variable in the models. The fit of the model was checked using the 'plot.manyglm' function, modelling residuals against fitted values and generating Q-Q plots.

Model selection was carried out, based on Akaike's Information Criterion (AIC), using R's 'step' command, which compares the AIC of all potential models to determine the best model to fit the data. The significance of terms in the final models was assessed using the 'anova' function. To examine which taxa contributed to dietary variation, univariate tests were run on each dietary item separately using the 'p.uni = "adjusted"' argument in the 'anova.manyglm' function. Two methods were used to visualise distribution of consumed genera: i) genus level bipartite plots were created using the *bipartite* package (Dormann 2019) to illustrate the overlap of genera consumed between countries; and ii) differences in genera consumed between seasons were visualised using non-metric multidimensional scaling (NMDS). For the purpose of NMDS analysis, genera occurring in a single dietary sample were removed, as this analysis is liable to outliers skewing the results. The command 'metaMDS', from the *vegan* package (Oksanen *et al.* 2020) was used to estimate differences between the presence of genera in individual samples, using Jaccard distance. 'Ordispider' was used to visualise results from nMDS via plotting with *ggplot2* (Wickham 2016).

Table 2.1: Details of data sets and variables used for different multivariate models generated. Terms marked with† were included in the final model

Data subset	Rationale	Independent variables
Senegal	The only site with mix of adult and juveniles, sampled over several years and sampled throughout winter (five month period). Assess temporal variation and effect of age class.	Year (2014/15, 2015/16, 2016/17, 2017/18) Age class (adult/juvenile/unknown) Body condition Julian date (using November 1st as start of sampling season) † Season:Age class interaction Season:Body condition interaction Season:Julian date interaction Condition: Age class interaction Condition:Julian date interaction
Adults	Compare adults across all sites to rule out variation being caused by the presence of juveniles at one site	Season (W1, W2, W3, W4, S1, S2) Body condition Season:body condition interaction
France	Multiple sites sampled, separated into two regions: island (Ile d'Oléron) and mainland (Chizé and Aulnay). Assess effect of sampling site.	Region Condition Region:condition

Full data set	Provide overall comparison of all birds across all seasons	Season (W1, W2, W3, W4, S1, S2) † Body condition Season:body condition interaction

2.3.6.2 Cultivated vs. wild seeds, and the effect on bird condition

An index of bird body condition was calculated using a generalised linear model (GLM) with Gaussian family and identity link function. Bird weight was the dependent variable, and wing length and time measurements were predictor variables, the latter to account for within-day variation in weight as there was an approximate linear increase in weight through the daily sampling period. Residuals from the model represented the condition of individual birds. Variation in condition between seasons was assessed using a one-way ANOVA, modelling condition as the dependent and season as the independent variable.

To test if variation in food types consumed in the different sampling countries influenced turtle dove condition, plants were categorised into “cultivated” (34 taxonomic units), consisting of crop plants, those cultivated for animal fodder and seeds commonly provided in supplementary feed, and “wild” (95 taxonomic units). For the most part, wild/cultivated status did not differ across sampling sites, with the exception of *Panicum* sp., which occur naturally in Senegal, but primarily as a constituent of bird feed in Europe, thus *Panicum* sp. were divided into those occurring in samples from Senegal (wild) or Europe (cultivated). The genus *Brassica* occurred in one instance where it could not be identified to species level. Due to the diversity of this genus including both wild and cultivated species within the area in this study, this taxonomic unit was excluded from the analysis of food type.

These classifications were used to analyse the effect of the type of food consumed on body condition. Condition and the proportion of wild seed taxa consumed were compared between seasons by generating a GLM with Gaussian family and identity link function, using body condition as the dependent variable and season, proportion of dietary items accounted for by wild seed and their interaction as predictor variables. Model selection was carried out using the ‘step’ function in R. In order to assess whether body condition changed as the wintering season progressed, the same analysis was carried out on a subset of data consisting of only birds sampled in Senegal, including Julian date (calculated from November 1st), and interactions between Julian date and season as additional dependent variables. To assess whether

sampling date varied between seasons, and if this could be driving between-season trends, a GLM modelling Julian date as the dependent variable and season as the independent variable was generated, using Poisson distribution and logarithmic link function.

All mean values are reported ± standard error.

2.4 Results

2.4.1 DNA amplification, sequencing and taxon identification

DNA was successfully amplified from 203 turtle dove faecal samples across three countries, including four winter sampling seasons and two spring sampling seasons (Table 2.2). The Illumina sequencing run yielded 17.1 million paired-end reads, which was reduced to 9.7 million reads following data processing (Table S1.5). The average read count per sequenced sample sampled was 43,586 ± 1322 reads (min = 323, max = 136,843). A total of 121 taxonomic units were present in the diet of turtle doves in this study (N=203, Table 2.2). Of these, 58.7% (n=71) were identified to species level, another 38.0% (n=46) to genus, 2.5% (n=3) to tribe or subtribe and the remaining 0.8% (n=1) to family (Taxa identified

Table S1.6).

Table 2.2: Number of faecal DNA samples successfully sequenced, per country and year sampled. Samples were collected from November to March in Senegal, hence two years.

Country	Year	Sampling season	Total N sampled
Senegal	2014/2015	Winter 1 (W1)	25
Senegal	2015/2016	Winter 2 (W2)	14
Senegal	2016/2017	Winter 3 (W3)	28
Senegal	2017/2018	Winter 4 (W4)	61
France	2018	Spring 1 (S1)	63
Hungary	2019	Spring 2 (S2)	12
Total			**203**

Despite a relatively small sample size in Hungary in this study, sampling completness analysis predicted relatively consistent level of available dietary items being detected across all three study sites (between 34% and 46%), with 39% of estimated available taxonomic units being detected in samples from Hungary (Table 2.3).

Table 2.3: Sampling completeness analysis based on Chao estimators for incidence dependent estimation of species richness for each country sampled

Country	Observed SR	Estimated SR (Chao±S.E.)	Sampling completeness	Sample size
Senegal	57	125.107±36.46	45.56%	128
France	49	143.575±55.11	34.13%	63
Hungary	26	67.365±28.80	38.60%	12

2.4.2 Dietary variation

The species richness (number of taxa identified in each bird sampled) ranged from one to seven overall, with a mean species richness of 2.827 ± 0.288 taxa consumed per individual (Table 2.4). Species richness in samples from Senegal differed between years (GLM: $Dev_{3,124}$ = 12.676, p = 0.005), consequently these years could not be combined to make a single 'country' variable. Species richness was therefore analysed based on sampling season, with significant variation being observed in the species richness between sampling seasons (GLM: Dev = $30.199_{5,197}$, p<0.001) (Table 2.4, Figure 2.2). Birds sampled from Senegal in the winter of 2017/18 (W4) had the lowest mean species richness (1.918), which was significantly lower than all other sampling seasons, apart from the winter of 2016/17 (W3), according to Tukey's post-hoc analysis (Figure 2.2). Birds in Hungary (S2) had the highest mean species richness, indicating the most varied diet. When supplementary food from bait was excluded in France, this site had the lowest average species richness, and lowest maximum species richness within a single individual, indicating a less varied use of natural resources.

Table 2.4: Species richness (SR) of taxa consumed by individuals at three sampling locations, including four sampling years in Senegal and a comparison in France when bait seeds were excluded from analysis.

	Season	Sample size	Minimum SR	Mean SR ± S.E.	Maximum SR
Senegal					
2014/15	W1	25	1	2.920 ± 0.237	5
2015/16	W2	14	1	3.214 ± 0.482	6
2016/17	W3	28	1	2.536 ± 0.260	5
2017/18	W4	61	1	1.918 ± 0.147	6
Senegal total		**128**	**1**	**2.391 ± 0.121**	**6**
France	S1	63	1	3.381 ± 0.350	7
France (bait excluded)	S1	38	1	1.711 ± 0.155	4
Hungary	S2	12	1	3.583 ± 0.288	6
Overall		**203**	**1**	**2.768 ± 0.100**	**7**

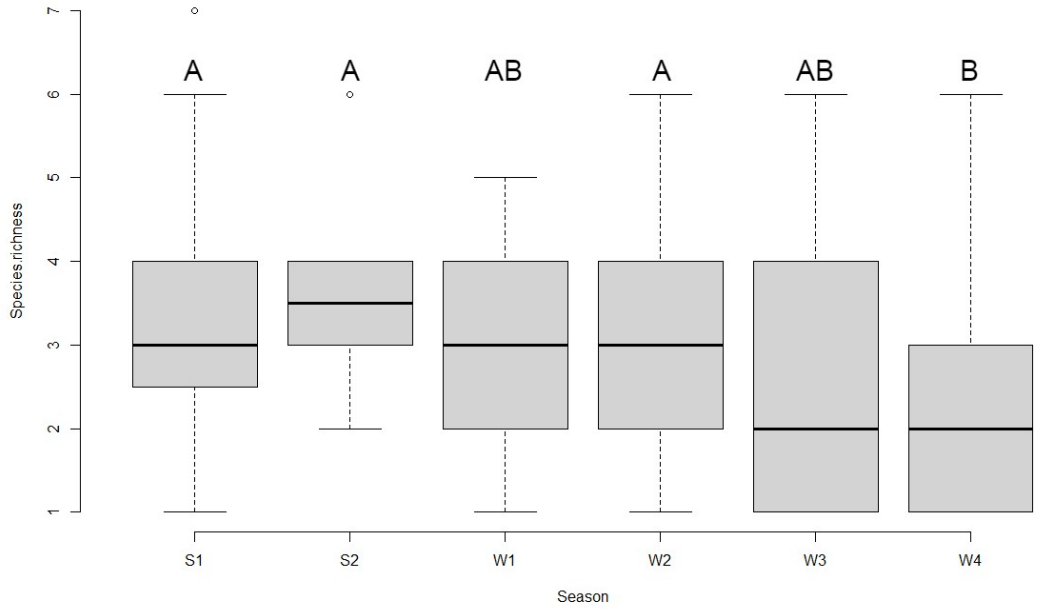

Figure 2.2: Variation in species richness of dietary items consumed by turtle doves across 6 sampling seasons. Letters (A-B) show significant difference according to Tukey's post-hoc test. Species richness was lower in W4 than in S1, S2, W1 and W2. For full breakdown of p-values associated with plot pairwise differences see Appendix 1, Table S1.7 .

The most frequent taxa in the diet of turtle doves differed across countries sampled. In Senegal, the dominant taxa consumed were *Cenchrus americanus* (pearl millet) and *Sorghum* sp., each being detected in 32.0% (n = 41) of birds sampled. Cenchrinae, the subtribe to which *C. americanus* belongs was detected in a further 14.1% (n=18) of turtle doves sampled in Senegal, however, it cannot be definitively stated that these are *C. americanus*. These findings are supported by field observations of a high proportion of caught birds having *Sorghum,* millet or both in their crop (Chris Orsman, *personal communication*). In France, the four most dominant taxa were *Triticum* sp*.* (74.6%, n=47), *Helianthus sp.* (41.3%, n = 26), Triticeae (the subtribe within which *Triticum* falls) (36.5%, n = 23), *Sorghum sp.* (33.3%, n = 21), all of which are present in the bird seed mix used to bait the potter traps in order to facilitate capture. *Triticum monococcum* (einkorn wheat) was also detected in 22.2% of birds (n = 14). Whilst the species of *Triticum* present in the bird seed mix could not be differentiated between three top hits on

GenBank, these were *T. turgidum, T. aestivum* and *T. dicoccoides*, and as *T. monococcum* is grown in France, this seed may have come from the environment or from garden bird feeders. Excluding items known, or likely, to be fed to the birds, the most common food resource was *Geranium molle* (dove's foot crane's-bill), a weed commonly occurring across Europe. In Hungary, the three most common taxa identified were *Euphorbia* sp. (50%, n = 6), *Euphorbia pseudoesula* (Figert's spurge) (50%, n = 6), and *Euphorbia cyparissias* (cypress spurge) (33.3%, n = 4), all of which are naturally occurring species.

When looking at the family level, Poaceae was the most frequently consumed taxon in both Senegal and France, occurring in 86.7% (n=111) and 90.5% (n=57) of turtle doves, respectively. Of the 14 taxonomic units within the Poaceae family detected in Senegal, 28% were cultivated including *C. americanus* and *Sorghum* sp.. However, the next two most commonly consumed taxonomic units in Senegal were *Panicum* sp., consumed by 22.6% (n=29) of birds sampled, and *Echinochloa* sp., consumed by 18.0% (n=23) of birds sampled. Both of these are widespread genera in the grass family, native to Africa. There was a greater proportion of cultivated taxonomic units within the Poaceae family identified in France (50%, n=6), and the wild genera (*Agrostis* sp., *Lolium* sp. and *Poa* sp.) were detected far less frequently than those cultivated or commonly provided in bird seed mixes (*Sorghum* sp., *Triticum* sp. and *Panicum miliaceum*). Poaceae was the second most commonly consumed family in Hungary, appearing in the diet of 41.7% of birds sampled (n=5). Fewer taxonomic units from this family were identified in Hungary (n = 5), but of those present 60% were cultivated.

Hungary was the only country where the dominant family consisted solely of wild food resources. Euphorbiaceae were detected in the diet of 58.3% (n=7) of birds, and consisted of five different taxonomic units: *Euphorbia* sp. and four distinct species of *Euphorbia,* all of which are naturally occurring. Furthermore, despite being used to bait the sites where birds were captured in Hungary, maize was not detected in the diet of birds caught in this location.

Asteraceae occurred in the top three families consumed by individuals in all countries sampled, being the third most commonly detected in Senegal (10.9%) and Hungary (25.0%) and second in France (41.3%). In France, this was dominated by *Helianthus* sp., a component of the bait used to catch birds, but also a common element in garden bird seed, which was detected in 41.3% of all birds sampled. Only one wild genus within this family, *Carduus* sp. (true thistles), was detected in the diet of three birds sampled in France. In contrast, in Hungary, *Helianthus* sp. was only identified in one individual. The other two genera identified were *Ambrosia* sp. (ragweeds) and *Carduus* sp., naturally occurring genera accounting for most of the occurrence of the Asteraceae family. In Senegal, the most commonly occurring member of the Asteraceae family was also naturally occurring genus, *Blainvillea.*

When looking at birds from Senegal to assess the effects of year, age class and Julian date on genera consumed by turtle doves, only Julian date was retained in the model. This indicates that age class and sampling year did not have a significant effect on the diet of birds sampled in Senegal, while the point in the season at which birds were sampled had a highly significant effect on genera consumed (MGLM: LRT = 192.2_{123}, p <0.001). When considering just adult birds across all sampling sites, no explanatory variables were retained in the model. Because age did not have a significant effect, it was not included in further modelling as the absence of juveniles from European samples confounded the effects of age and season.

In the full data set, season was the only variable retained in the final model following model simplification based on ‘step’, and had a significant effect on the genera consumed by turtle doves (MGLM: LRT = 647.4_{5}, p <0.001; Figure 2.3). This is likely driven by the country rather than the year, as when testing the effect of year sampled on the diet composition in Senegal, year was not a significant term. Furthermore, there was little overlap of consumed taxa between countries sampled (Figure 2.4). Following adjusting for multiple testing, season had a significant effect on 12 dietary items (N = 85 genera), indicating that these are the genera responsibly for a large proportion of the seasonal variation (Table S1.8). Of these genera, seven occurred only in Senegal (*Cenchrus, Echinochloa, Dactyloctenium, Senna, Balinvillea,*

Melochia and *Nymphaea*) and two occurred only in France (*Triticum* and *Geranium*), supporting the idea that country drove dietary variation.

For the purpose of investigating the effect of removal of bait genera from analysis, model simplification using step was omitted, as the aim was to compare the effects of genera included on the diet, rather than finding the simplest model to explain the data. The significant terms in the model did not change, with both the full data set and that with bait excluded producing the same level of significance for the effect of season, body condition and the interaction between the two on dietary items consumed by turtle doves (Table 2.5). There was some variation between the two data sets when adjusting for multiple testing, with differences in the genera consumed responsible for the largest portion of dietary variation (Table S1.9). Two items found in bait (*Triticum* and *Helianthus*) significantly contributed to dietary variation when bait was included, but these were no longer significant following the exclusion of bait. Two genera which did not significantly drive variation when bait was included did have an effect when bait was excluded. Both were wild seeds, one found only in Senegal (*Urochloa*) and on found only in France (*Mercurialis*).

The greatest variation between data sets with and without bait items included was observed in NMDS analysis (Figure 2.3). When bait was excluded, there is greater distance between the centroids for France and Senegal, demonstrating a greater difference in the diets consumed between breeding and wintering grounds.

Table 2.5: Result of MGLM on full model when assessing dietary data including and excluding taxa found in bait

	Full data set			No bait		
	LRT	df	*P*	LRT	df	*P*
Season	647.4	5	0.001	536.6	5	0.001
Condition	114.9	1	0.006	120.3	1	0.01
Season:condition	72.3	5	0.034	74.1	5	0.021

When considering multiple sites sampled in France, following model simplification, region was the only explanatory variable retained in the model. There was a significant difference between the diet of birds sampled in mainland France and Ile d'Oléron (MGLM: LRT_1 = 121.0, p <0.001). This difference was likely driven by the different bait used between the island and mainland, as following univariate analysis, the only significant genera were *Panicum* sp. (LRT = 12.5, p = 0.003) and *Sorghum* sp. (LRT = 48.7, p<0.001), both of which were present in the seed mix used on the mainland, but not on the island.

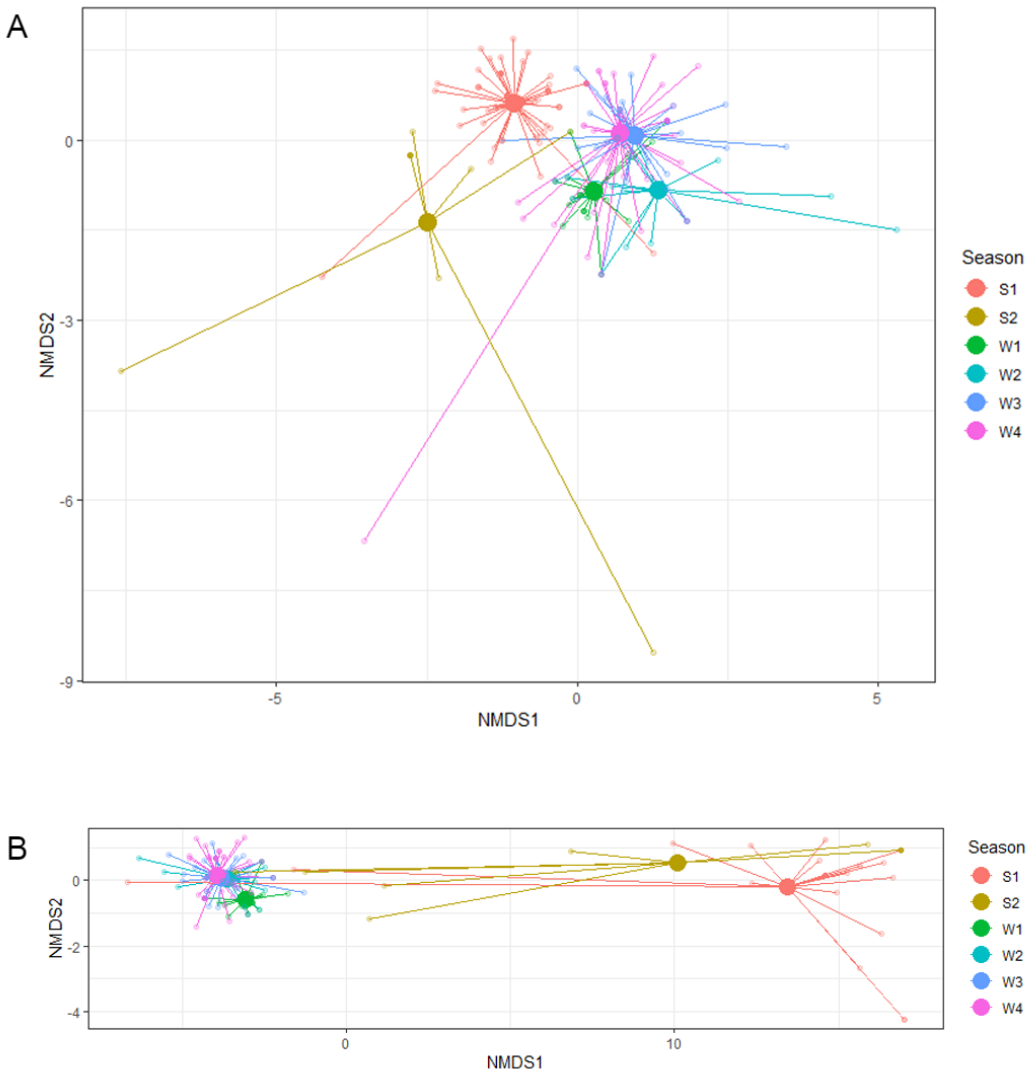

Figure 2.3: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves, differentiated by sampling season. A) Full data set; B) Bait items excluded. Ordination of multidimensional data condenses information into two-dimensional axes, representing variation in the diet. Each small point is representative of the diet of a single turtle dove sampled, and the distance between the points is representative of the similarity of diet composition between individual (a shorter distance between points indicates greater similarity between diets). The larger points forming the centroid of each group (sampling season) represent the mean co-ordinates per group. Stress: A = 0.058; B = 0.02

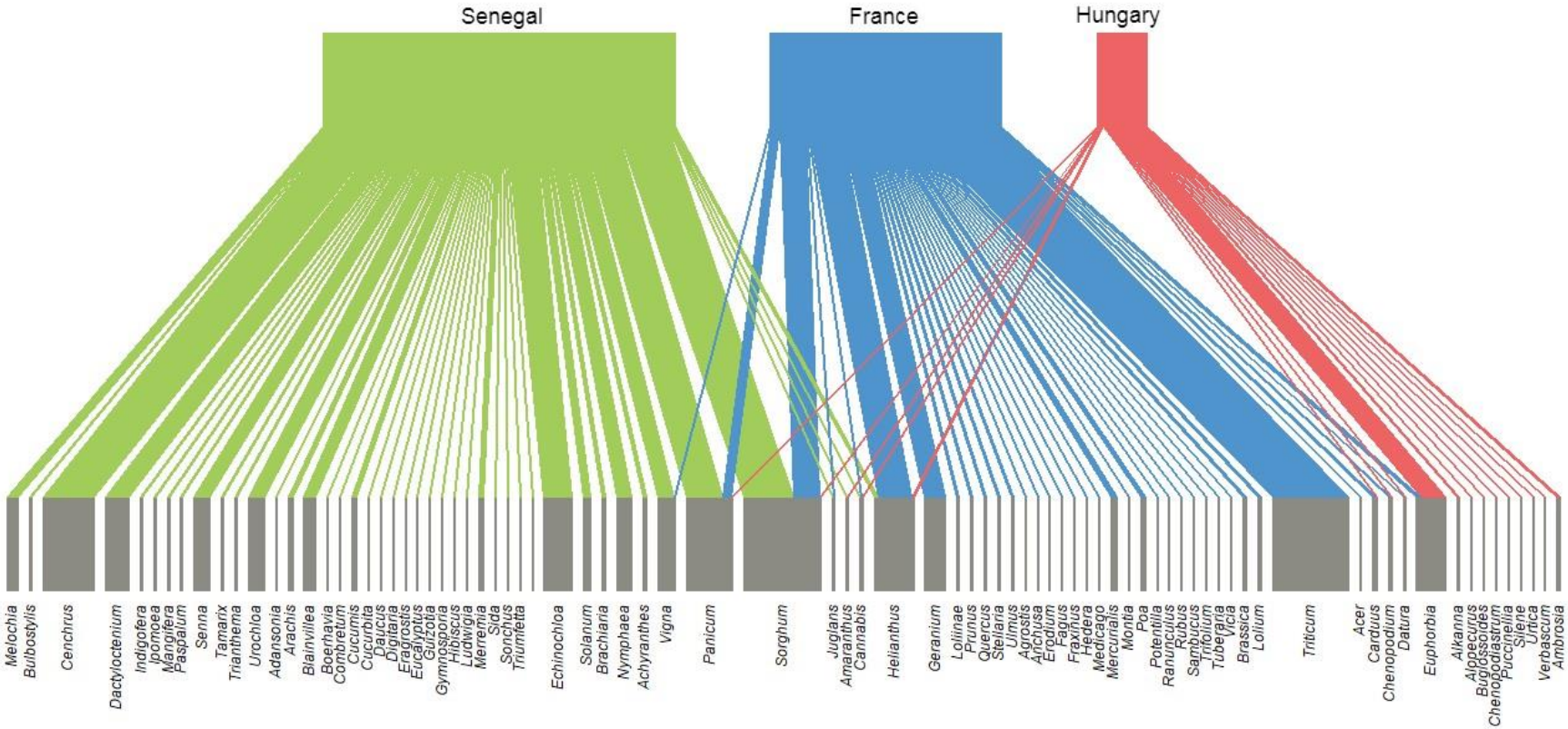

Figure 2.4: Bipartite plot illustrating the distribution of consumed genera across countries sampled. The width of coloured bars is representative of the number of birds sampled per country (Senegal n = 128, France n = 63, Hungary n = 12) and width of grey bars represents the number of times each genera was detected in the diet.

2.4.3 Food type and its effect on body condition

Hungary had the greatest proportion of wild taxonomic units recorded in the diet (80.6%; Table 2.6). France had the highest proportion of cultivated seeds occurring in the diet, accounting for 72.0% of taxonomic units detected, and being present in the diet of 95.2% of birds sampled (Table 2.6). This is unsurprising due to the use of bait to capture birds. When excluding seeds present in the bird seed mix, 25 individuals (39.7%) sampled in France had no taxonomic units recorded in their diet, indicating that this feed mix is an important part of the diet. In Senegal, levels of wild and cultivated seeds detected in the diet varied across the years sampled, with the percentage of diet accounted for by wild taxa decreasing with each year sampled, whilst the percentage of diet made up by cultivated seeds increased year on year (Table 2.6, Figure 2.5). In W4, 54.1% of birds sampled had a diet consisting only of cultivated seeds, the highest rate observed across all sampling seasons.

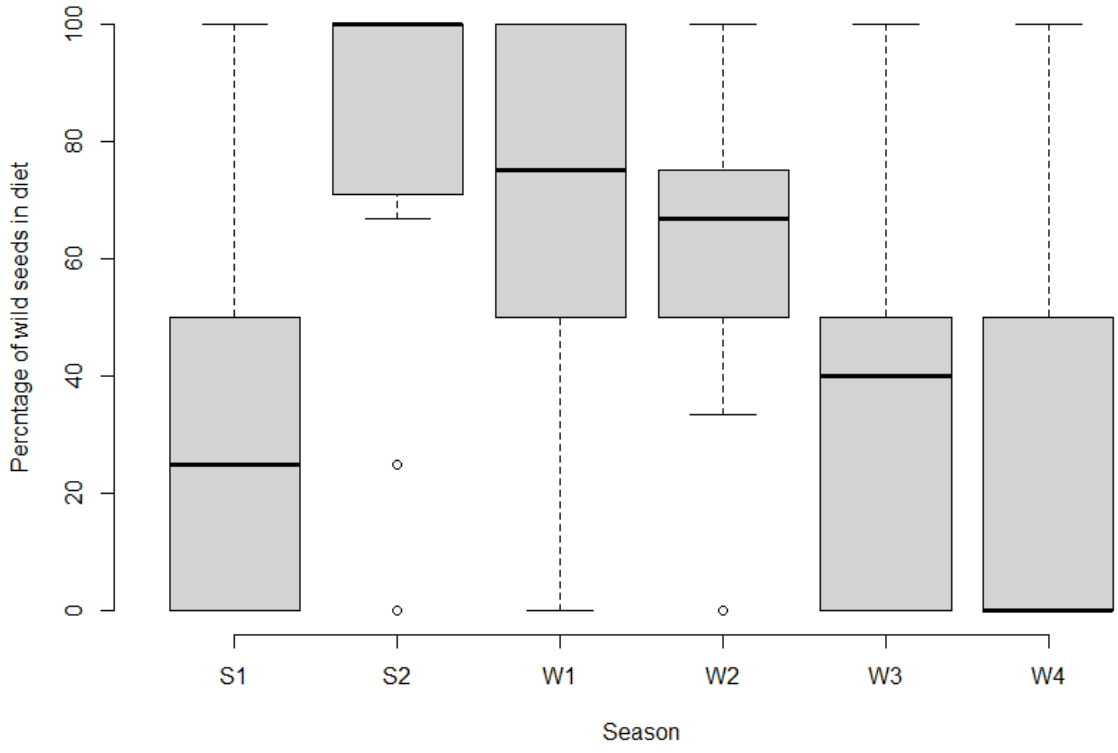

Figure 2.5: Boxplot illustrating the median and interquartile range of percentage of diet accounted for by wild seeds (n=93) across six sampling seasons

Table 2.6: Summary of levels of wild plants (n=93) and cultivated plants (n=30) consumed in each sampling season in Senegal (W1-W4), France (S1) and Hungary (S2).First two columns represent the percentage of individual birds in which wild or cultivated taxonomic units were detected. Middle two columns show the percentage of individual birds in which the diet consisted solely of either wild or cultivated seeds. Last two columns show mean percentage of diet (±S.E) accounted for by wild and cultivated plants

	% Individuals sampled with wild/cultivated taxa detected in diet		% Individuals sampled with **only** wild/cultivated taxa detected in diet		% Dietary items accounted for by wild/cultivated taxa	
	Wild	**Cultivated**	**Only wild**	**Only cultivated**	**Wild**	**Cultivated**
W1	88.0%	68.0%	32.0%	12.0%	67.2 ± 6.604	32.8 ± 6.604
W2	85.7%	78.6%	21.4%	14.3%	58.9 ± 8.580	41.1 ± 8.580
W3	67.9%	96.4%	3.6%	32.1%	36.3 ± 5.643	63.7 ± 5.643
W4	45.9%	86.9%	13.1%	54.1%	30.7 ± 4.746	69.3 ± 4.746
S1	91.3%	95.2%	4.8%	39.7%	28.0 ± 3.636	72.0 ± 3.649
S2	91.7%	33.3%	66.7%	8.3%	80.6 ± 9.857	19.4 ± 9.857

Both the proportion of wild food resources consumed (GLM: $F_{1,194}$=8.524, p=0.004) and season (GLM: $F_{5,189}$=26.500, p<0.001) separately affected turtle dove body condition, but the interaction between these two terms was not significant. Birds feeding on a higher proportion of cultivated food were in better condition than those feeding on wild seeds (Figure 2.6). There was a significant difference in body condition between sampling seasons (ANOVA: F=27.210$_{5,191}$, p<0.001), with birds sampled in France (S1) having a significantly higher body condition than all other seasons sampled (Figure 2.7)

However, this was not solely driven by the presence of supplementary food, as when individuals whose diet consisted solely of bait seeds were removed from analysis, the effect of body condition was still significant (ANOVA: F=23.150$_{5,167}$, p<0.001), and the significant pairwise differences did not change (Figure S1.1, Table S1.11). The remaining birds sampled in France, whose diet consisted of a mix of wild and cultivated seeds still had a significantly higher average body condition score than those sampled in all other seasons. Bait could not be excluded entirely for comparison of body condition as only four birds had not consumed any bait.

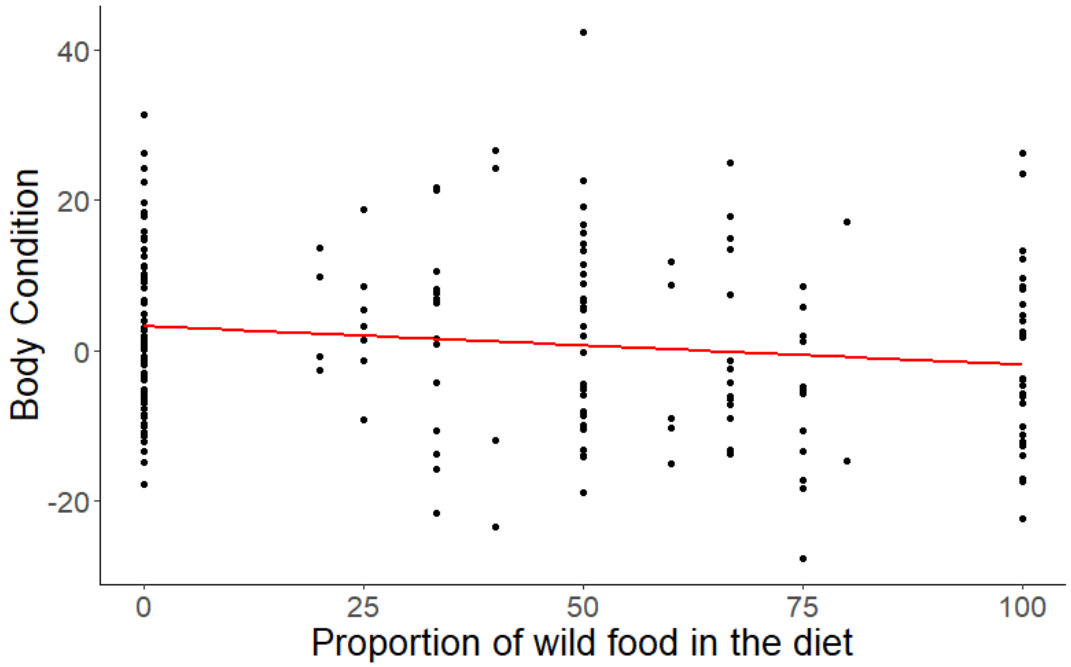

Figure 2.6: Linear regression illustrating the relationship between turtle dove body condition and the percentage of dietary items detected accounted for by wild food resources, such as weed seeds.

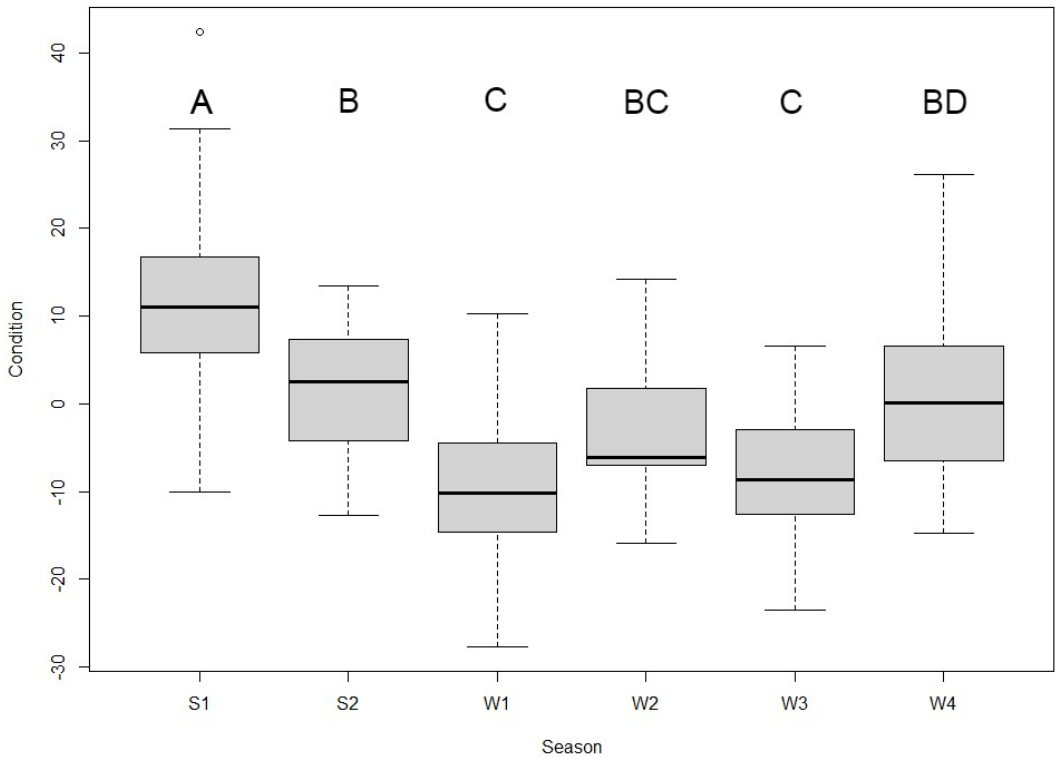

Figure 2.7: Variation in body condition of turtle doves across six sampling seasons (ANOVA: $F=27.210_{5,191}$, *p<0.001*). Significant pairwise differences in mean body condition determined by Tukey's post-hoc test and are indicated by letters. For full breakdown of p-values associated with plot pairwise differences see Table S1.10.

In Senegal, the proportion of wild food resources consumed (GLM: $F_{1,123}=6.294$, $p = 0.014$), season (GLM: $F_{3,120}=12.338$, $p<0.001$) and Julian date (GLM: $F_{1,119}=26.447$, $p<0.001$) all influenced an individual's body condition. Body condition increased as the wintering season (Figure 2.8A) progressed, as did the percentage of wild seeds in the diet (Figure 2.8B), indicating increased used of wild seeds later in the season. Mean Julian date differed between the four seasons (GLM: $Dev_3= 588.44$, $p<0.001$; Figure 2.9), with earlier average Julian date of bird sampling in W3 (41.89±7.505) and W4 (64.05±5.996) than in W1 (88.96±0.680) and W2 (93.67±11.952).

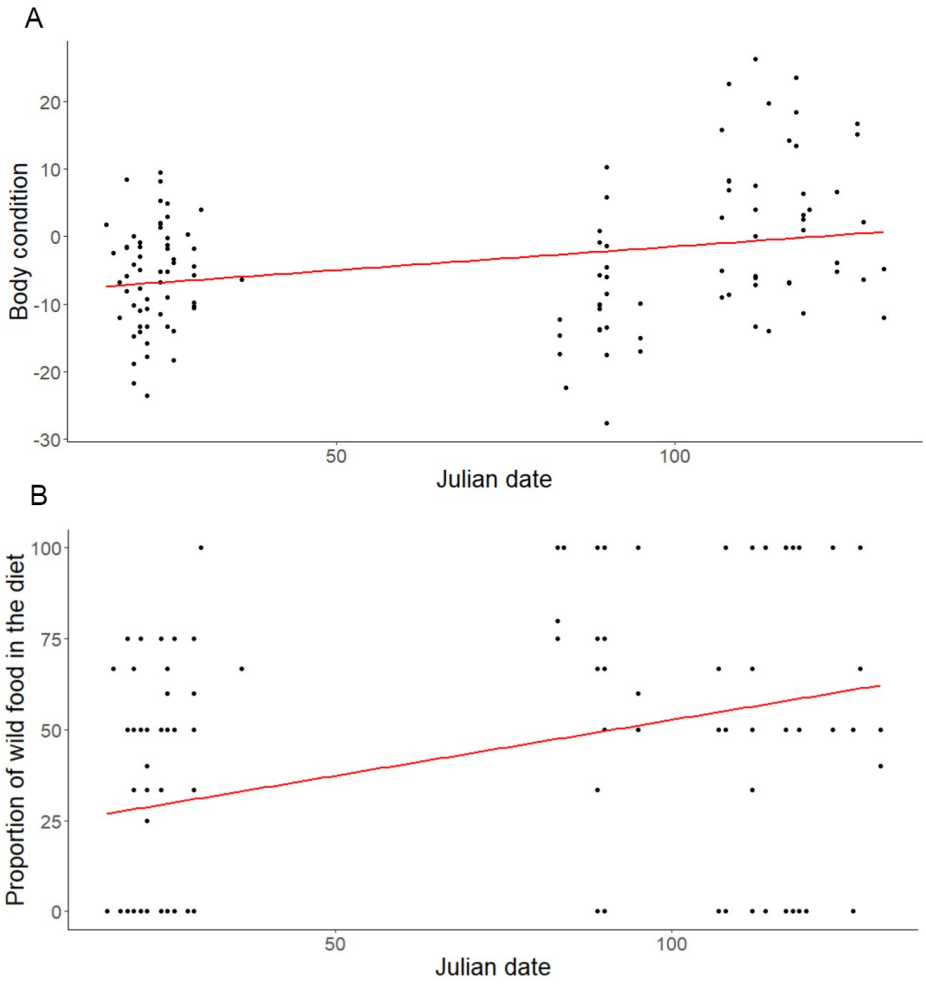

Figure 2.8: Change in A) body condition, B) proportion of the diet accounted for by wild food resources as the non-breeding season progressed, represented by Julian date (number of days since November 1st)

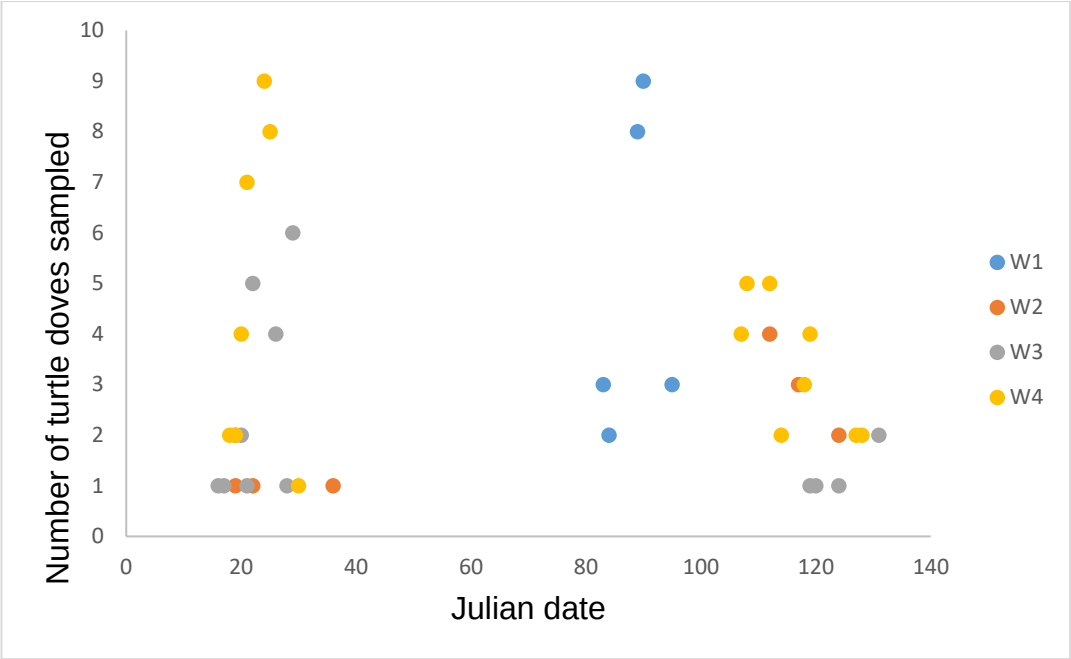

Figure 2.9: Distribution of the number of birds sampled as the non-breeding season progressed (from November 1st) in each winter sampled.

2.4.4 Repeatability

Sixteen repeated faecal DNA samples were included to assess the consistency of sequencing within a single run. Results were consistent in 43.75% of repeated samples, and in those that did vary, 43.75% had only one taxon occurring in just one repeat and 12.5% had two taxa occurring in only one of the repeats. All taxa occurring in only one of the two repeats after cleaning occurred at relatively low read count. Variation between repeats could not be mitigated by selection of a higher threshold for data cleaning. Increasing the filtering threshold to 5% did not alter the variation in repeats. Further increasing to higher thresholds, resulting in the removal of a greater number of reads removed inconsistency in some repeats, but introduced inconsistency in others as new taxa were removed at higher thresholds.

2.5 Discussion

A wide range of dietary items consumed by turtle doves were detected across breeding and wintering grounds, with 121 taxonomic units identified. Consistent with results described in similar dietary studies, a small number of taxonomic units accounted for the majority of the diet, whilst many other units were observed infrequently (Murton *et al.* 1964; Browne and Aebischer 2003a; Gutiérrez-Galán and Alonso 2016). The mean number of different taxonomic units detected in the diet of individuals (2.827 ± 0.288) was lower in this study than previously described when analysing turtle dove diet using HTS (10.4 ± 0.61) (Dunn *et al.* 2018).

There was significant dietary variation across the three regions sampled, with little overlap observed between the genera consumed in the different countries. This is unsurprising given the geographical distance between the European sites and the African site, with many of the genera detected in Africa not being present in Europe, and vice versa. The only genera found in all three regions were *Panicum* sp., *Sorghum* sp., *Cannabis* sp., *Helianthus* sp.. With the exception of *Panicum* sp., which is native to West Africa (National Research Council, 1996), these genera are not naturally occurring within the regions, rather they are cultivated crops or common components of bird seed. Such supplementary resources are increasingly being detected in the diet of turtle doves (Browne and Aebischer 2003a; Dunn *et al.* 2018).

2.5.1 Turtle dove diet in the wintering grounds

A mix of cultivated and wild seeds were found in individuals sampled in Senegal, although the former were more frequent (between 60.8% and 96.4%). The most common seeds consumed in this study were *Cenchrus americanus* (pearl millet) and *Sorghum* sp., which were common crops in the landscape surrounding the roost site where the birds were caught (Chapter 3). Field observations of a high proportion of caught birds having *Sorghum,* millet or both in their crop also support these findings (*unpublished data*). Both of these species have previously been shown to be important constituents of turtle doves' diets (Morel and Morel 1979; Morel and Roux 1966), and the annual production of these crops (along with rice *Oryza* sp.) in west Africa has

been correlated with the annual survival of turtle doves (Eraud *et al.* 2009). *Cenchrus americanus* is a domesticated crop plant, which was abundant in my sampling region (Chapter 3), and is now widely grown across the Sahel due to being well-adapted to infertile soils and withstanding drought (National Academic Council 1996; POWO 2019). Furthermore, based on GPS data, turtle doves were known to be present within fields growing this crop in the Senegalese sampling area in this study (Chapter 3 – Habitat and Homerange in Senegal). Whilst commonly regarded as a key component of turtle dove diet (Jarry and Baillon 1991), cultivated rice itself was absent in the present study. This probably reflects the geographical variation in the different crops grown, as very few areas of rice cultivation were present in my study area (Chapter 3).

Wild grass seeds were also an important constituent of turtle doves' diet in Senegal, especially *Panicum* and *Echinochloa* sp. which, although often harvested for food (National Research Council 1996), are not cultivated crops. As there is currently no ITS2 region sequence for *P. laetum* on Genbank, this species could not be confirmed in the present study; however, it is commonly found in Senegal, and has been shown to be an important food source for turtle doves on the wintering grounds (Jarry and Baillon 1991; Morel 1987). Evidence of *P. laetum* was present in previously wet areas around Beer Sheba, but it was not abundant, and was observed to be more abundant at sites along the Senegal river than at my study site (*unpublished data*). Two more wild grasses, *Dactyloctenium aegyptium* and *Urochloa mosambicensis* were also detected relatively frequently in the diet, further suggesting that wild grass seeds are an important element of the diet of turtle doves in their wintering grounds.

The proportion of cultivated seed species in the diet increased with each winter sampled. Cultivated seeds accounted for just 32.8% of the diet in W1, but by W4, they made up 69.3% of the diet of birds sampled. It is possible that this is a result of variation in seed availability, which was investigated using data on crop production in Senegal, obtained from the Food and Agricultural Organization of the United Nations (FAOSTAT 2012). In 2017 (W4) crop production was at its highest of all of the years sampled, with 215,419 tonnes

of sorghum and 875,484 tonnes of millet produced. This supports the findings of a high level of cultivated crop consumption in W4, as such a strong year for production would yield wide-scale availability of this resource. Conversely, the lowest productivity for both sorghum and millet occurred in 2014 (W1), when cultivated seeds made up the lowest proportion of the dietary items consumed. However, production of these crops was 33% higher in 2015 (W2) than 2016 (W3), with a combined produced of 938,374 tonnes and 619,855 tonnes respectively, which does not align with the greater consumption of cultivated crops in W3. It is possible that the lower production of cultivated crops reported for W3 was a result of poor weather conditions, which would also impact the availability of wild seeds. Therefore, this increased consumption of cultivated seeds in W3 may be reflective of reduced availability of wild seeds, as opposed to a preference for cultivated seeds.

Alternatively, the higher levels of cultivated crops occurring in W3 and W4 may be a result of yearly sampling variation. When comparing the distribution of birds sampled across season, there was a significant difference in average Julian date between sampling seasons, with an earlier average Julian date in W3 and W4 than W1 and W2. With the exception of W1, when all birds were captured in January and February, there were generally two capture periods, an early-season (November-December), and a late-season (February-March) period. As number of sampling sessions early and late in the season were kept relatively consistent, the variation in the distribution of birds sampled across the season is a result of varied capture success and DNA extraction or PCR failure. The two most prevalent cultivated crops, millet and sorghum, are harvested from September to November in Senegal (GIEWS 2020), resulting in an abundance of spilt grain early in the wintering season, which decreases as these resources are consumed post-harvest. As more birds were sampled during this earlier period in W3 and W4, this may, at least in part, explain the higher prevalence of cultivated seed in the diet in these years.

As the wintering season progressed, it appears birds became more heavily reliant on wild seeds than cultivated, as the proportion of wild seeds in the diet increased with increased Julian date. This increase in wild seeds in the diet as the wintering season progresses may be a result of a reduction in availability

of cultivated crops, as harvesting generally commences in September or October (GIEWS 2020) resulting in a reduction in the availability of cultivated crops later in the wintering season.

2.5.2 Turtle dove diet in the breeding grounds

The relative contributions of cultivated and wild seeds to the diet differed between the countries surveyed. While turtle doves sampled in France consumed the highest proportion of cultivated seeds, much of this would have consisted of the bait used to attract birds as, pre-harvest, when trapping occurred, these seeds would not have been readily available in the surrounding farmland (Hervé Lormee, *personal communication*). Due to the necessity to use bait to capture birds in this region, and the repeated provisioning of sites each year for long-term monitoring purposes, it is difficult to discern the extent to which birds would utilise alternative cultivated versus wild resources if this feed were not provided. Although bait (ground maize) was used to attract birds to capture sites in Hungary, it was not detected in their diet, despite evidence that UniPlant primers detect this species, both in *in vitro* primer testing, and in HTS studies (Dunn *et al.* 2018, Moorhouse-Gann, 2017). Unlike in France, the site in Hungary was not baited to attract birds every year, and the bait was not put out every day during sampling. This suggests that birds in France may have become dependent on, or acclimated to, the use of this source of anthropogenic food.

Although cultivated seeds formed a considerable part of the diet of turtle doves captured in France, there was also a diverse range of wild seeds detected in the diet, with 33 wild taxonomic units identified in this region. This may be a reflection of the higher floral diversity in the forest habitat where they were caught, compared to heavily managed agricultural land. Gutiérrez-Galán and Alonso (2016) investigated the diet of turtle doves in forests in Spain, and found a greater number of wild species in the diet than previous studies performed in farmland. However, turtle doves forage over distances of several kilometres (Browne and Aebischer 2003a), thus would likely still utilise food resources beyond the forest, such as agricultural fields.

The most commonly detected wild species in France was *Geranium molle*, which was present in 20.6% of birds sampled. Commonly referred to as dove's foot crane's-bill, this annual herbaceous weed occurs across Europe, and has previously been detected in the diet of turtle doves in Spain (Gutiérrez-Galán and Alonso 2016) and the UK (Dunn *et al.* 2018). Gutiérrez-Galán and Alonso (2016) found *G. molle* to be among the most frequently consumed species in their study, and recognised it as a potential species of importance due to its availability early in the breeding season. This is consistent with my finding of it being the most abundant wild seed in the diet in France, with sampling occurring early in the breeding season, during May.

In the UK in the1960s, *Fumaria officinalis* (common fumitory) and *Stellaria media* (common chickweed) were important components of turtle doves' diet (Murton *et al.* 1964). In contrast *F. officinalis* was not detected in this study, and *S. media* only in the diet of two birds in France (3.2%). The decline of these species in the diet of turtle doves is similar to findings of recent studies in the UK (Browne and Aebischer 2003a; Dunn *et al.* 2018). These plant species, once common in arable land across Europe, are declining as farming practice such as inorganic fertilisers and pesticides, less diverse cropping rotations and removal of field margins, reduce arable plant biodiversity across Europe (Andreasen *et al.* 1996; Dunn *et al.* 2015; Storkey *et al.* 2012). For example, *Stellaria media,* previously one of the most abundant arable weeds, declined significantly between 1979 and 2009 in one study in France (Fried *et al.* 2009). The loss of such species from the diet of turtle doves found in this study, and previous, further illustrates the resulting dietary switch away from naturally occurring weed seeds which is threatening the population.

The proportion of the diet accounted for by wild seeds was highest in Hungary (80.6%), with wild seeds being detected in the diet of over 90% of birds sampled, as opposed to cultivated seeds, which only occurred in the diet of one third of birds sampled. This is supported by a previous study in Hungary (Haraszthy 1998). When investigating the gut contents of 36 turtle doves, wild seeds such as *Sinapsis arvensis, Chenopodium* sp., *Steraria* sp. and *Fumaria* sp. dominated the diet, accounting for 83% of seeds identified, whilst common crops and animal fodder such as *Alfalfa* sp. and wheat accounted for just 13%

of seeds present (Haraszthy 1998). Birds in Hungary also had, on average, the greatest richness of taxonomic units per individual (3.583±0.288), indicating a more varied diet, although this was still lower than previously observed in turtle doves in the UK (10.400±0.61) (Dunn *et al.* 2018). This may reflect a higher diversity of food available in the UK than sampling sites of this study, or variation caused by different bioinformatic pipelines and methods of data cleaning used in the two studies. Due to the inclusion of different control samples, it was not possible to directly compare the two data sets with the current pipeline, illustrating the need for a more standardised approach to data cleaning (Drake *et al.* 2021).

Euphorbia species were the most abundant taxonomic units identified in Hungary, including *E. pseudoesula, E. cyparissias. E. seguieriana* and *E. stepposa.* Taxa from the *Euphorbia* genus and Euphorbiaceae family have previously been detected in the diet of turtle doves in small amounts in the UK and France (Murton *et al.* 1964; Dubois 2002; Dunn *et al.* 2018), but to my knowledge, this is the first identification of this genus as a dominant food resource. Despite a relatively small sample size in Hungary in this study, sampling completion analysis predicted relatively consistent level of available dietary items being detected across all three study sites (between 34% and 46%), with 39% of estimated available taxonomic units being detected in samples from Hungary.

The frequency of wild seeds in the diet in Hungary is consistent with the hypothesis that birds would consume a higher proportion of wild food in areas of lower agricultural intensity. Agricultural intensity varies across Europe, but is generally lower in eastern European countries which are either not members, or new members of the EU (Donald *et al.* 2001). Whilst agriculture covers a total of 86.4% of the total land area of Hungary, the use of traditional farming methods, such as smaller field sizes, lower use of chemical herbicides, and retention of semi-natural field margins (Tryjanowski *et al.* 2011), may have contributed to smaller declines in farmland bird populations than observed in western Europe (Báldi and Faragó 2007). However, it is important to note that since countries like Hungary have joined the EU, agriculture has increased, putting them at risk of experiencing similar declines to those observed across

western Europe (Verhulst *et al.* 2004). Hungary now has a mixed system, with intensive agriculture occurring alongside traditional farming (Tryjanowski *et al.* 2011), therefore it is important to consider the potential effect of changing practices. For example, it has been observed that extensively managed vineyards in Hungary, which are heterogeneous, providing a variety of landscape elements such as fruit trees, hedgerows, forested slopes and grassland, had higher bird species richness and abundance than the more homogenous, intensively managed vineyards (Verhulst *et al.* 2004). The variation in land use between Hungary and France, along with the support for our hypothesis regarding agricultural intensity provides an indication of the impact of agriculture between sites. However, as logistical constraints within this project meant we were only able to assess one country in eastern Europe, it would be beneficial to conduct dietary studies across a range of sites with lower agricultural intensity, in order to assess whether this observation is consistent across eastern Europe. The findings from such studies would make valuable contributions in understanding the relationship between diet and land use to help inform agri-environment and other land management measures to ensure the declines observed across western Europe do not spread across the whole continent.

As well as less intensive agriculture, the habitat in which turtle doves reside may also contribute to high proportion of wild seeds within the diet observed in this study. Wooded areas are important for turtle doves in Hungary, with the density of turtle doves in forest habitats being twice that of farmland (Szép *et al.* 2012; Fisher *et al.* 2018). As in France, birds were captured in a forest in Hungary, therefore potentially benefiting from the more varied flora in woodland habitat than farmland. The finding of a high occurrence of wild weed seeds is consistent with that of Gutiérrez-Galán and Alonso (2016), who identified a greater proportion of wild seeds in the diet of birds captured in forest habitat in Spain.

2.5.3 Effect of age on diet

Adults and juveniles were compared in the wintering ground and age was not found to have a significant effect on the genera consumed by turtle doves. There are a number of possible explanations for this. It may be a result of the

granivorous nature of turtle doves, as one factor commonly observed to have an effect on the dietary variation between adults and juveniles in other species is the skill required to capture different types or sizes of invertebrate prey (Wunderle 1991). This will not affect turtle doves as they exclusively feed on grains, thus no hunting skill is involved in obtaining food (Dunn *et al.* 2015). It may also be a result of the way turtle doves handle seeds. Finches, for example, remove the outer husks of seeds, requiring pressure to crack seed's outer coating, resulting in another restriction for juvenile birds: the maturation of the beak (Marchetti and Price 1989). It has been shown in a number of passerines, including hawfinches (*Coccothraustes coccothraustest*), chaffinches (*Frigilla coelebs*) and Darwin's medium ground finches (*Geospiza fortis*), that juveniles feed on smaller, softer seeds than their adult conspecifics, due to their skull and musculature not being fully developed (Kear 1962; Price and Grant 1984; Marchetti and Price 1989). However, as doves swallow seeds whole, this factor is unlikely to restrict juvenile turtle doves (Welty, 1975, in: De Nagy *et al.* 2015). It is also possible that this lack of variation is due to the fact that these birds are caught in the wintering grounds. In their review of differences in adult and juvenile foraging, Marchetti and Price (1989) suggested that, unlike many other vertebrate and invertebrate taxa, juvenile and adult birds do not occupy substantially different niches, rather birds appear to have evolved such that they reach adult form and function as rapidly as possible (Marchetti and Price 1989). Therefore, as birds caught in Senegal are not newly fledged, and juveniles have to be sufficiently developed to complete a migration of up to 4000km, it is likely that any foraging differences observed in newly fledged birds would no longer be observed. It is possible that if adults were compared with younger juveniles, during the breeding season, then diet may be significantly affected by age.

2.5.4 Effect of diet on body condition

Overall, the proportion of cultivated food resources present in the diet was positively correlated with turtle dove body condition, which supported my hypothesis that a higher proportion of cultivated seed in the diet would result in higher body condition score, as well as similar findings of Dunn *et al.* (2018). However, it is important to note that Dunn *et al.* (2018) also observed that

nestlings in their study had a higher body condition when the diet included a higher proportion of naturally occurring weed seeds, indicating that the availability of wild seeds is should be considered important for the successful breeding and survival of this species.

An important contributor to the decline of turtle doves is a reduction in breeding attempts, with a 61% decline in the number of young successfully fledged per pair observed between the 1960's and 2000 (Browne and Aebischer 2004). This has been suggested to be linked to reduced food availability, making it harder for adults to reach and maintain adequate body condition for breeding, resulting in a shorter breeding season (Browne and Aebischer 2001). Whilst this has not been proven in turtle doves (Browne and Aebischer 2003b), a reduction in wild food availability early in the season, when nutritional demands to reach breeding condition are highest, occurred concurrently with a dietary switch to a greater proportion of cultivated seed (Browne and Aebischer 2003a), making this a plausible cause for reduced breeding attempts. As crop plants do not provide seeds until later in the breeding season, provision of supplemental feed is a potential strategy for species management, to help make up for the shortfall of wild food availability early in the season. This method of management is a key aspect of many agri-environmental schemes focussed on supplementary feeding (UK Government 2021a; UK Government 2021b).

During the breeding season, when there are energy demands for producing and laying eggs (Robb *et al.* 2008a), and pre-migration when birds need to fuel their migratory journeys (Newton 2006), it benefits birds to have higher fat reserves. However, in the non-breeding season, birds often carry less weight, as they only need to obtain enough food to satisfy their own subsistence needs to survive, and lower weight can enable more effective predator avoidance (Lima 1986; Kullberg *et al.* 1996). Birds were found to have lower body condition scores in winter, however, in Senegal, body condition increased with Julian date, suggesting that birds were investing more in increasing body condition later in the season in preparation for migration, one of the most energetically demanding periods of the annual cycle (Cooper *et al.* 2015). This increase was concurrent with an increase in consumption of wild resources.

This may be a result of a lack of cultivated seeds in the environment following harvest earlier in the season, or alternatively, that turtle doves utilise wild seeds for their nutritional value to increase fat reserves in preparation for migration. Whilst I cannot determine the cause of this correlation, it highlights that wild seeds are a key constituent of the diet in the build-up to migration, highlighting their importance on the wintering grounds, as well as for nutrition on the breeding grounds.

2.5.5 The inclusion of bait in dietary analysis

The need to use bait to attract rare species for capture in order to analyse diet comes with the inherent problem of how to work with bait items occurring in the diet. In some studies, bait items are simply removed from analysis as a known item that was fed to individuals (da Silva *et al.* 2020). However, I argue that this is not an appropriate response, as the abundance of these bait seeds in the birds sampled from France highlight that it is an important component of the diet of these birds. Furthermore, a previous dietary study of turtle doves in the UK found that 89% of birds sampled had consumed seed commonly occurring in garden bird feed (Dunn *et al.* 2018) showing that turtle doves are commonly consuming this type of resource. It is arguable that turtle doves would not need to use such resources if an abundance of wild resources were available in their environment.

We therefore conducted analysis both with and without the bait seed included to assess the impacts this provisioned food may have on ecological inferences made in dietary studies. Overall, the inclusion of bait did not affect the level of significance of terms in the full model. Some variation was observed in specific dietary items driving the majority of the variation in season, however, the majority of the seeds determined to be driving the dietary variation remained the same. *Triticum* and *Helianthus* were two components of bait which significantly drove dietary variation. When bait was excluded, *Triticum* was absent from analysis as this was only consumed from bait. *Helianthus* was consumed in Hungary and Senegal as well as from bait, but when bait was excluded from analysis, the consumption of this genus no longer significantly contributed to dietary variation. The biggest ecological difference observed when removing bait was that of the similarity between diets, as represented by

NMDS. When bait was removed, there was greater difference evident from plots between the diets of birds sampled in Europe and Africa, which will be driven by the use of seeds which are widespread in the environment in Senegal (*Sorghum* and *Panicum*) in bait used in France. Removing birds whose diet consisted solely of bait seed from analysis did not change the variation in body condition across countries sampling season, with birds caught in France still being in significantly better condition that those sampled in all other season.

Whilst there is some variation in the results, these comparisons of data with and without bait indicate that ecological inferences made in this study were not heavily impacted by the inclusion of bait seed in the analysis. Due to the abundance of bait in the diet detected in this study, and that of Dunn *et al.* (2018), as well as the known switch to an increase in consumption of cultivated seed in absence of wild resources observed in turtle doves (Browne and Aebischer 2003a), I believe ecological inferences of diet would be less representative of the true nature of the foraging behaviour of turtle doves if bait were excluded.

2.5.6 Conclusions

I have shown that there is significant variation in the diet of turtle doves not only between the breeding and wintering grounds, but also between two different breeding sites within Europe with differing levels of agricultural intensity. As hypothesised, I found a greater abundance of wild seeds in the diet of birds sampled from the eastern European site, where agricultural intensity is not as high. However, as only one country was assessed, it would be of benefit to further investigate the diet of turtle doves in a wider range of countries subject to less intense agriculture to ensure the findings of this study are representative of low agricultural intensification more broadly. I also focused sampling on less frequently studied forest habitats in Europe, rather than farmland habitats, providing further information on dietary components occurring within this environment.

As most diet studies of turtle doves focus on breeding sites, comparatively little is known about the feeding ecology of this migrant species in their wintering site. However this is crucial information in establishing the threats facing this

species across its range, as food availability in the wintering ground is an important factor in survival due to the need to build up sufficient fat reserves to complete a long migration (Dubois 2002; Eraud *et al.* 2009). I have shown that, whilst turtle doves in this region do appear to be heavily dependent on cultivated crops, the diet is varied and there are also a wide range of wild seeds present in the diet. Furthermore, consumption of wild food resources appears to increase as the wintering season progresses, in line with an increase in body condition.

I also found support for my hypothesis that birds feeding on a higher proportion of cultivated seeds would be in better condition. The observed negative relationship between the proportion of wild seeds in the diet and body condition suggests that the provision of supplemental resources and availability of cultivated seeds are important for turtle dove survival. However, this study only considers adult birds early in the breeding season, not nestlings, which are more reliant on natural resources. Furthermore, continuous supplemental feeding with cultivated seeds in not a sustainable long-term plan, thus management systems which aim to mitigate the need for supplemental resources by increasing wild seed availability, particularly early in the breeding season, should be devised.

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Chapter 3 - Home-range, habitat use and diet of the European turtle dove on its sub-Saharan wintering grounds

Photograph by Rebecca Young – sunrise over Beer Sheba

Contribution of collaborators involved in this chapter was mainly support in collection of samples and environmental survey data from the field. Samples used in this project were collected from Senegal in three years prior to my PhD commencing by the RSPB and Nature-Communautés-Développement. The main team involved in sample collection was Chris Orsman (RSPB), Moussa Ka (NCD) and Mamadou Diallo (NCD). I worked with Chris Orsman, Moussa Ka and Mamadou Diallo and master's student Coen van Tuijl to collect samples from Senegal in 2017.

3.1 Abstract

Long distance migrant birds are declining more rapidly than their sedentary counterparts. This is likely, at least in part, due to environmental degradation on their wintering quarters, as poor conditions on the wintering grounds, such as a lack of food availability, can have implications for survival and subsequent breeding success. With land use in Africa changing, and grassland and wooded areas being increasingly converted to agricultural land, it is crucial to understand the way animals, such as the declining European turtle dove, interact with their environment on the wintering ground. Despite this, the

majority of research is conducting on the breeding grounds, and consequently relatively little is known about feeding ecology on the wintering grounds. Here I use a combination of dietary metabarcoding, environmental surveying and GPS data to investigate how turtle doves interact with the environment in Senegal, and the how this is associated with their diet. The home-range of individuals was variable, ranging from 4.73km^2 to 204.95km^2, with a mean of 33.94km^2 (±6.96). The average distance individuals travelled in a single day was 8.11km (±1.31km), and the daily distance travelled by birds was positively correlated with body condition. When considering the type of land use selected by individual birds for foraging, grassland was the most selected, and tree cover had the greatest effect on the probability of turtle doves' presence, indicating the importance of natural aspects of the landscape. However, cultivated crops did also play a key part in the diet, with sorghum and millet being the most highly selected food sources when considering seeds presence in the diet in relation to abundance in the environment.

3.2 Introduction

Across the planet, habitat degradation poses a major threat to biodiversity, driven by a range of anthropogenic causes, including urbanization, deforestation and agricultural intensification (Butler *et al.* 2007; Hurtt *et al.* 2011; Hayhow *et al.* 2019). Habitat degradation accounted for almost half of all threats within each terrestrial taxonomic group in the World Wildlife Fund Living Planet report (WWF 2018). Consequences of habitat degradation include a loss of nesting sites, adequate shelter and food resources (Wilson *et al.* 1999; Johnson 2007). For this reason, an understanding of how species interact with their environment is crucial in order to gain greater insight into how any environmental changes will affect their demography. Every year, over winter, millions of animals across a wide range of taxa undertake long migrations in order travel to warmer climates to avoid the harsh conditions and lack of resources that comes with the change of season (Alerstam *et al.* 2003). In the case of these migratory species, the consideration of interaction with the environment is more complex, as it must consider a geographical range spanning multiple locations.

3.2.1 Conservation of migratory species

Planning conservation interventions can be particularly challenging for migratory species as their range covers a wide area, and species are frequently on the move along different migratory passages (Lovejoy *et al.* 1987), therefore, interventions addressing threats at just one location may be insufficient to halt or reverse species declines (Runge *et al.* 2014). For example, many migratory ungulates, including the Saiga antelope (*Saiga tatarica*), are in decline as a result of habitat degradation and fragmentation, and hunting pressures, resulting in calls for the establishment of protected areas (Harris *et al.* 2009; Singh and Milner-Gulland 2011). However, this is complex for migratory species, as the whole migration route cannot be incorporated into a single protected area, potentially causing declines by disrupting migration (Bolger *et al.* 2008). In addition, it may be that species face different threats at different locations. The drivers of contributing to the decline of leatherback sea turtles (*Dermochelys coriacea*) differ on the breeding grounds (egg poaching) compared to along the migratory route (mortality from fisheries due to long-line fishing), thus effective conservation relies on interventions addressing the drivers of species decline across a wide spatial range (James *et al.* 2005).

Widespread declines have been observed in European birds, in particular migratory species, which are declining at a higher rate than resident birds (Sanderson *et al.* 2006; Vickery *et al.* 2014). This is likely, at least in part, due to the fact that resident birds face threats in just one location: their year-round habitat. Migrant species on the other hand, face threats in three locations: the breeding grounds, the wintering grounds and the passage areas (Berthold *et al.* 1998; Bairlein 2003; Newton 2004a). Poor conditions in any of these locations can have implications for species survival and subsequent breeding success, and conditions on the wintering ground, such as annual rainfall, have been linked to annual species survival rates and population trends (Peach *et al.* 1991; Szep 1995; Bairlein 2003; Eraud *et al.* 2009; Atkinson *et al.* 2014; Norman and Peach 2013).

Several European species spend the non-breeding season in Africa, with Afro-Palaearctic (A-P) migrant species being those that breed in the Palaearctic

region of Eurasia, and spend winter in sub-Saharan Africa (Vickery *et al.* 2014). Whilst declines in bird species have been widely recorded across the world, these long-distance A-P migrants have experienced larger declines than short-distance migrants and non-migratory species, suggesting the importance of conditions in their African wintering sites, as well as passage areas (Sanderson *et al.* 2006; Vickery *et al.* 2014; Zwarts *et al.* 2018). When investigating the population trajectory of long-distance migrants in Europe, the Pan-European Common Bird Monitoring Scheme identified an average 23% decline in long-distance migrants between 1980 and 2009, compared to just 7% for both resident and short-distance migrants combined (PECBMS, in Vickery *et al.* 2014). Furthermore, in their continent-wide analysis of European breeding birds, Sanderson *et al.* (2006) identified significantly greater declines in inter-continental migrant species than short-distance migrant or resident species between 1970 and 2000. Observed declines vary across species and wintering regions, with an apparent separation between those spending winter in more arid regions, compared to more humid regions of Africa (Hewson and Noble 2009). During the 1960's and 70's, the Sahel experienced widespread droughts, which were linked to declines of several avian species over-wintering in the northern tropics (Kanyamibwa *et al.* 1990; Baillie and Peach 1992; Newton 2004a; Sanderson *et al.* 2006). In more recent years, partial recoveries have been observed in some species wintering in these more arid regions, with population stabilization coinciding with rainfall returning to levels close to those observed before the drought (Vickery *et al.* 2014; Mallord *et al.* 2016). For example, monitoring of sand martin (*Riparia riparia*) population trends in Cheshire, UK, identified that increased recruitment and adult survival were correlated with higher levels of rainfall in sub-Saharan wintering grounds (Norman and Peach 2013). However, A-P migrants spending the non-breeding season in more southern, humid zones, such as the tree pipit (*Anthus trivialis*) and willow warbler (*Phylloscopus trochilus*), have been observed to be declining at a faster rate than resident birds since around 1990 (Thaxter *et al.* 2010).

Understanding the ecology of a species and how it interacts with its habitat is fundamental in conserving and managing species, as not only the habitat itself,

but also the ecological requirements of species, will greatly vary between the wintering and breeding grounds (Legagneux *et al.* 2009; Bairlein 2003). Despite this, research has tended to focus on the breeding grounds (but see Vickery *et al.* 2014), with relatively little information available on habitat use and ecological requirements on the wintering grounds (Bairlein 2003; Sanderson *et al.* 2006; Mallord *et al.* 2016).

It is a well-supported hypothesis that the availability of adequate food resources is the most important ecological constraint during the non-breeding season for birds (Brown and Sherry 2006). Possible implications of reduced food availability include poorer body condition and later migration, potentially having consequences for subsequent breeding success (Robb *et al* 2008b). In response to experimental reduction of food availability in over-wintering mangrove habitat, American redstarts (*Setophaga ruticilla*) maintained fat deposits, an insurance against starvation, at the expense of maintaining pectoral muscle (Cooper *et al.* 2015). This lack of muscle was suggested as the cause for the observed delay of, on average, one week in spring migration departure (Cooper *et al.* 2015). When considered alongside previous research which detected lower reproductive success in American redstarts arriving later to the breeding grounds, the availability of food in wintering grounds has important implications for species survival (Tonra *et al.* 2011). This has also been demonstrated conversely, with food provisioning for blue tits (*Cyanistes caeruleus*) over winter increasing their breeding success the following spring (Robb *et al.* 2008b).

As the UK's only migratory columbid and a year round obligate granivore, the European turtle dove is ecologically unique, and one of the UK's fastest declining bird species (Dunn *et al.* 2015; Hayhow *et al.* 2019). The diet of this species is likely to contribute to its decline, as granivorous species are particularly vulnerable to habitat change (Dunn *et al.* 2015). Similar drastic declines in granivorous species have been observed elsewhere, including the loss of the three most abundant granivorous species, the Sudan golden sparrow (*Passer luteus*), the black-crowned (*Eremopterix nigriceps*) and chestnut-backed (*Eremopterix leucotis*) sparrow larks from parts of North West Senegal (Zwarts *et al.* 2018). Furthermore, Zwarts *et al* (2018) found an

average decline of 80% in seed-eating resident birds between the early 1960's and 2014/2015, estimating a loss of 24 million African granivorous birds. An even larger relative decline was observed in the European turtle dove, attributed to a combination of adverse effects in wintering and breeding grounds (Zwarts *et al.* 2009; Zwarts *et al.* 2018).

3.2.2 Agricultural intensification on the wintering grounds

Agricultural intensification is one of the leading causes of habitat degradation, resulting in highly detrimental effects on farmland biodiversity, particularly avian species (Chamberlain *et al.* 2000; Newton 2004b; Butler *et al.* 2007). Changing farming practices have had severe implications for the availability of wild seeds, a factor considered to be a key driver in the dietary switch observed in turtle doves towards cultivated crops (Browne and Aebischer 2003a). The effects of such agricultural practices have been widely studied on European breeding grounds (Wilson *et al.* 1999; Hole *et al.* 2002; Hart *et al.* 2006; Reif *et al.* 2008), but fewer detailed studies have been carried out into changes in habitat in wintering grounds, despite the importance of adequate food resources for winter survival and subsequent breeding success (Bairlein 2003; Sanderson *et al.* 2006; Robb *et al.* 2008b).

Land use in sub-Saharan Africa, a region used by a wide range of avian species as wintering grounds, is changing, with a 57% increase in agricultural land between 1975 and 2000, much of which comes from the conversion of natural grassland and forest to farmland (Brink and Eva 2009; Mallord *et al.* 2016). Furthermore, the Sahel region is home to the fastest loss of natural non-forest vegetation and increase in agricultural land in sub-Saharan Africa, following widespread droughts at the end of the 20th Century (Brink and Eva 2009). There is much evidence of the potential detrimental effects of this habitat degradation on wintering birds. In Mexico, it was estimated that the destruction of 69,240 hectares of grass and shrubland, as a result of agricultural expansion, has reduced the winter carrying capacity for grassland birds by over 355,000 individuals (Pool *et al.* 2014). In addition, an 80% decline in ground feeding birds was detected in the western Sahel region, when comparing surveys conducting between 1964 and 1990, and those conducted in 2014-201 (Zwarts *et al.* 2018). This study deduced that increased pressure

from livestock grazing had caused a large reduction in the soil seed bank, thus reducing food availability for granivorous species (Zwarts *et al.* 2018). A change in avian species composition was also observed in relation to the loss of tree cover, following prolonged drought in the Sahel, where in the driest zones, 90% of woody cover was lost between the 1960's and 2000's (Zwarts *et al.* 2018). Correlations with woody habitat has been identified in species such as the wood warbler (*Phylloscopus sibilatrix*), where a positive correlation was identified between the probability of occupancy of a site and woodland cover, suggesting that the removal of trees in favour of cropland may have negative implications for this species (Mallord *et al.* 2016).

Turtle doves feed on a mixture of wild and cultivated seeds in the African wintering grounds. Key food resources have been identified as rice (*Oriza* sp.) and sorghum (*Sorghum* sp.) crops, and *Panicum laetum*, a wild grass (Jarry and Baillon 1991; Dubois 2002; Eraud *et al.* 2009), although it is not known to what extent they rely upon different food resources during this period of the annual cycle. Work on the breeding grounds suggests that newly-fledged young favour seed-rich habitats, including semi-natural grassland, fallow and areas of low intensity grazing, whilst adults favour lightly or ungrazed grasslands (Dunn *et al.* 2017; Dunn *et al.* 2020). Gleaning a greater understanding of how birds use the habitat on the wintering grounds and the effects that changes to the environment are having on populations will help inform the development of appropriate management plans.

As well as land use, seed food availability in this region will be impacted by the climate, with annual rainfall being associated with food availability (Newton 2004a). The Sahel region of sub-Saharan Africa experiences a long, hot dry season, with the average maximum temperature reported in Dakar, Senegal exceeding 25°C every month from September – December, the earlier half of the non-breeding season (World Weather Online 2022). Whilst temperatures fall in the latter part of the non-breeding season, the average maximum temperature each month does not fall below 22°C between January and March. Furthermore, as the wintering season progresses, average monthly rainfall decreases from 15 days in September (173.7mm of rainfall) to, on average, zero or one day of rain (0.1-3.1mm of rainfall) from November to

March (World Weather Online 2022). This climate during the time when turtle doves are over-wintering in Africa results in arid conditions, with vegetation desiccating and dying back, and crops being harvested, reducing the availability of seed food resources in the environment (*personal observation*). Whilst migrant birds have evolved with these arid conditions in winter, thus would have adapted to finding food in poor conditions, the change in land use and clearing of natural grasslands, which would also be adapted to winter conditions, in favour of cultivated crops, alter this ecosystem and the access to wild seed resources.

3.2.3 Using home-range to understand habitat use in the wild

By studying the movement patterns of animals, we can gain insight into habitat selection to help understand how individuals use the landscape available to them (Aebischer *et al.* 1993; Legagneux *et al.* 2009). An animal's home-range is defined by 'the area traversed by the individual in its normal activities of food gathering, mating and caring for young' (Burt 1943). Thus, the home-range does not constitute all areas traversed by an animal, rather the area within which it normally moves (White and Garrott 2012). Whilst several aspects are known to impact an animal's home range, including age, sex, body condition and habitat structure, food availability is often considered to be the primary influence (Rolando 2002; Legagneux *et al.* 2009).

Several studies, covering a wide array of taxa, have supported the prediction that home-range size is correlated with resource availability, with animals occupying the minimum area required to meet their needs (in Williams *et al.* 2016). For example, it has been observed that female roe deer (*Capreolus capreolus*) adjusted the size of their home-range in response to decreasing food supply, and that high density of food was one of the attributes observed to influence habitat selection within the home-range (Tufto *et al.* 1996). There is also empirical evidence supporting this theory, as when provided with supplementary food resources, a reduction of 43% in home-range size was observed in female African striped mice (*Rhabdomys pumilio*). Furthermore, in a review analysing published work on bird home-range, considering eleven influencing factors including food availability, habitat structure and predator-

prey interactions, food availability was determined to be the primary determinant of home range in birds (Rolando 2002).

With advances in technology and the miniaturization of transmitters, it has become possible to remotely monitor free-ranging animals via radio and GPS tracking (White and Garrott 2012). This has allowed researchers to gain a greater understanding of an animal's daily movements and activities, resulting in an abundance of studies reporting survival and habitat use of a wide array of species (Kenward 2001). Furthermore, with developing statistical techniques, home-range analysis has become more detailed, accurate and accessible (Seaman and Powell 1996). Marine biology, where terrestrial boundaries make it particularly difficult to study the movement of animals, has benefitted greatly from being able to use telemetry to track animals under water. For example, movement data obtained through satellite tracking has been used to advise on protected area planning for conservation of dugongs (*Dugong dugon*) and green turtles (*Chelonia mydas*) in Australia (Gredzens *et al.* 2014). GPS tags have also been beneficial in informing the conservation management needs of wide ranging species, such as the Andean condor (*Vultur gryphus*), which crosses country borders on a daily basis when travelling between breeding sites and feeding sites, highlighting the need for transboundary conservation policies (Lambertucci *et al.* 2014).

Despite radio and satellite telemetry facilitating an increase in home-range analysis, such information is still lacking in the wintering grounds for many avian species (Legagneux *et al.* 2009; Mallord *et al.* 2016). Turtle doves spend two-thirds of their annual cycle either along migration routes or in sub-Sahelian wintering quarters (Lormée *et al.* 2016). A recent study have utilised geologgers and satellite transmitters to determine the migration routes and discern wintering home-ranges of European turtle doves (Eraud *et al.* 2013; Lormée *et al.* 2016). Due to the nature of the tags used in this previous study, home-ranges are likely to have large error margins, as geologgers intended for the study of long-distance migration are accurate to "<250m" to ">1500m" (Lormée *et al.* 2016). By using data collected from highly accurate GPS tags, I aim to complement these studies and gather further insight into habitat use by turtle doves in their African wintering grounds.

A range of data were collected on turtle doves over-wintering in Senegal for four consecutive winters, including fixes from GPS tags to reveal the movement of birds during the day and diet data obtained through high throughput sequencing (HTS) of DNA from faecal samples. These were used, along with habitat surveys, to investigate how turtle doves interact with their wintering environment. I hypothesise that i) Variation in the diet of turtle doves will correlate with food available in the environment; ii) Turtle doves will use a mixture of natural and cultivated land for foraging, but will show a positive preference for grassland, due to the its high quality natural resources; and iii) Birds will travel a greater distance from communal rooststo forage as the season progresses and food resources become more scarce.

3.3 Methods

3.3.1 GPS data collection

Over three wintering seasons, a total of 23 GPS tags were fitted to European turtle doves caught within a roosting area at the Beer Sheba Project (14°38'N, 16°80'W, Beer Sheba Project 2002), an agricultural resource centre that encompasses a 100 ha *Acacia* woodland near Sandiara in Senegal. Full details of the field site and capture methods are described in Chapter 2 (2.3.1). GPS tags (NanoFix® GEO+RF, Pathtrack Ltd, Otley, UK) were glued to the synsacrum of birds after trimming of feathers in this area. GPS tags were programmed to record the location of birds every hour between 7am and 8pm. These times were intended to capture at least one fix in the morning and the evening within the roost, with fixes during the day reflecting foraging locations (Chris Orsman, *personal communication*). Fixes were remotely downloaded when birds came within a 500 m range of a portable receiver, located within the roost at Beer Sheba. Therefore, if birds did not return to this roost, i.e. through mortality or moving to a new roost, GPS fixes would not be downloaded. The two main uses of these GPS data in this study were to i) identify turtle dove foraging locations, where environmental surveys were then conducted, and ii) calculating the home range of birds in the wintering ground.

3.3.2 Environmental data collection

Environmental information used in this study was collected from 3,141 points over three winters (Table S2.1). Data were collected from two types of site: GPS and Control. GPS points were locations recorded to have been visited by birds fitted with GPS tags and control points were locations based on a 709m x 709m grid (Figure S2.1), which represented the habitat available to turtle doves in the analysis. These control points are an independent sample of locations across the range where environmental data were collected. Data on a range of environmental variables were collected from each of these points in order to describe the composition and structure of habitat in a 100m radius surrounding GPS and control points. For full details of environmental data collected, see Table S2.2. As habitat comparisons containing large numbers of unused habitat types can bias statistical analysis (Browne and Aebischer 2003a), some categories recorded in environmental surveys were grouped together, resulting in eight groups for dominant landscape, five groups for dominant vegetation and eight groups for crop type (Table S2.2). A sample of seeds was collected from the ground at each location, trying to collect a representative of each seed species present in approximately a 5m radius of the GPS point, during a collection period of maximum 10 minutes. Where there were no obvious seeds, a soil scoop was taken for subsequent use to identify if seeds were present. Seed samples were collected a total of 2,007 locations. Due to time constraints, a subsample of these were subsequently analysed, consisting of 432 samples covering winters two to four, both types of sample (GPS and control) and all habitat types (Table S2.1). As very few samples were collected in the first winter (2014/2015), this season was not included in dietary selection analysis.

Seeds were identified based on morphotypes, due to a lack of seed taxonomy expertise, and subsequently each morphotype was identified by DNA barcoding. DNA was extracted from seeds of each morphotype following the salting out method (Randall *et al.* 2015). If seeds were small, DNA was extracted from multiple seeds in order to obtain sufficient DNA. Prior to extraction, seeds were rinsed in polished water. TNES DNA extraction buffer was used, consisting of 200mM TRIS, 250mM NaCl, 25mM EDTA and 0.5%

(w/v) SDS. 400µl of TNES was added to seed samples, and they were manually homogenised using a sterile pestle. Samples were briefly vortexed, then centrifuged at 13,000 rpm for 1 minute. A 300µl aliquot of supernatant was removed and transferred to a new tube, to which 300µl isopropanol (-20°C) was added and incubated at room temperature for 2 minutes to allow DNA precipitation. Following incubation, samples were centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded and 500µl 70% ethanol was added and centrifuged at 13,000rpm for 5 minutes to rinse the pellet. Excess liquid was removed and the pellet allowed to air dry, before resuspension of the DNA pellet in 100µl TE buffer (10mM Tris, 1mM EDTA).

Following extraction, DNA was amplified, sequenced, and species assigned as described for bait seed identification in Chapter 2 (Appendix 1, 1.1). During taxonomic assignment, Kew Gardens Plants of the World (POWO 2019) was used to confirm whether the taxa identified occurred in Senegal. This was also used if multiple top hits occurred, where possible, species assignment was made by selecting only those occurring within my sampling region. In instances where more than one species occurring in Senegal were the closest match, assignments were made to the highest taxonomic rank in common. Following assignment, any morphotypes assigned to the same taxon were aggregated.

3.3.3 Statistical analysis

All statistical analyses were carried out in R, version 4.0.1 – “See Things Now” (R Core Team 2020).

3.3.3.1 Movement and home-range of individuals

Home-range analysis was carried out using the *adehabitatHR* package (Calenge 2019). Analysis of home-ranges was carried out using areas calculated by the kernel density estimator (KDE) method. Kernel methods are increasingly used for spatial analysis over traditional methods such as minimum convex polygon (MCP) (Worton 1995; Seaman *et al.* 1999), as they are able to identify more heavily used areas, thus providing more accurate estimates of home-range (Calenge 2019). Furthermore, MCPs incorporate areas which were not used, just traversed (Mohr 1947). The full home-range of birds was defined at the 95% density isopleth (KDE_{95}), which will remove

just the most extreme points from the data set (Figure 3.1) (Castaño *et al.* 2019). A core area, indicating a relatively small area used most intensively by individual birds (Seaman and Powell 1996) was defined by the 50% density isopleth (KDE_{50}), (Figure 3.1) (Castaño *et al.* 2019). Both 95% and 50% MCPs were also calculated in this study for the purpose of making direct comparisons with previously published data ($MCP_{95,}$ MCP_{50}) (Browne and Aebischer 2003a; Lormée *et al.* 2016; Dunn *et al.* 2020). In order to compare the performance of the MCP and KDE methods, the mean home-range and core area sizes calculated by each method were compared using a paired *t*-test on log-transformed area.

As per Seaman *et al.* (1999), a minimum of 30 fixes was required to calculate the home-range of an individual. Sufficient GPS fixes to calculate home-range were obtained for 21 birds, across three seasons. The period of collection varied between birds: it was generally between two weeks and one month, but four birds greatly exceeded this time-frame (Françoise: 89 days; Sokone: 107 days; XXX: 90 days; Ziguinchor: 108 days) (Table 3.1). In order to assess whether the of number of days over which GPS fixes were recovered was correlated with the size of the home-range, the home-range was calculated for each individual across the full season. The correlation between number of fixes used to calculate home-range and KDE_{95} was assessed using Pearson's correlation. Variables were considered highly correlated if *r* exceeded 0.5 (as per Dunn *et al.* 2020). In addition, an alternative home-range was calculated, whereby the four individuals with fixes reported over periods greater than 30 days were divided into monthly increments, based on the first fix date for each bird individually, to account for the variation in collection period (Table 3.1, Table S2.3). One bird (Françoise) moved roost part way through the season, then returned to Beer Sheba, thus GPS fixes were subdivided based on dates this bird was inhabiting the two roosts in order to account for the change in location and prevent over-estimation of home-range. The correlation between number of fixes and KDE_{95} was assessed using these alternative home-ranges as above.

The relationship between KDE_{95} and KDE_{50} was assessed using areas calculated with sub-divisions into monthly increments. When multiple home-

ranges were calculated per individual (as for Dewi, Françoise, Sokone, Tampa, XXX and Ziguinchor), the mean area was used. Log-transformed KDE_{95} and log-transformed KDE_{50} were compared using a linear model.

The kernel smoothing value (*h*) was selected using the *ad hoc* approach ($h_{ad\ hoc}$), rather than using the commonly applied reference (h_{ref}) approach, due to the latter's tendency to result in over-smoothing (Berger and Gese 2007). Firstly, kernels were plotted for each bird using the 'kernelUD' command with h_{ref} as the smoothing parameter, and *h* values obtained (Table S2.4). As long as the kernel produced a single area, h_{ref} was reduced by 10% and the kernel plotted again. This was repeated until the *h* value used resulted in a non-contiguous kernel. Once this point was reached, the lowest *h* value yielding a contiguous kernel was used to calculate home range (Figure 3.2). This process was repeated to obtain an *h* value specific to each home range calculated (Table S2.4, Table S2.5).

The daily distance travelled by individuals was calculated using the 'as.ltraj' function in *adehabitatLT* package (Calenge 2011). GPS fixes were collected at 1 hour intervals, with the number of relocations recorded in a single day ranging from 10-12, between the hours of 07:00 and 20:00. Trajectories in this study were of Type II, as each point consisted of an x and y co-ordinate, and the time at which that location was recorded (Calenge 2011).

In order to assess variation in daily distance travelled through time, a generalised linear mixed model (GLMM) was constructed using log-transformed daily distance travelled as the dependent variable. As the number of days sampled varied between individuals (3-110 days), a random effect of Bird ID was included to avoid pseudo-replication (Legagneux *et al.* 2009). The continuous variable Julian date (measured from November 1st each year), sampling year, as a factor, and the interaction between them were included as independent variables in the full model. Significance was determined by removal of terms and comparison to the full model, using a likelihood ratio test. Mean daily distance travelled per individual was considered with respect to body condition (calculated as described in Chapter 2: 2.3.6.2), by producing a linear model using mean daily distance travelled per individual as the

dependent variable and body condition as the independent variable. All means are presented ± standard error.

Table 3.1: Summary of GPS fixes collected for each tagged bird used for home range analysis. Individuals highlighted in bold had fixes over a period greater than one month, therefore were divided into shorter increments for home range analysis. See supplementary information for full breakdown of fixes used in home-range analysis (Table S2.3).

Bird ID	Home range ID	Year	Season	Fix start date	Fix end date	N days	N fixes
Adama	A2	2016/17	2	01/12/2016	07/12/2016	6	69
Bakel	B2	2016/17	2	01/03/2017	16/03/2017	15	141
Dewi	D2	2016/2017	2	02/03/2017	21/03/2017	18	235
Françoise		**2015/2016**	**1**	**21/11/2015**	**20/02/2016**	**67**	**215**
Jeremy	J3	2017/2018	3	20/11/2017	17/12/2017	27	242
Kousmar	K1	2015/2016	1	22/02/2016	26/02/2016	4	38
Linguère	L1	2015/2016	1	22/02/2016	15/03/2016	22	137
N'Dioum	N1	2015/2016	1	27/02/2016	15/03/2016	17	121
Nianing	N3	2017/2018	3	23/11/2017	02/12/2017	9	59
Ouro Sogui	O1	2015/2016	1	05/03/2016	16/03/2016	11	153
Palmarin	P1	2015/2016	1	05/03/2016	15/03/2016	10	133

Quincy	Q1	2015/2016	1	05/03/2016	16/03/2016	11	142
Ranerou	R2	2016/2017	2	18/11/2016	14/12/2016	26	165
Richard-Toll	R3	2017/2018	3	27/11/2017	17/12/2017	20	204
Sandiara	S2	2016/2017	2	19/11/2016	24/11/2016	5	53
Sokone		**2017/2018**	**3**	**27/11/2017**	**17/03/2018**	**107**	**1300**
Tamba	T3	2017/2018	3	17/02/2018	12/03/2018	22	272
Vieux	V3	2017/2018	3	20/02/2018	04/03/2018	12	54
XXX		**2016/2017**	**2**	**28/11/2016**	**01/03/2017**	**90**	**857**
Zeinab	Z3	2017/2018	3	28/02/2018	16/03/2018	16	201
Ziguinchor		**2016/2017**	**2**	**01/12/2016**	**23/03/2017**	**109**	**1086**

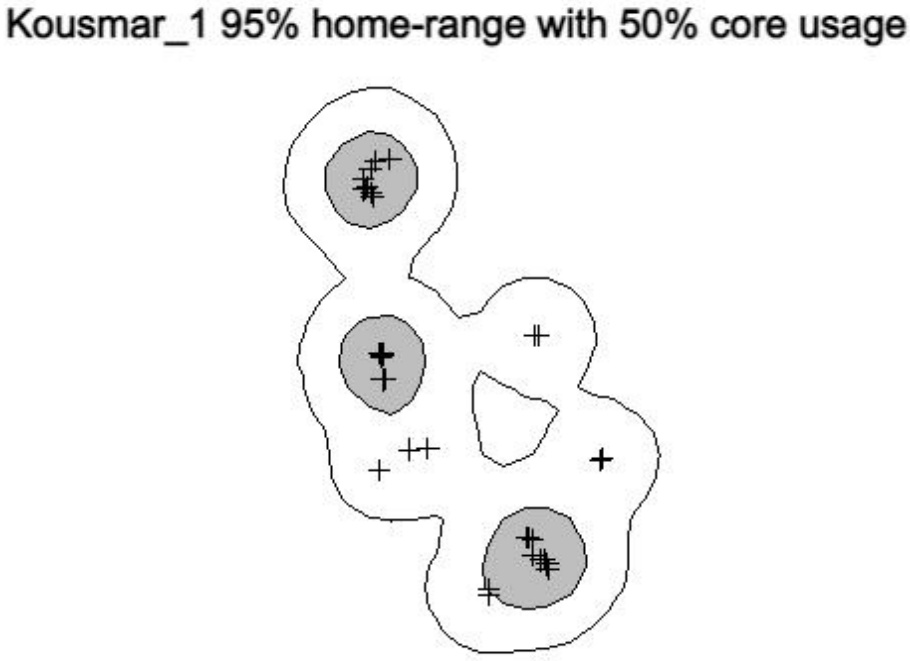

Figure 3.1: 95% home-range kernel and 50% core usage kernel for Kousmar, tagged in season 1

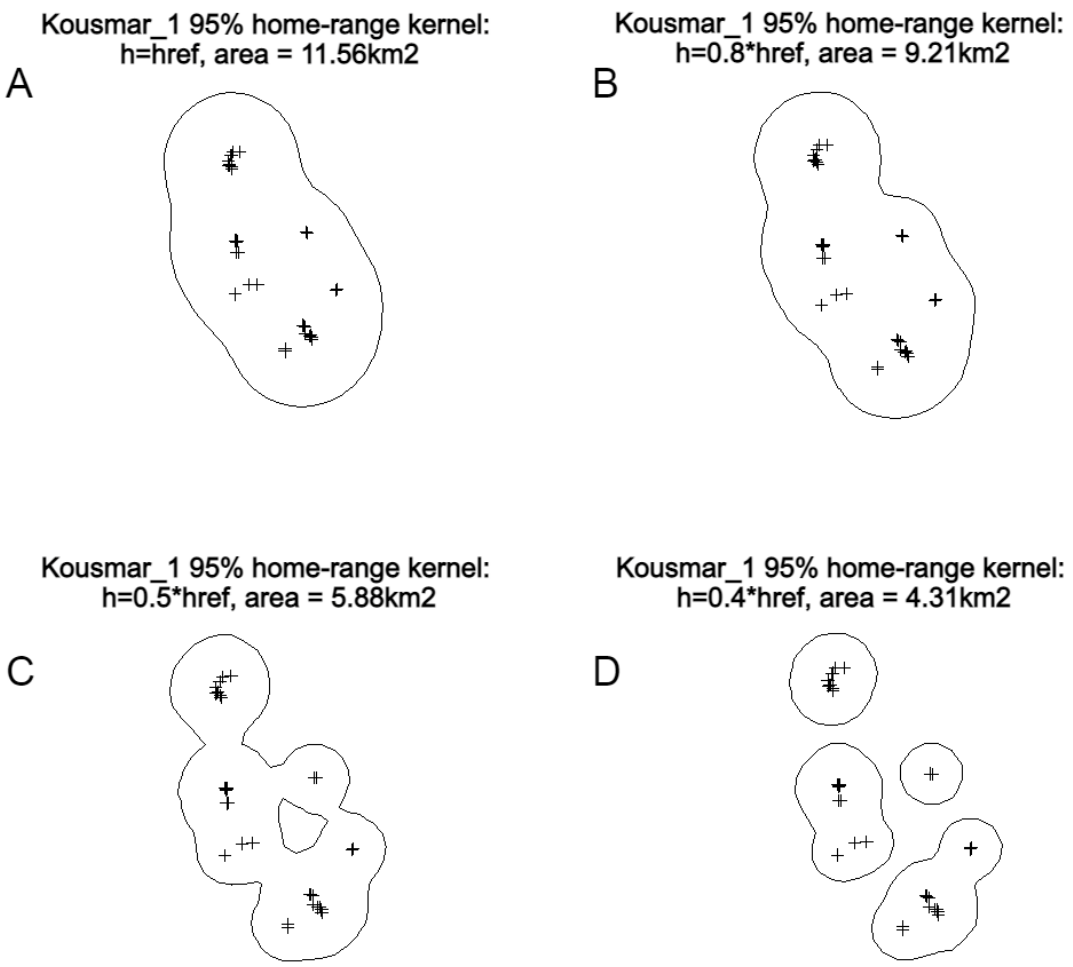

Figure 3.2: Selection of h value based on the ad hoc method ($h_{ad\,hoc}$). A) Kernel plotted when h_{ref} value is used, B-D) Progressive iterations following the ad hoc approach of reducing h value by 10%. D shows a fragmented kernel, therefore this h value is not used for the home range, and the lowest h value resulting in a contiguous kernel is used (C), in this instance, h_{ref}*0.5

3.3.3.2 Habitat selection

Habitat selection by birds was analysed by comparing the used and available habitat, using compositional analysis for more broad scale analysis and Generalised Linear Mixed Models (GLMMs) for finer scale analysis.

Compositional analysis was conducted using the 'compana' function in the *adehabitatHS* package (Calenge 2020) in order to compare used habitat with available habitat, using Type II design (distribution of animals (use) within a study area (availability)) (Thomas and Taylor 1990; Aebischer *et al.* 1993).

Habitat information from previously described environmental surveys was used, with locations of fixes from GPS tagged birds representing ‘used habitat’ and locations from the grid of control points representing ‘available habitat’. For the purpose of this analysis, points located within Beer Sheba were removed, as this was the primary roosting location, thus GPS fixes within this area were unlikely to represent foraging locations.

Three aspects of the habitat were considered in separate analyses: dominant land use, dominant vegetation and crop type. The categories within each of these habitat types are presented in Table 3.2. (For more detailed description, see Table S2.2).

Table 3.2: Categories used in compositional analysis

Dominant landscape	Dominant vegetation	Crop type
Arable	Bare ground	Bean
Bare earth	Forbs	Hibiscus
Grassland	Grasses	Fallow
Natural/agricultural mosaic	Shrubs	Millet
Shrub land	Trees	Peanut
Trees		Sorghum
Village		Other crop
Water		Natural area

When collecting habitat information from environmental surveys, for dominant land use and dominant vegetation, the three categories accounting for the greatest area of land within a 100m radius of the point were recorded. These were scored from three (most extensive) to one (least extensive) at each GPS point. For each bird, habitat use was calculated by summing the scores for each habitat type across all of the GPS fixes and then dividing by the total score (number of GPS fixes x 6 (i.e. the sum of scores at each point)). The same was carried out using control points, instead of individual bird GPS fixes, to calculate available habitat.

In environmental surveys, up to six crops within a 100m radius of each point were recorded, including the percentage of the area accounted for by that crop. The percentage of area covered by these categories was used to calculate an overall proportion of available and used habitat consisting of different crops.

For each aspect of habitat (dominant land use, dominant vegetation, crop type), two matrices were produced (used and available). These contained the proportion of total GPS points for each bird represented by each habitat type, and the proportion of total control points represented by each habitat type, to give the proportion of habitat used by, and available to individuals, respectively. There were a small number of unused, or unavailable habitat types, resulting in zero values. Zero values are not appropriate for use in compositional analysis, as it utilises logarithmic transformation, assuming each animal uses all habitat types (Aebischer *et al.* 1993). Therefore, Aebischer *et al.* (1993) recommend replacement of such values with small, positive values, less than the smallest nonzero value in the used and available matrices, and values ≤0.001 have commonly been used in compositional analysis to prevent bias (Chamberlain *et al.* 2000; Hartke and Hepp 2004; Dunn *et al.* 2017). However, use of smaller substitution values has been found to lead to an increase in the error rate (Dasgupta and Alldredge 2002; Bingham and Wildlife 2004; Bingham *et al.* 2007). Therefore, in this study, zero values in the matrix were replaced with 0.007, as per (Bingham *et al.* 2007), as substitution of zero with values greater than 0.07% were found to minimise misclassification. The significance of habitat selection was identified using Wilk's lambda test, based on 1000 repeats. In instances where habitat selection was determined to be significant, habitats were ranked according to their use.

Generalised Linear Mixed Models (GLMMs) were generated using the 'glmer' function in the R package *lme4* (Bates *et al.* 2015). Using the presence or absence of birds at a location as the dependant variable, a binomial GLMM was fitted using the logit link function to determine which habitat variable affected the presence/absence of turtle doves. Environmental surveys provided a range of potential variables to include in this analysis pertaining to land use and habitat structure (Table S2.2). Due to the distribution of data across the habitat types which defined the levels in factorial variables, some

levels contained very few data points, resulting in unstable models. For this reason, certain habitat types were grouped together based on landscape/vegetation structure or proportion of land covered (Table S2.2). Environmental data for a GPS location was only included in the model if it was collected within 14 days of the bird being recorded at this site, in order to use environmental data which more closely reflects the habitat at the time of the bird's presence. As environmental information on the habitat used was collected based on GPS locations of 23 birds, bird ID was included as a random variable to account for repeated measures.

Prior to fitting the model, collinearity between pairs of variables was tested using Pearson's correlation. Pearson's correlation coefficient exceeded the threshold of 0.5 in two instances (proportion of crop harvested/bare earth within crop area and normally bare earth/bare earth within degraded grasslands). In these cases, only one variable was considered for inclusion in the model (bare earth within cropland and bare earth in degraded grassland), selected as the most likely to be influential based upon turtle dove feeding ecology. A model was generated including all terms considered potentially influential to turtle dove habitat use - the 'full model'. The full model was fitted using the 'bobyqa' optimizing function. Significance of variables was then assessed by removing each term individually, and testing against the full model. Any terms which at this stage were not found to significantly improve the fit of the model ($p>0.05$) were excluded from the final model.

In order to assess the effect of each term within the model on the presence of turtle doves, the 'predict' function was used to evaluate the effect of each term individually, controlling all other variables in the model by setting them to the mode (for factors) of the data set. Repeated measures were controlled for by including the argument "re.form = NA" which makes predictions for the entire population represented by the random terms. As standard error calculations are not available when using the 'predict' function with merMod objects generated by GLMMs in the *lme4* package, standard error was calculated from the equivalent GLMs (i.e. excluding the random term), which will provide a more conservative estimation of standard error.

3.3.3.3 Foraging selection

In order to investigate the use of resources in relation to their availability in the environment, the *econullnetR* package (Vaughan *et al.* 2018) was used. This compares a resource matrix containing data on the availability of resources, in this case seeds, to a consumer matrix containing the information obtained from metabarcoding of the seeds present in faecal samples from birds sampled in Senegal in the winters of 2015– 2017 (Chapter 2). By using the 'generate_null_net' function, this analysis uses null models to determine whether taxa were consumed more or less frequently than expected based on their availability, suggesting that birds are selecting or avoiding particular seed species. The model was run with 9999 iterations. As species level differentiation was not always possible when identifying both seeds from the environment and the diet, the taxonomic rank used was genus to maximise the data available for analysis. Seed sampling recorded only the absence/presence of seeds from each sample, rather than the abundance which would normally be used with *econullnetR*. In lieu of abundance data, the proportion of samples in which each seed genus was detected was calculated as a proxy for their relative abundance at a landscape-scale. As not all seed morphotypes found in samples from Senegal could be identified by Sanger sequencing (45.7% of morphotypes identified to genus level), this analysis was run on a subset of seeds successfully identified. This meant that any genus detected in the diet through metabarcoding, but not found in environmental samples were not included in consumer matrix as they may have been present in the environment as one of the morphotypes unable to be identified.

3.4 Results

3.4.1 Home range

Home-range was calculated for 21 birds, across three seasons and, with GPS fixes collected from birds over a period of 4-108 days (mean 7.17 days ± 7.53) (Table 3.1, Table 3.3). When home-range and core usage were calculated for each individual across all days of GPS data collection (Table S2.6), there was a significant correlation between the number of GPS fixes and KDE_{95} (r = 0.502). When home-ranges were calculated from data divided into monthly

increments to account for the variation in collection periods, number of fixes was no longer significantly correlated with home-range (r = 0.382), therefore subsequent analyses were carried out on these sub-divided data. KDE_{95} was variable, ranging from 4.73km^2 to 204.95km^2, with a mean of 33.94km^2 (±6.96) (Table 3.4). KDE_{50} representing the most used area of the home-range had a mean area of 5.19km^2 (±1.20), and ranged from 0.57km^2 to 35.09km^2 (Table 3.4). KDE_{95} and KDE_{50} were positively correlated (LM: $F_{1,19}$=187.7, p<0.001) (Figure 3.3).

The mean MCP_{95} was 15.90km^2 (±3.09) and the mean MCP_{50} was 2.39km^2 (±0.62). Area calculated using KDE was significantly greater than that obtained by MCP for both home-range (t=8.710$_{30}$, p<0.001) and core usage (t=5.691$_{30}$, p<0.001).

The majority of the birds appeared to return to Beer Sheba to roost throughout the sampling period, however, one individual (Françoise) appeared to be using a second roost, approximately 22km from Beer Sheba, from 11th-29th December 2015 (Figure 3.4).

Daily distance travelled was calculated for 14 individuals over a period ranging from 3-110 days, depending on the return of GPS fix information. The mean daily distance travelled was 8.11km (±1.31km), and ranged from 0.25km to 49.19km travelled in a single day (Table 3.5). When controlling for individual bird ID, sampling year did not affect the daily distance travelled, but Julian date did have a significant effect, with the distance travelled decreasing as the season progressed (GLMM: χ^2_1=8.134, p=0.005) (Figure 3.5). The dates for which daily distance travelled was calculated ranged from 27th November – 23rd March (26-142 days from November 1st as Julian date). The daily distance travelled was positively correlated with individual body condition (LM: $F_{1,13}$=7.848, p=0.015) (Figure 3.6).

Table 3.3: Summary of calculated areas of home-range and core area for each individual using both minimum complex polygon (MCP) and kernel density estimator (KDE) methods. Where individuals were broken down into multiple shorter time increments, the average home-range per individual is presented in bold, with full details of all home-range calculations available in the supplementary material (Table 2.7).

Bird ID	$h_{ad\ hoc}$	MCP_{95} (km^2)	MCP_{50} (km^2)	KDE_{95} (km^2)	KDE_{50} (km^2)
Bakel_2	499.53	2.98	1.88	13.70	2.57
Dewi_2	473.21	2.91	0.24	4.73	0.57
Francoise_1		**3.78**	**0.53**	**11.74**	**2.15**
Jeremy_3	592.46	5.23	0.59	17.94	5.68
Kousmar_1	240.19	2.96	1.67	5.88	1.11
Linguère_1	836.18	11.29	4.27	44.03	7.73
N'Dioum_1	805.05	15.61	6.80	34.64	6.40
Nianing_3	1092.52	10.45	0.18	47.24	9.33
Ouro Sogui_1	623.61	5.49	2.85	18.53	3.43
Palmarin_1	835.69	9.23	0.91	30.59	5.48
Quincy_1	484.81	5.15	1.40	13.47	2.92
Ranerou_2	588.84	26.30	1.28	51.18	3.85
Richard-Toll_3	1098.31	47.15	16.59	98.57	19.63
Sandiara_2	457.41	4.66	2.40	13.02	2.74
Sokone_3		**15.42**	**2.01**	**30.78**	**3.17**
Tamba_3	1035.27	38.18	1.74	61.49	8.45
Vieux_3.2	298.96	1.92	0.02	5.44	0.95
XXX_2		**37.13**	**2.17**	**55.03**	**5.74**
Zeinab_3	480.43	8.64	0.15	15.21	1.84
Ziguinchor_2		**26.60**	**3.73**	**61.69**	**10.27**

Table 3.4: Summary of the minimum, maximum and average of all home-ranges calculated when divided into 30 day increments.

	Home range measure			
Bird ID	MCP_{95} (km^2)	MCP_{50} (km^2)	KDE_{95} (km^2)	KDE_{50} (km^2)
Minimum	0.47	0.02	4.73	0.57
Mean±S.E.	15.90±3.09	2.39±0.62	33.94±6.96	5.19±1.20
Maximum	74.29	16.59	204.95	35.09

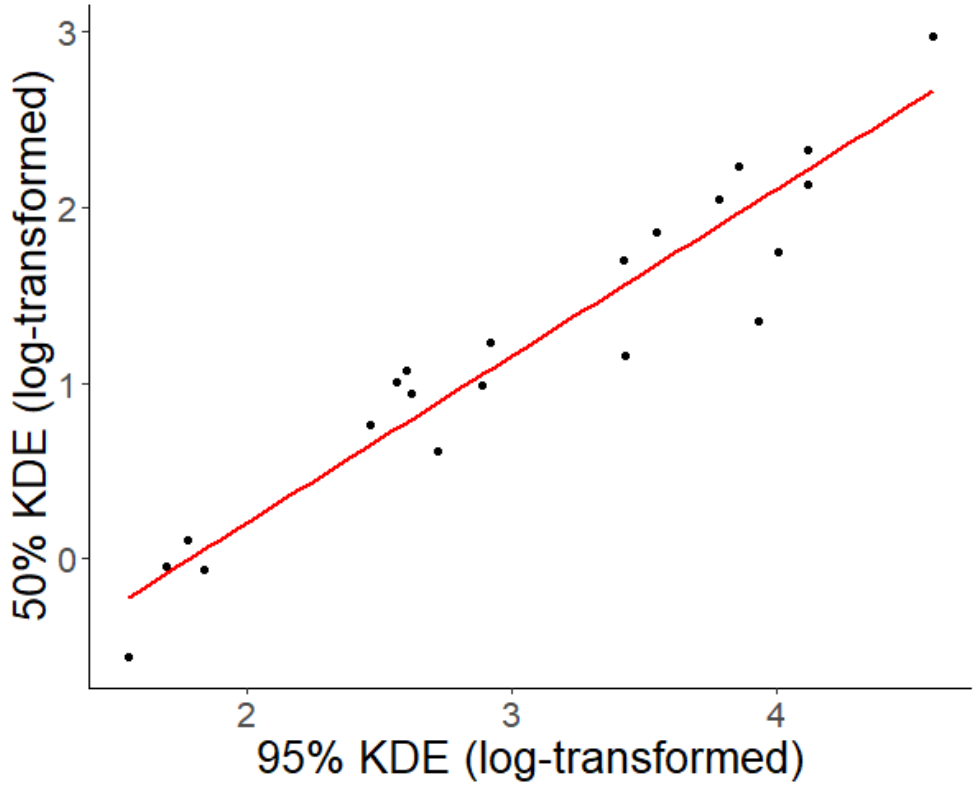

Figure 3.3: Linear regression comparing KDE_{95} and KDE_{50}

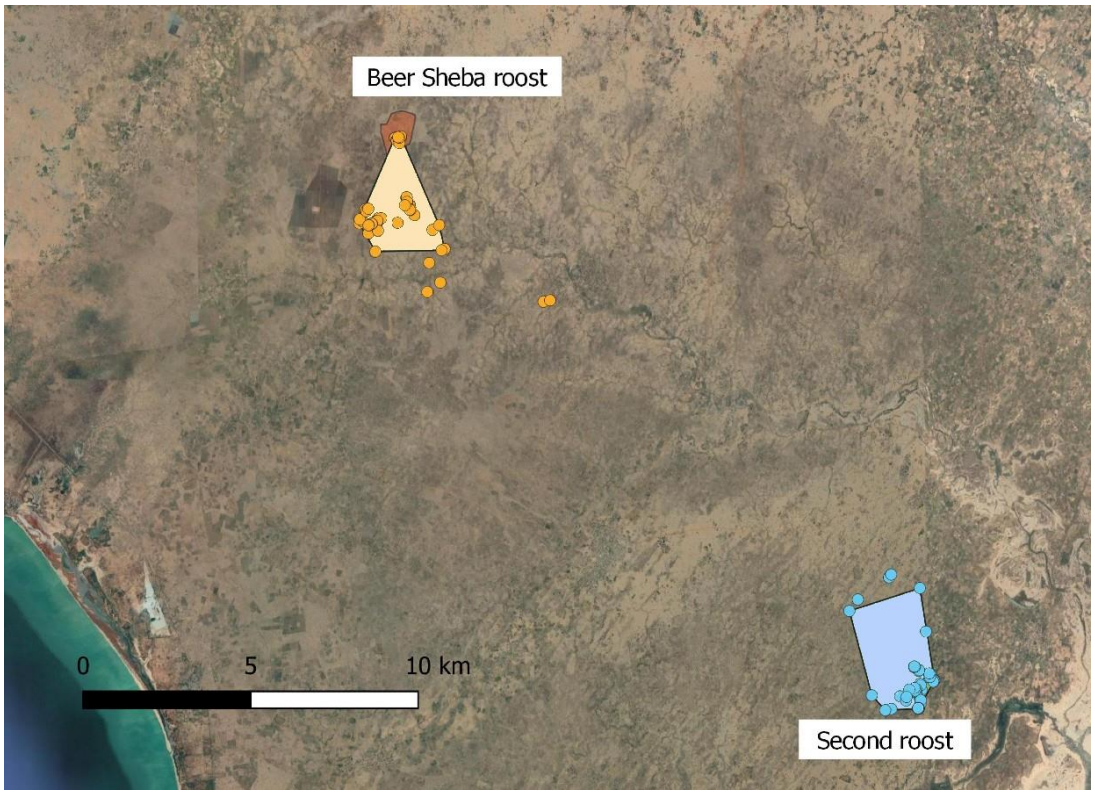

Figure 3.4: 95% MCPs calculated for home-range of Francoise, separated into the two roosts

Table 3.5: Minimum, maximum and mean (± S.E.) daily distance travelled by birds

Bird ID	Mean distance (±S.E.)	Minimum distance	Maximum distance	Number of days
Adama	2.74 ± 0.78	0.79	5.54	6
Bakel	3.62 ± 0.63	1.13	6.30	9
Dewi	5.30± 0.42	2.23	11.48	20
Jeremy	7.81 ± 0.54	4.50	10.16	13
Ouro Sogui	4.96 ± 0.93	1.63	6.71	5
Palmarin	10.34 ± 1.36	6.88	12.51	4
Quincy	4.12 ± 1.18	2.01	6.11	3
Richard-Toll	17.21 ± 0.53	9.51	30.39	13
Sandiara	8.09 ± 1.10	7.24	8.62	3
Sokone	13.84 ± 0.18	2.24	49.19	110
Tamba	17.54 ± 0.40	0.31	25.33	22
XXX	8.27 ± 0.21	0.72	16.26	84
Zeinab	4.34 ± 0.46	0.25	8.70	17
Ziguinchor	5.35 ± 0.20	1.46	12.29	90
Overall	8.11 ± 1.31	2.74	17.54	

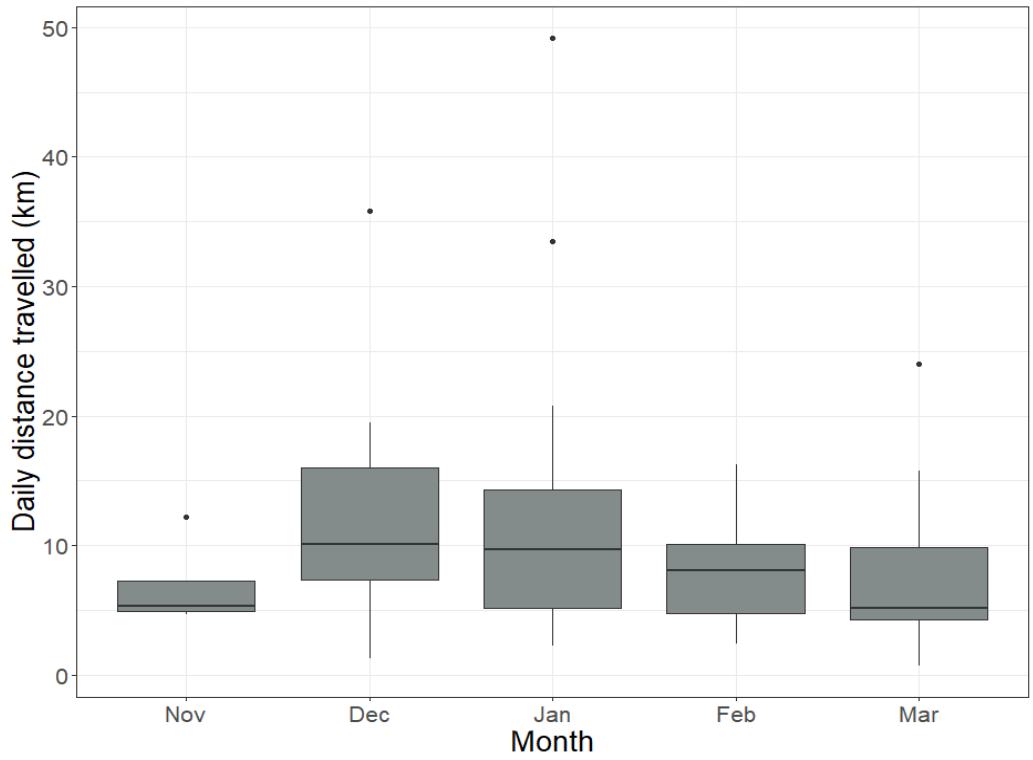

Figure 3.5: Average daily distance travelled as the wintering season progressed. Boxes represent the upper and lower quartiles, intersected by the median. Whiskers extend to 1.5 times the interquartile range (IQR) above and below boxes, points indicate values lying outside of the 1.5 IQR

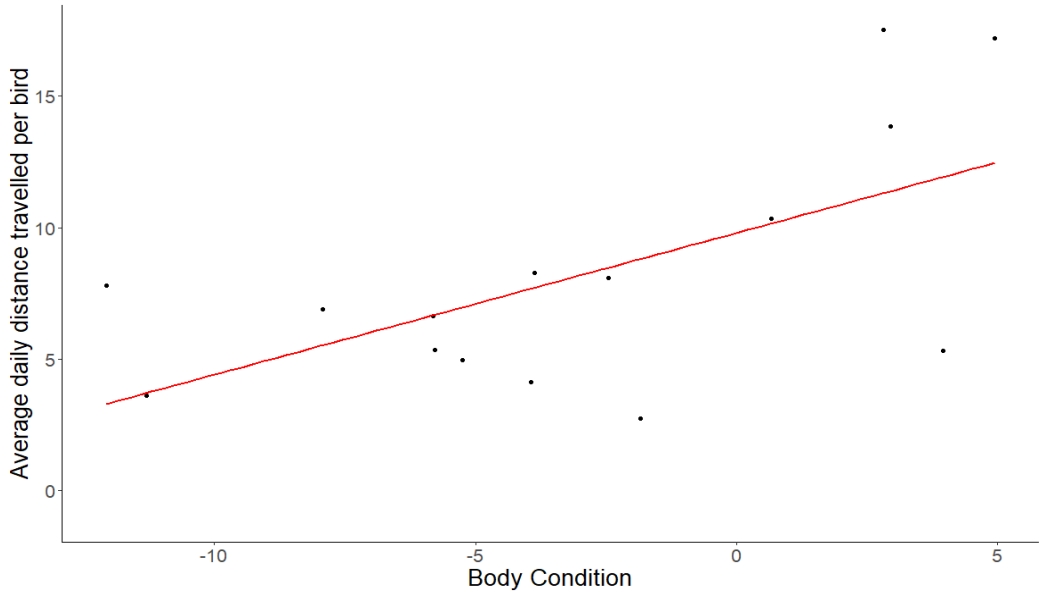

Figure 3.6: Body condition compared to mean daily distance travelled (km) per individual bird (LM: $F_{1,13}$=7.848, p=0.015)

3.4.2 Habitat selection

Three broad scale habitat variables were considered in compositional analysis: the dominant landscape, the dominant vegetation type and the crop composition. Comparison of selected foraging habitats with those available suggested significant habitat selection at the landscape level (Wilks's λ = 0.116, p = 0.001; Table 3.6; Figure 3.7A). Grassland was used most relative to availability (ranked 7, Table 3.6), being significantly selected over all other land uses, apart from water (compositional analysis ranked 0-7 from least to most selected habitat). Significant selection was also identified within dominant vegetation (Wilks's λ = 0.428, p = 0.020; Table 3.7; Figure 3.7B), with trees being ranked the most selected habitat.

No significant selection was identified within crop composition (Wilks's λ = 0.475, p = 0.054)

Table 3.6: Ranking matrix from the compositional analysis, comparing availability and use of different types of landscape. Significant preference or non-preference at $p<0.05$ of the landscape in the row vs. the column is indicated by triple signs (+++/---). Single signs indicate preference/non-preference, but not to a significant degree. Land use selection is ranked from 0-7, indicating the lowest to greatest selection of habitat type by turtle doves, respectively, based on the number of positive associations within a row.

	Arable	Bare earth	Grassland	Natural/ agricultural mosaic	Shrubs	Trees	Water	Village	Rank
Arable	0	+	---	---	-	---	---	-	1
Bare earth	-	0	---	---	-	---	---	---	0
Grassland	+++	+++	0	+++	+++	+++	+	+++	7
Natural/agricultural mosaic	+++	+++	---	0	+++	---	---	+	4
Shrubs	+	+	---	---	0	----	---	-	2
Water	+++	+++	-	+++	+++	+	0	+++	6
Village	+	+++	---	-	+	---	---	0	3
Trees	+++	+++	---	+++	+++	0	-	+++	5

Table 3.7: Ranking matrix from the compositional analysis, comparing availability and use of different vegetation types. Significant preference or non-preference at $p<0.05$ of the landscape in the row vs. the column is indicated by triple signs (+++/---). Single signs indicate preference/non-preference, but not to a significant degree. Vegetation type selection is ranked from 0-4, indicating the lowest to greatest selection of vegetation type by turtle doves, respectively, based on the number of positive associations within a row.

	Grass	Shrub	Tree	Forb	Bare earth	Rank
Grass	0	+	---	-	-	1
Shrub	-	0	---	---	---	0
Tree	+++	+++	0	+++	+++	4
Forb	+	+++	---	0	-	2
Bare earth	+	+++	---	+	0	3

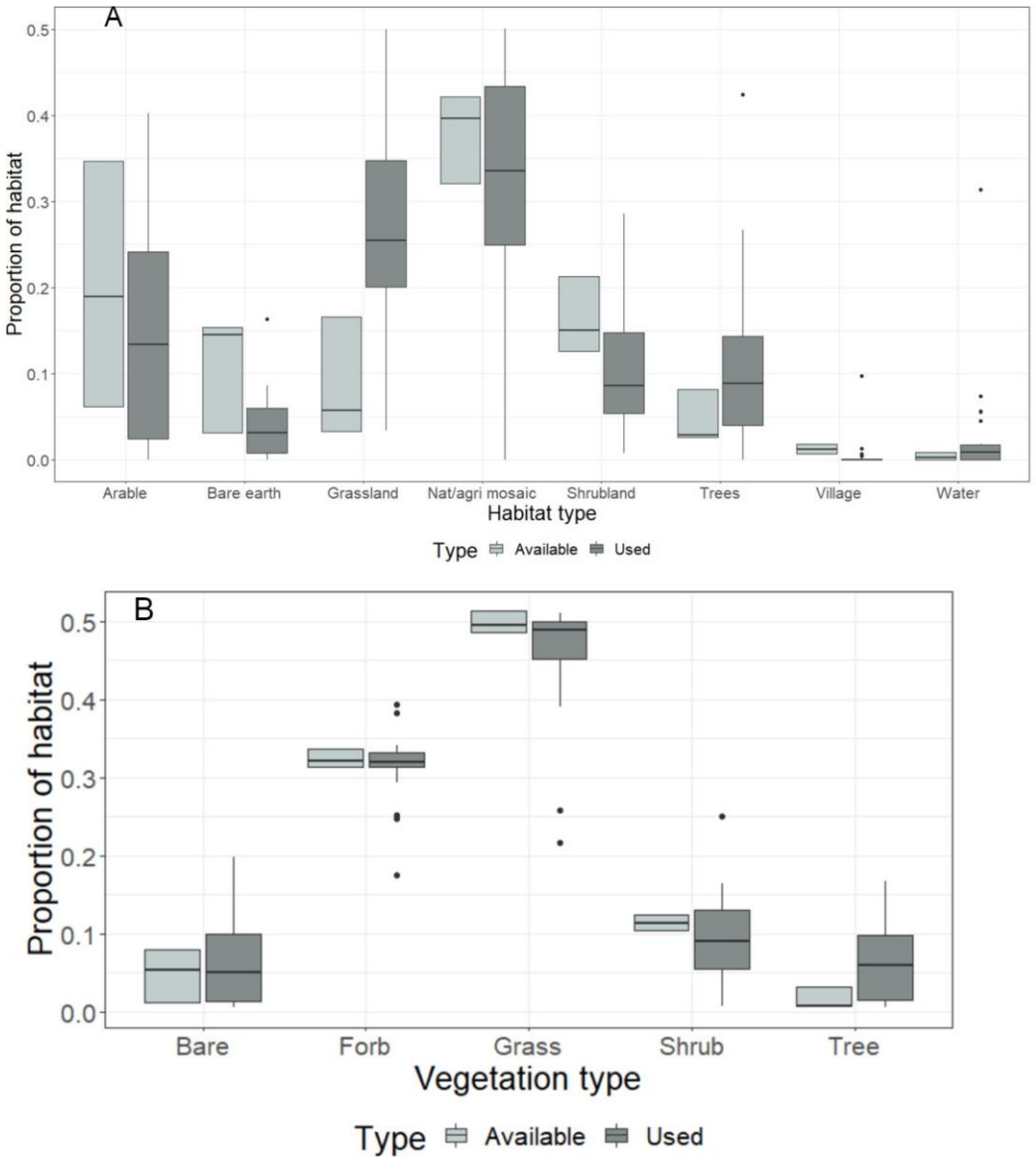

Figure 3.7: The proportion of habitat used by and available to turtle doves: A) dominant landscape and B) dominant vegetation. Boxes represent the upper and lower quartiles, intersected by the median. Whiskers extend to 1.5 times the interquartile range (IQR) above and below boxes, points indicate values lying outside of the 1.5 IQR

For GLMMs, the final model included the following categorical variables: dominant land use; dominant vegetation; proportion of bare earth within grassland; percentage tree cover; percentage shrub cover; percentage forb cover; percentage grass cover and the random variable bird ID. All of the predictor variables had a significant effect on the presence of birds (Table 3.8). When using predictive models, tree cover had the greatest effect on the probability of turtle doves' presence, with a 0.76 (±0.02) chance of birds being present in areas with 15-40% tree cover, and a 0.51 (±0.12) chance of turtle dove presence in areas with 40-65% tree cover (Figure 3.8). When considering dominant vegetation, trees also had an important effect, with shrubs/trees having the greatest effect on the likelihood of turtle dove presence out of the four vegetation types (0.029 ± 0.12; Figure 3.9)

When predicting the effect of dominant landscape on the probability of turtle doves being present, the highest probability of presence was predicted for arable land (0.18 ± 0.02; Figure 3.10), followed by grassland (0.14 ± 0.02; Figure 3.10).

Table 3.8: Significance of the environmental variables on the presence of turtle doves, according to GLMM

Variable	χ^2	Df	*p*
Dominant land use	33.879	4	<0.001
Dominant vegetation	10.899	1	0.012
Class of bare grassland	43.367	4	<0.001
Class of tree cover	742.300	5	<0.001
Class of shrub cover	29.931	3	<0.001
Class of forb cover	57.156	4	<0.001
Class of grass cover	84.384	3	<0.001

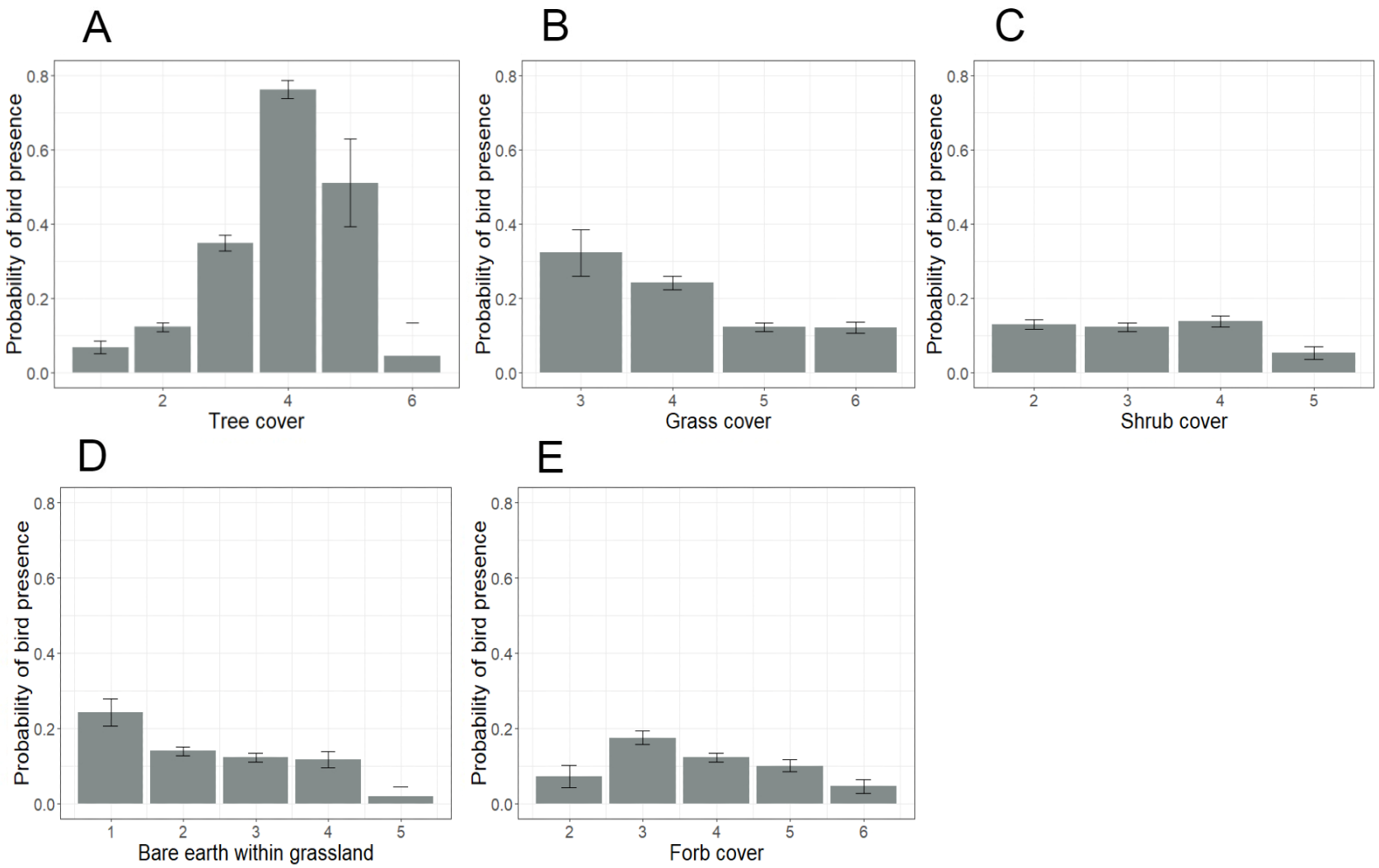
A
B
C
D
E
Probability of bird presence
0.0
0.2
0.4
0.6
0.8
Tree cover
Grass cover
Shrub cover
Bare earth within grassland
Forb cover

Figure 3.8: Probability of birds being present in different habitat structures, calculated using predict on GLMM results. As standard error calculations are not available when using the 'predict' function with merMod objects generated by GLMMs in the lme4 package, standard error was calculated from GLMs. Proportion cover was divided into levels as follows: 1 = 0%, 2 = 1-4%, 3 = 4-15%, 4 = 15-40%, 5 = 40-65%, 6 >65%. Some levels were grouped in order to make GLMM stable, for full information see Table S5. Significance of the effect of different environmental variables included in GLMM on the presence of turtle doves is presented in Table 3.8.

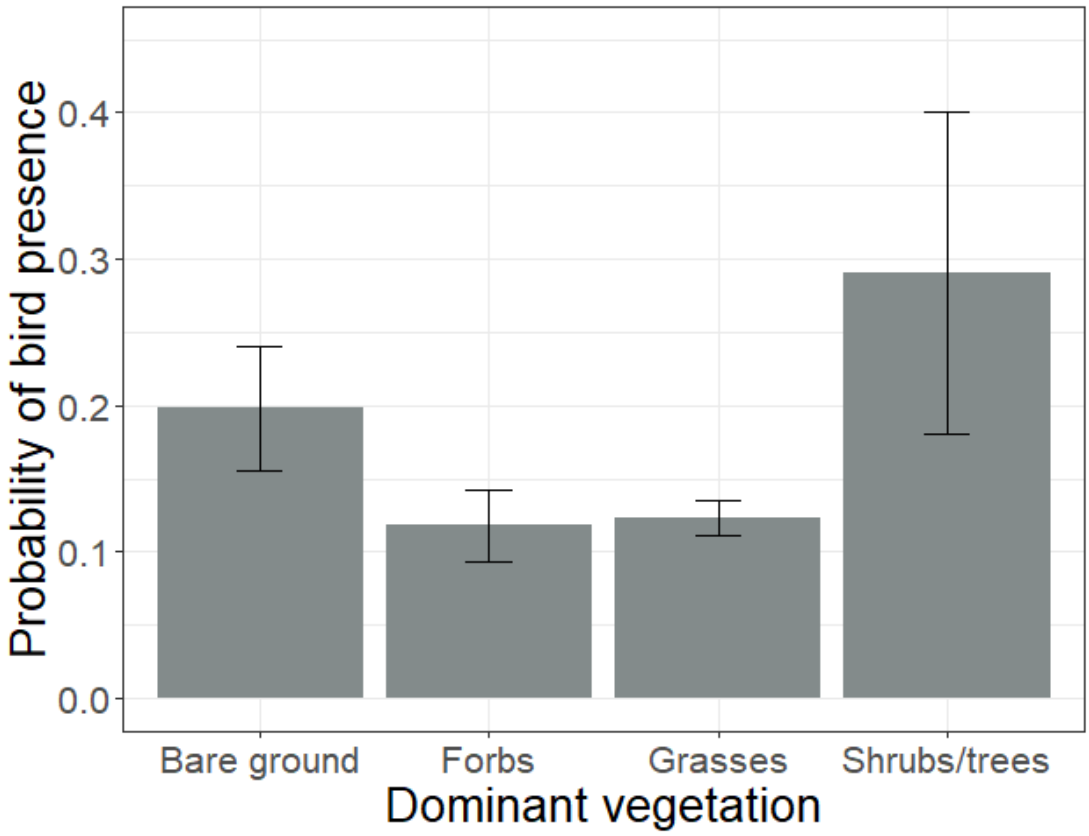

Figure 3.9: Probability of turtle doves being present based on different vegetation types, calculated using predict on GLMM results. As standard error calculations are not available when using the 'predict' function with merMod objects generated by GLMMs in the lme4 pack

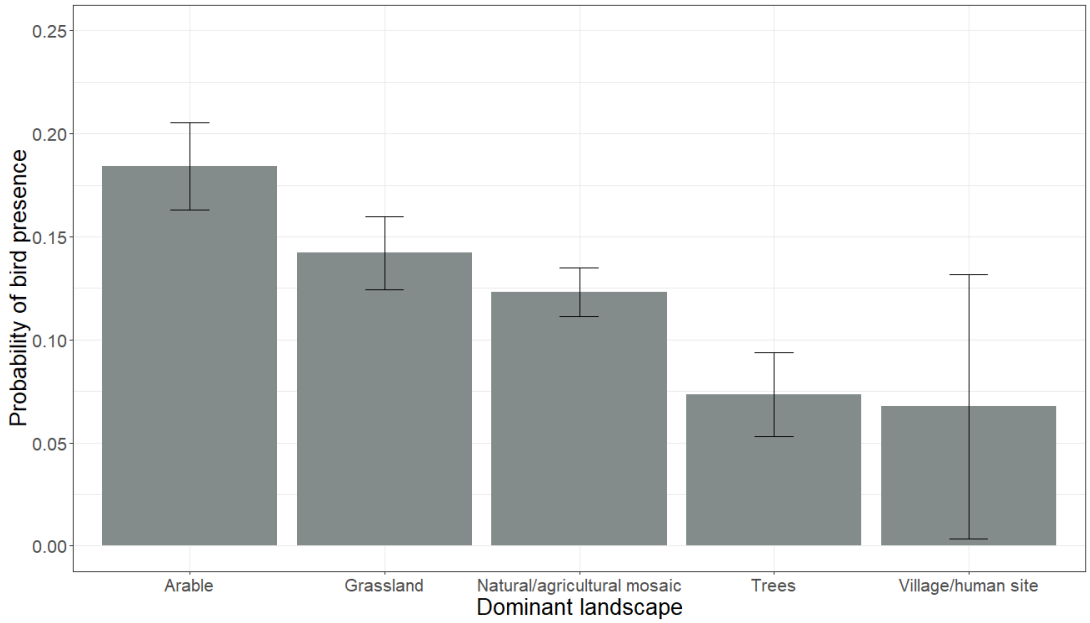

Figure 3.10: Probability of turtle doves being present in different landscape types, calculated using predict on GLMM results. As standard error calculations are not available when using the 'predict' function with merMod objects generated by GLMMs in the lme4 package, standard error was calculated from GLMs.

3.4.3 Feeding preference

DNA was extracted from 151 seed morphotypes, and 83.4% (n=126) were successfully amplified by PCR and sent for Sanger sequencing. Of these, 38.9% (n=49) were successfully identified to species level, and a further 15.9% (n=20) to genus and 5.6% (n=7) to family level.

Of the 45 genera assessed to compare occurrence in the environment with occurrence in the diet, 14 significant interactions were identified (Figure 3.11). Seven of these interactions were positive, suggesting selection, as seeds were consumed more often than would be expected based upon their occurrence in the environment. The strongest selection was for two genera of crop plants *Sorghum* and *Cenchrus.* Two legumes, *Vigna* and *Senna* were consumed more than expected, but to a lesser extent. *Urochloa*, a genus within the grass family, and the flowering plants *Melochia* were also selected. The six remaining significant interactions were negative, suggesting an avoidance of these genera. Despite their presence in the environment, all but one of the seeds exhibiting negative interactions were absent in the diet of turtle doves. *Panicum* sp. did occur in the diet of turtle doves, but less frequently than would be expected based on its availability.

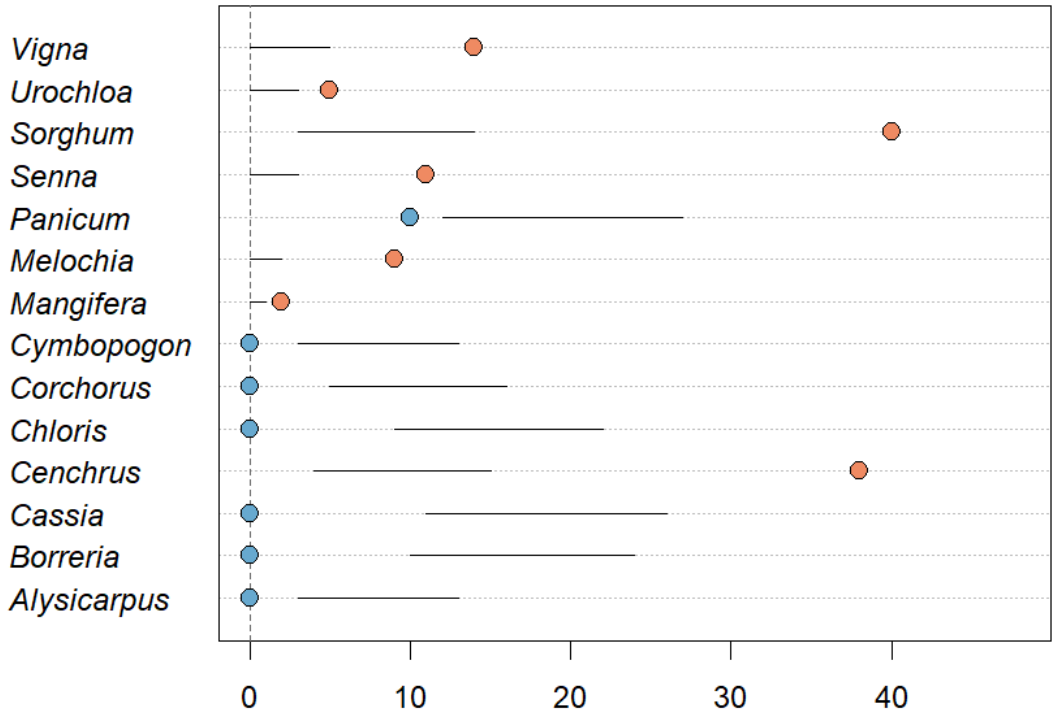

Figure 3.11: Subset of genera with significant results showing the use of dietary resources compared to their availability in the environment, as calculated by EconullnetR, using 9999 iterations. Lines represent the 95% confidence limit of the null model, dots represent the number of times each taxonomic unit was consumed. Results are significant when dots fall outside of the 95% confidence limit range, with selection represented by red dots and avoidance represented by blue dots.

3.5 Discussion

In this study, the home-range of 21 turtle doves was calculated and the daily distance travelled assessed for 14 turtle doves in the wintering grounds. This is one of relatively few studies to analyse avian home-range and habitat use on the wintering grounds, and to my knowledge, the most extensive analysis of movement data for turtle doves on their wintering grounds to date. I observed smaller home ranges than previously described for turtle doves during the non-breeding season, with a mean KDE_{95} of 33.94km^2 (±6.96), and mean KDE_{50} 5.19km^2 (±1.20).

I observed a mean daily distance travelled of 8.11km (±1.31km), with a maximum distance of 49.19km travelled in a single day, and birds in better body condition tending to travel further each day. Significant habitat selection was detected, with grassland being the most used habitat type in relation to its

availability in the landscape. Trees were also found to be highly ranked for habitat selection, and analyses predicted the highest probability of turtle doves being present in areas of 15-40% tree cover. There was significant habitat selection within dominant vegetation types, with trees and shrubs being ranked most selected.

3.5.1 Home-range on the wintering ground

The home-range of migratory avian species has not been widely studied in the wintering grounds, but the significance of wintering grounds in species conservation is becoming increasingly addressed with more work being done in this area of avian ecology (Atkinson *et al.* 2014; Vickery *et al.* 2014; Zwarts *et al.* 2018). For example, studies investigating the home-range of warbler species over-wintering in Africa indicated relatively small mean home-ranges of these songbirds, including the aquatic warbler (*Acrocephalus paludicola*) (KDE_{95} = 0.04km^2), wood warbler (MPC_{100} = 0.12km^2) and willow warbler (*Phylloscopus trochilus*) (MPC_{100} = 0.12km^2) (Arbeiter and Tegetmeyer 2011; Mallord *et al.* 2016; Willemoes *et al.* 2018). In contrast, birds of prey wintering in Africa can have vast home-ranges, as observed in wintering Monagu's harrier (*Circus pygargus*), where the home-range covered, on average, around 200km^2 (Trierweiler *et al.* 2013).

Whilst some mapping of turtle dove home-range has been completed on the breeding grounds, relatively little is known about the home-range and daily distance travelled to forage by birds on the wintering grounds. One study tracking the migration of birds from France was able to determine two wintering sites from one individual (Lormée *et al.* 2016). Due to the nature of the tags used in the previous study being designed to assess long distance migration, as opposed to high-precision local movement, the accuracy of the GPS fixes in the present study will produce a truer representation of home-range within the wintering period. Furthermore, this study uses information for 21 birds, allowing an assessment of how home-range varies between individuals, unlike the previous study, which only analysed the wintering home-range of a single bird (Lormée *et al.* 2016). I calculated both KDE and MCP home ranges, but due to the variation observed between the methods, for the purpose of comparing home-range areas calculated in this study with those from previous

work on turtle doves, I will discuss the MCP results, as this was the method used by previous authors.

Lormée *et al.* (2016) identified two wintering home-ranges used by a single turtle dove (Marcel), covering 57.9km^2 (65 days: 2nd October 2013 - 1st December 2013) and 87.3km^2 (138 days: 3rd December 2013 – 21st April 2014) when calculated using MCP_{95} . The mean MCP_{95} in the present study was considerably smaller, at just 15.90km^2 (±3.09, n=31), and apart from one, (Ziguinchor_2.1: 74.29km^2), all MCP_{95} areas calculated in the study were less than 50km^2. The mean core usage area estimate (MCP_{50}) of 2.39 km^2 (±0.62, n=31) was more similar to that observed by Lormée *et al.* (2016): MCP_{50} of 3 km^2 and 2.3 km^2 at the two wintering sites. One possible explanation for this difference in home-range estimates, as previously alluded to, is the accuracy of the GPS locations of the two types of tag used. The accuracy estimates for locations from the tags in the previous study ranged from "<250m" to ">1500m" (Lormee *et al.* 2016), whereas those used in the current study estimate accuracy to of 10-30m (Chris Orsman, *personal communication*).

Alternatively, it is possible that this disparity in home-range size is a result of data being used to calculate Marcel's home-range covering a much longer time span than those of this study. It was reported that for the two distinct wintering sites, the first was used for 65 days, and the second for 128 days (Lormée *et al.* 2016). The number of days over which GPS fixes were recorded was, on average, considerably shorter (30.7 days ± 7.5) in this study, and given the correlation between home-range area and number of fixes recorded, it is possible that my home-range calculations may underestimate the true size of the area used by turtle doves across the whole wintering period. This being said, when all fixes in the present data set were used to calculate the home-range of an individual without dividing them into shorter time scales, the mean home-range only increased slightly, to 20.00km^2 (±4.80).

In addition, the individual exhibiting the largest overall home-range in the present study (Françoise: 75.8km^2) appeared to utilise two roosts. When these were split (as described in section 3.3.3.1), the home-range around the two sites was much smaller (4.82km^2 and 6.06km^2). Therefore, it is possible that in the previous study, the bird may have been utilising multiple roosting sites

within each wintering site, accounting for the larger home-range. The location of the second roost site used by Françoise was able to be obtained because this bird returned to Beer Sheba. Other individuals, particularly those for whom GPS fixes were recorded over a shorter period of time, may have moved to an alternative roost and not returned to Beer Sheba, therefore not come back into range of the receiver. Thus, the home-range calculations in this study are generally focussed around a single roost, rather than a single bird, as per Lormée *et al.* (2016), potentially accounting in part for their smaller area.

Within the breeding grounds, work carried out on the home-range of turtle doves in the UK has showed some variation in home-range sizes, but they are consistently much smaller than those reported from the wintering grounds. One study assessed the home range of birds living in two sites with differing habitat, using 100% MCP (Browne and Aebischer 2003a). They observed a mean home range of 0.84km^2 (±0.24) at one site, and 4.97km^2 (±2.23) at the other, with the largest home-range reported covering 11.30km^2. Dunn *et al.* (2020) used a 90% MCP to obtain a mean home-range in the breeding grounds of 0.86km^2 (±0.16). There are a number of potential reasons for the smaller home-ranges in the breeding ground. Whilst breeding, turtle doves will exhibit territorial behaviour, defending a small area around the nest, with average territory seizes identified at two sites in the UK being 0.02km^2 and 0.3km^2 (Browne and Aebischer 2004). A difference in home-range size on the wintering grounds has been observed among songbirds that exhibit territorial behaviour and those that don't, with winter home-range estimates for territorial individuals of less than 0.02km^2, compared to up to 0.15km^2 in non-territorial individuals (Brown and Long 2007; Brown *et al.* 2000). During the non-breeding season, turtle doves roost in large groups, rather than defending their own territories, potentially resulting in the larger home-ranges observed.

In addition, previous studies of turtle dove breeding home-range have suggested food availability to be a driving factor in the size of home-range. Browne and Aebischer (2003) identified the main reason for the larger home-range at one site to be the greater geographical distance between nesting and foraging sites, and showed that cropped arable habitats were avoided, whilst Dunn *et al.* (2020) observed a relationship between smaller home ranges and

a greater proportion of non-farmed habitat. This is consistent with the inference that food availability is the most important ecological constraint on the wintering grounds (Brown and Sherry 2006). As a larger home-range can be indicative of poorer habitat quality (Williams *et al.* 2016), I suggest that the larger home-ranges observed on the non-breeding grounds reflect the need to travel a greater distance to obtain sufficient food in the arid environment in the wintering ground. High levels of nomadism has previously been identified in granivorous birds inhabiting desert environments due to dry weather conditions causing patchy distributions of seed resources, and therefore a need to travel to find such resources (Davies 1984; Dean 1997). Whilst turtle doves are not nomadic species, this described behaviour does support the suggestion that foraging across larger areas may be driven by sparse food availability.

In a study of home-range of great spotted cuckoos (*Clamator grandarius*) on the breeding and wintering grounds, results apparently contrasted the findings of this study, with home-ranges being significantly larger in the Spanish breeding grounds than African wintering grounds (Rühmann *et al.* 2019). However, Rühmann *et al.* (2019) identified a lower availability of food in the breeding grounds than in the wintering grounds, suggesting that cuckoos were travelling further to forage, thus supporting my suggestion of food availability driving home-range size. In addition, studies on a range of taxa demonstrate larger home-ranges to be linked with scarcer food resources, including the leopard tortoise (*Stigmochelys pardalis*), roe deer (Capreolus capreolus) and polar bears (Ursus maritimus) (Ferguson *et al.* 1999; McMaster and Downs 2009; Morellet *et al.* 2013).

Given the climate of the wintering grounds, the availability of water will also contribute to habitat quality, and likely play a role in determining the home range of individuals. In Senegal, whilst birds were observed drinking from ephemeral, rain-fed pools (Chris Orsman, *personal communication*), such seasonal pools would disappear as the non-breeding season progressed through the driest months of the year, leading to a need to travel to more reliable water sources. At Beer Sheba, birds travelled to a small, local reservoir to drink, approximately 6km from the roost when alternative water resources

were scarce (Chris Orsman, *personal communication*). The distance to such water sources could therefore also contribute to the larger home ranges observed in this study than those conducted on the breeding grounds.

I have shown in this study that birds can travel close to 50km in one day, with the maximum daily distance travelled recoded for Sokone (49.19km). Overall, the daily distance travelled was, on average, 8.11km (± 1.31), but the mean distance travelled by a single individual ranged from 2.74km (±0.78) to 17.54km (±0.53). A positive correlation was detected between the distance birds travelled and body condition. This may support the idea that food availability influences how far individuals will travel. It has been shown in breeding turtle doves in the UK that larger home-ranges were associated with a relatively high proportion of seed-rich habitat, such as semi-natural grassland and fallow, but a smaller proportion of unfarmed habitat than smaller home-ranges (Dunn *et al.* 2020). This suggests that breeding individuals would travel further to reach high quality food resources when the immediate home-range was of poorer quality (Dunn *et al.* 2020). It is possible that something similar is occurring in the wintering grounds, with birds travelling a greater distance to utilise better foraging habitat, resulting in the better body condition observed in birds travelling further. However, as a causal relationship cannot be determined, this relationship could either indicate that birds flying further benefit from a more diverse or plentiful food supply, resulting in improved body condition, or simply that individuals in better condition are able to, and thus tend to fly further.

In their study of three species of dabbling duck (mallard *Anas platyrhynchos,* Eurasian teal *A. crecca crecca* and northern pintail *S. acuta*), Legagneux *et al.* (2009) observed an increase in foraging distance over winter. Dabbling ducks are largely granivorous over winter, and it was suggested that the longer distances travelled to forage as winter progressed were a result of food depletion, as resources were not renewed (Legagneux *et al.* 2009). I expected to see a similar relationship, however, Julian date was negatively correlated with daily distance travelled, suggesting that as the season progressed, birds did not travel as far from the roost to forage. This did not support my hypothesis that birds would need to travel further to access sufficient resources as the

season progressed and food supplies became limited. It is possible that, as suggested regarding home-range size, birds may have relocated to an alternative roost as food resources became scarcer closer to Beer Sheba. In order to ascertain if this were the case, it would be necessary to use alternative GPS tags, which are not reliant on individuals returning to a receiver.

3.5.2 Methodology in calculating home range

In this study, 95% home-range and 50% core usage area were calculated using both MCP and KDE methods. KDE is becoming an increasingly popular alternative to MCP due to an increased accuracy in home-range estimation. MCPs are built by connecting outermost points in a location data set using straight lines, in order to create a shape encompassing all of the spatial points (Mohr 1947), including areas traversed by animals between foraging areas and thus, potentially unused habitats. The KDE method uses utilization distributions of individuals to identify more heavily used areas, allowing models to consider multiple centres of activity, thus making them more ecologically meaningful (Hemson *et al.* 2005; Calenge 2019). Furthermore, they are less influenced by outlying points, allowing a more accurate depiction of habitat use by animals (Hemson *et al.* 2005).

An important consideration when using KDE method for estimating home-range is selection of a smoothing parameter (*h*), as it will influence the size of the home-range estimate, with a larger *h* value resulting in less detailed home-range estimates (Seaman and Powell 1996; Worton 1989). Least-squared cross-validation (LSCV) parameter has been commonly suggested as the best method (Seaman *et al.* 1999; Girard *et al.* 2002; Gitzen and Millspaugh 2003), however, I did not feel it was appropriate for my data as this parameter may overestimate home-ranges when there are fewer than 50 locations (Seaman and Powell 1996). Furthermore, consistent use of the same core areas increases the failure rate of the LSCV method, or causes under-smoothing, resulting in 'islands' rather than a contiguous home-range, with the fragmentation of these islands being highly dependent on sample size (Hemson *et al.* 2005; Kie, 2013; Schuler *et al.* 2014). Whilst it could be suggested that home-range may not be contiguous, the definition of a home range as *'that area traversed by the individual in its normal activities of food*

gathering, mating, and caring for young' dictates that, while there may be non-contiguous areas of core usage, the home range should be contiguous (Kie, 2013). The reference bandwidth is used in ecological studies (Mallord *et al.* 2016; Castaño *et al.* 2019; Mitchell *et al.* 2019), but can result in over estimation of home-range due to over-smoothing of data (Seaman and Powell 1996). The *ad hoc* method of calculating *h* was used in the present study to mitigate these potential over or under-smoothing issues by using an *h* value adapted to each measure of home-range (Berger and Gese 2007; Jacques *et al.* 2009).

Both home-range and core area calculated were significantly larger when using the KDE method, than the MCP method. This larger area estimated by KDE is consistent with previous work comparing the use of MCP and KDE (Olsen *et al.* 2017).

3.5.3 Habitat selection and foraging preference in the wintering grounds

Habitat selection was identified when considering the dominant landscape within an area, with grassland being ranked highest for selection. This is consistent with similar findings in the breeding grounds (Dunn *et al.* 2017; Dunn *et al.* 2020) and supports my hypothesis that, whilst a mixture of habitat types is used for foraging, natural grasslands are positively selected. The higher selection of this land type over natural/agricultural mosaic, which ranked fourth, and arable land, which ranked third lowest, indicates the importance of unmanaged land, containing wild food resources for turtle doves on the wintering ground. This is supported by previous studies identifying *Panicum laetum*, a wild grass, to be an important element of turtle dove diet (Jarry and Baillon 1991), as well as findings from this study. When investigating diet using metabarcoding (Chapter 2), four genera from within the grass family (*Panicum* sp.*, Echinochloa* sp.*, Dactyloctenium* sp. and *Urochloa* sp.) were frequently detected in the diet of turtle doves sampled in Senegal, which is consistent with using grassland for foraging. Furthermore, when using *EconullnetR* to identify selection and avoidance of resources in the environment, *Urochloa* sp. were consumed more than would be expected based on availability. Natural grassland is also an important environment for other species, with seeds from the grasses *Panicum laetum, Brachiaria hagerupii* and *Dactyloctenium*

aegyptium being identified as predominant food resources for resident dove species in the Sahelian region (Zwarts *et al.* 2018).

Surprisingly, a negative result was detected for *Panicum* sp. when analysing dietary preference, suggesting an avoidance of this food resource. This was unexpected given that it has been reported as an important component of the diet on the wintering grounds (Jarry and Baillon 1991), and it occurred frequently in the diet of birds in this study, being detected in the diet of almost a quarter of birds sampled in Senegal (Chapter 2, Taxa identified

Table S1.6). One explanation for this may be that this apparent avoidance could be an indirect result of a stronger preference for other available food resources, such that individuals may not be actively avoiding this genus, rather they have such a strong preference for alternative genera, *Panicum* is appearing less frequently in the diet. *Panicum* sp. was detected at a consistently high proportion in environmental sampling (accounting for 11-14% of seeds present across three winters). However, despite consistent availability, occurrence of this genus in the diet did fluctuate between years. It was most common during the winter of 2014/215 (W1), where it was detected in the diet of 76% of birds sampled. In subsequent years, it was a less commonly occurring dietary item, being detected in the diet of 29% of birds caught in 2015/16, and only 7% and 6% of birds caught in 2016/17 and 2017/18, respectively. Therefore, whilst it was consumed, the higher proportion of alternative dietary items and its consistent availability in the environment suggests that this resource was not a preference over other resources such as cultivated *Cenchrus* sp. and *Sorghum* sp.

As well as grassland, trees were highly ranked in the compositional analysis for habitat selection, identified as third most important when analysing dominant land use, and most selected when analysing dominant vegetation. The proportion of tree cover also had a significant effect in the GLMM and, based on predicted models, there was a higher probability of turtle doves being present in areas with greater tree cover, providing further support for the importance of trees in the environment. In a large participatory study, bringing together published literature and expert knowledge, Atkinson *et al.* (2014) used principal component analysis and clustering to assign migratory species to the

habitats with which they are most associated in the African wintering grounds. The European turtle dove was assigned to 'Terrestrial habitats with shrubs and trees', which is consistent with this relatively high importance of trees within the compositional analysis. Additionally, Browne and Aebischer (2003) found that, in the breeding grounds, wooded areas were used more frequently relative to their availability. In Senegal, birds were frequently observed loafing in trees, particularly during the hottest hours of the day (*personal observation*), which may account for their relative importance.

Wooded areas have been identified as important habitat components for other migratory birds over-wintering in Africa. One study identified a preference for habitats containing tall trees for willow warblers (*Phylloscopus trochilus*), melodious warblers (*Hippolais polyglotta*) and pied flycatchers (*Ficedula hypoleuca*), with larger home-ranges observed in human disturbed sites being attributed to a lower density of tall trees (Willemoes *et al.* 2018). Similarly, a preference for sites with greater tree density and taller trees was detected in wood warblers (Mallord *et al.* 2016). The findings of this study, alongside previous work, highlights the importance of retaining trees and woodland in the wintering grounds, where deforestation and removal of shrubs in favour of agricultural land is increasingly common (Brink and Eva 2009). Many reforestation projects are being established across Africa (AFR100; GGW; Trillion Trees) which, as well as having benefits for combating climate change through carbon sequestration, reducing erosion and improving soil fertility, will provide crucial habitat to support declining species inhabiting this region.

One unexpected finding from this study was that grass was ranked lowest for selection when considering dominant vegetation, the opposite of what was observed when looking at dominant land use. Visualising the proportion of used and available locations accounted for by different habitat types (Figure 3.7) helps put this into the context of ecological importance. When looking at the habitat at a fine scale, grass accounted for the greatest proportion of available habitat, close to 50%, whilst the proportion of used habitat with grassy vegetation was slightly lower. This shows that, whilst it is a very commonly used habitat type, it is less so in comparison to its availability. Trees on the other hand, which were ranked highest, accounted for a very low

proportion of the available habitat surveyed. Whilst they only accounted for just over 5% of used habitat, this is proportionally much greater than their availability, contributing to their high rank of selection.

Whilst compositional analysis did not detect an overall significant selection in crops, some crop plants were identified as being selected when comparing dietary items detected with their availability in the landscape. *Sorghum* sp. and *Cenchrus* sp. (millet) were both strongly selected for, which is consistent with the findings of Chapter 2, as these crops were prominent in the diet of turtle doves sampled in Senegal. The abundance in the diet and high selection of these crops demonstrates the importance of cultivated seeds in the diet of this declining species, providing food resources in a highly modified landscape, following conversion of natural grassland and woodland into agricultural land (Brink and Eva 2009). This finding is consistent with the dietary switch from more wild to cultivated seeds observed on the breeding grounds of both European turtle doves, and the African sub-species *Streptopelia turtur aronicola*, breeding in Morocco (Browne and Aebischer 2003a; Dunn *et al.* 2018; Mansouri *et al.* 2019). Previously observed dietary change has been attributed largely to a loss of wild food resources due to increased agriculture, and my results showing selection of cultivated seeds suggests that this change in feeding ecology is consistent across their range.

3.5.4 Conclusion

This is, to my knowledge, the most detailed analysis of home range and habitat use of European turtle doves on their wintering grounds to date. I provide a more in depth representation of home-range use by turtle doves in the wintering grounds, showing a much larger area of use than those observed in the breeding grounds. The negative correlation between daily distance travelled and progression of the non-breeding season was surprising, but perhaps alludes to birds moving on to an alternative roost as food supplies become diminished. The positive correlation detected between daily distance travelled and body condition indicates that birds may be travelling greater distances to obtain higher quality or more abundant food resources. This analysis of habitat use supports the dietary metabarcoding work (Chapter 2) in highlighting the importance of natural areas, such as grasslands, in the

survival of turtle doves in the wintering ground, as well as demonstrating the importance of trees in the environment. I also found evidence of high levels of selection for cultivated seeds, suggesting that these are important aspects of the diet, perhaps to buffer the loss of wild resources.

3.6 Acknowledgements

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Chapter 4 - Assessment of *Trichomonas gallinae* prevalence and strain composition in Turtle Doves on breeding and wintering grounds

Photograph by Nicolas Viacrroze

Contribution of collaborators involved in this chapter was mainly support in collection of samples from the field. Samples used in this project were collected from Senegal in three years prior to my PhD commencing by the RSPB and Nature-Communautés-Développement. The main team involved in sample collection was Chris Orsman (RSPB), Moussa Ka (NCD) and Mamadou Diallo (NCD). I worked with Chris Orsman, Moussa Ka and Mamadou Diallo and master's student Coen van Tuijl to collect samples from Senegal in 2017. I was supported in sample collection in France by Hervé Lormée and Cyril Eraud of the Office Français de la Biodiversité, Unité Avifaune Migratrice and in Hungary by Dr Orsolya Kiss, of the University of Szeged. During sample collection, all birds were handled under the licences of the aforementioned collaborators. The custom pipeline used for bioinformatic analysis used in this work was designed by Lorna Drake and Jordan Cuff (modified from that provided by Helen Hipperson and the NBAF team at the University of Sheffield).

4.1 Abstract

Trichomonas gallinae is a widespread protozoan parasite infecting a diverse range of avian orders, in particular, free ranging Columbiformes and Falconiformes, and has been found to cause mortality in the rapidly declining European turtle dove (*Streptopelia turtur*). At least 23 strains of *T. gallinae* have been identified, with certain strains found to be more virulent, resulting in a high rate of mortality in infected individuals, whilst other, less virulent stains often result in asymptomatic infection. I investigate several key aspects of the community ecology of this parasite, including prevalence, strain composition and co-infection with multiple strains, in turtle doves from both the breeding and wintering grounds. This study is one of only two, to my knowledge, utilising high throughput sequencing to identify the presence of multiple strains of *T. gallinae* within a single host. The prevalence of *T. gallinae* infection varied between sampling season, ranging from 50% to 83%, and ten distinct strains were detected, four of which have not been previously identified. A higher rate of co-infection with multiple strains of *T. gallinae* was detected in this study than in the only previous study, with multiple strains being detected in 16% of birds sampled. Assessment of the rate of co-infection between the four most commonly occurring strains identified that frequency of co-occurrence was lower than would be expected if infections occurred at random. These findings suggest that there is a mechanism acting to reduce the presence of multiple stains within the host.

4.2 Introduction

4.2.1 Trichomonas gallinae

Whilst underappreciated as a driver in species decline for a long time (Tompkins and Jakob-Hoff 2011), infectious diseases are increasingly recognised as an important conservation issue, contributing to the decline of species across a wide range taxa worldwide (Berger *et al.* 1998; Cunningham and Daszak 1998; Warner 1968; McCallum 2012). A widespread and important parasite affecting bird populations is the protozoan *Trichomonas gallinae*, the causative agent of avian trichomonosis: a disease found in a diverse range of avian orders, in particular free ranging Columbiformes and

Falconiformes (Bunbury *et al.* 2008a; Sansano-Maestre *et al.* 2009; Amin *et al.* 2014). With records dating as far back as the 1500s, this is one of the oldest documented wildlife diseases (Forrester and Foster 2009). Often referred to as 'Frounce' or 'Canker', it was described in the first English falconry books long before the discovery of the *T. gallinae* parasite (Stabler 1954). In 1878, *T. gallinae,* isolated from the upper alimentary tract of a rock dove squab (*Columba livia*), was first identified as the aetiologcal agent of this disease (Stabler 1954). Affecting primarily the upper digestive and respiratory tracts, *T. gallinae* causes a wide range of symptoms of varying severity. In mild cases, infections may result in inflamed mucosa; excess salivation causing drooling; and difficulty swallowing leading to regurgitation of food (McBurney *et al.* 2015). More severe instances can result in necrotic ingluvitis and the formation of caseous lesions in the throat which may block the oesophageal lumen if they grow to a sufficient size, potentially resulting in death by asphyxiation or starvation (Stabler 1954). Less commonly, trichomonosis may manifest itself in the liver tissue, air sacs and parts of the cranium (Stabler 1954; Narcisi *et al.* 1991).

Trichomonas gallinae has a global distribution, with cases reported from most major land masses across the world (Forrester and Foster 2009). This parasite distribution closely mirrors that of the rock dove, the primary host of *T. gallinae,* and considered the source of its worldwide distribution (Stabler 1954; Harmon *et al.* 1987). Infections in rock doves are reported in at least 31 countries, across every continent except Antarctica, where rock doves are not found (Forrester and Foster 2009). Whilst it is widely believed that Columbiformes are the natural host for this parasite (Stabler 1954)*, T. gallinae* has been identified in a wide range of orders, including Galliformes, Strigiformes, Passeriformes and Psittacines (McKeon *et al.* 1997; Anderson *et al.* 2009; Forrester and Foster 2009; Sansano-Maestre *et al.* 2009; Park 2011; Lawson *et al.* 2012;). This indicates a low host specificity, facilitating interspecific transmission between birds from several orders, potentially by pathogen spillover (Lawson *et al.* 2011b).

4.2.2 Trichomonosis epidemic

One of the most damaging epidemics for wild bird populations of recent years is the widespread epidemic of trichomonosis in greenfinches (*Chloris chloris*) and chaffinches (*Fringilla coelebs*), which is believed to be the result of pathogen spillover from columbids to passerines (Lawson, *et al.* 2011a). This novel infection of passerines began in the UK in 2005, when it was first recognised as an emerging infectious disease (EID) (Lawson *et al.* 2006). The resulting epidemic, termed 'finch trichomonosis', caused widespread passerine mortality, and several studies have identified a single, clonal strain of *T. gallinae* to be responsible (Lawson *et al.* 2011b; Chi *et al.* 2013; McBurney *et al.* 2015). Beginning in central and western England, and Wales in 2005, high levels of mortality were recorded within this region in 2006, with an estimated 500,000 greenfinches alone succumbing to trichomonosis during the first year of the epidemic (Robinson *et al.* 2010). The disease spread into eastern English counties in 2007, and has resulted in mortalities in wild British passerines every year since (Lawson *et al.* 2012). Following its emergence in the UK, trichomonosis spread into mainland Europe, with cases confirmed at multiple sites in Fennoscandia in 2008 (Lawson *et al.* 2011b). From these sites, parasites were isolated and found to exhibit no genetic variation when compared to the British strains of *T. gallinae* (Lawson *et al.* 2011b), indicating that the spread of this disease was facilitated by migration. The free-flying nature of avian hosts makes it very difficult to contain disease outbreaks, and this identification of strain homogeneity between British and Fennoscandian infection is the first documented case of the spread of a protozoan EID via bird migration, with chaffinches being identified as the most likely vector (Lawson *et al.* 2011a). As a consequence of this avian movement, subsequent outbreaks of trichomonosis in finches occurred in Ireland and across mainland Europe, in Northern Germany, Austria, France and Slovenia (Amin *et al.* 2014; McBurney *et al.* 2015; Marx *et al.* 2017). Concurrent with the spread into Europe, trichomonosis was first recognized in purple finches (C*arpodacus purpureus*) in Nova Scotia, Canada, in 2007 (Forzan *et al.* 2010). Over the following three years, it spread throughout three Canadian Maritime provinces, also causing mortalities in American goldfinches (*Carduelis tristis*) and pine

siskins (*Carduelis pinus*) (McBurney *et al.* 2015). With such a wide host and geographical range and potential conservation implications, study of the occurrence in wild avian populations is crucial.

4.2.3 Genetic diversity of *Trichomoans gallinae*

Several studies have genotyped isolates from a range of avian taxa and at least 23 distinct strains of *T. gallinae* are reported on NCBI GenBank (Clark *et al.* 2016). This includes isolates from columbids, birds of prey and passerines, sampled from a wide geographical distribution, covering Europe, Asia, North America and Africa, (Sansano-Maestre *et al.* 2009; Lawson, Cunningham, *et al.* 2011; McBurney *et al.* 2015; Thomas 2022). Amplification by polymerase chain reaction (PCR) of the 5.8S rDNA region and its flanking internal transcription spacer regions (ITS1 and ITS2) is a well-established method for detection of the presence of *T. gallinae* infection (Chi *et al.* 2013). Subsequent sequencing of this ITS1/5.8S/ITS2 (hereafter ITS region) can identify genetic heterogeneities between isolates, due to the diversity of non-coding ITS sequences (Katiyar *et al.* 1995; Kleina *et al.* 2004). It is possible to gain a higher resolution of strain differentiation by further sequencing the Fe-hydrogenase gene (hereafter *Fe-hyd* region), a common house-keeping gene in amitochondrial protists, allowing the identification of diversity within defined genotypes (Lawson *et al.* 2011b).

Across the literature, there are two commonly occurring genotypes, classified by the ITS region (A and B) which form two distinct branches when incorporated into a phylogenetic tree (Sansano-Maestre *et al.* 2009). Extensive phylogenetic analysis of the Trichomonadidae using the ITS region as a genetic marker by Kleina *et al.* (2004) was the first to identify the pathogenic G7 strain of *T. gallinae*, which falls under genotype A. Since then, isolates with 100% identity to this strain have been identified from a diverse array of species around the world, including UK greenfinches and chaffinches (Robinson *et al.* 2010), pink pigeons (*Columba mayeri*), Madagascar turtle doves (*Streptopelia picturata*) from Mauritius (Gaspar da Silva *et al.* 2007) and mourning doves, a house finch and a Cooper's hawk from the USA (Gerhold *et al.* 2008).

Lawson *et al.* (2011b) assessed the genotypic variation in British avifauna using isolates from post-mortem from 17 species of affected birds found dead, including 11 passerines, two columbids, three birds of prey and an owl. By using samples collected between 2005 and 2009 from a range of sites across England and Wales, they were also able to assess spatio-temporal variation. All of the isolates from UK birds examined in the study, regardless of species, location and year, were genetically identical in the ITS and *Fe-hyd* regions (Lawson *et al.* 2011b). The homologous nature of isolates from over 50 diseased passerines, and the lack of heterogeneity between populations where disease has emerged indicates the presence of a single clonal strain causing the widespread trichomonas outbreak in the UK. Furthermore, isolates from columbids collected before and after the emergence of the disease in finches supported the idea of pathogen spillover from free-ranging columbids rather than genotypic strain variation (Lawson *et al.* 2011b). To provide a comparison with British birds, the *Fe-hyd* gene was also amplified from *T. gallinae* isolated from three columbid species in the Seychelles. Unlike in the British samples, heterogeneity was observed in sequences from the Seychelles columbids, indicating a diversity of strains in Seychelles avifauna (Lawson *et al.* 2011b).

4.2.4 Transmission and virulence of *Trichomonas gallinae*

There are both intrinsic and extrinsic factors that influence the likelihood of parasitic infection becoming established within a population, and the severity of resulting infections. (Behnke *et al.* 2005). Such factors include feeding ecology (influencing exposure to parasites) and genetic variation in both the host and the parasite, affecting susceptibility and virulence respectively (Carius *et al.* 2001; Knudsen *et al.* 2004). *T. gallinae* commonly occurs apathogenically, particularly in its columbid hosts, however there are also known strains which cause mortality in over 75% of cases, indicating variation in the virulence of strains (Gaspar da Silva *et al.* 2007), consistent with that observed in the closely related *T. vaginalis* (Vanáĉová *et al.* 2007). This variation in virulence was first hypothesised over 70 years ago, when Stabler (1948) observed disparity between infection levels and recovery rates amongst pigeons, indicating some strains were more likely to cause severe symptoms

and death. Sansano-Maestre *et al.* (2009) identified isolates from pigeons and birds of prey, and observed that all birds exhibiting macroscopic lesions were infected with the same genotype (genotype A), leading to the hypothesis that there was a connection between this genotype and clinical disease (Sansano-Maestre *et al.* 2009). Support for this comes from a study conducted by McBurney *et al.* (2015) on the spread of trichomonosis in finches in Canadian Maritime provinces. They found that all individuals exhibiting clinical symptoms were infected with the same genotype A: the same clade as the strain identified in at least 11 UK passerine species (Lawson *et al.* 2011b).

As well as variation in the parasite itself, the virulence of a pathogen may also be impacted by other factors, including host immune status, or previous exposure to the pathogen (Carius *et al.* 2001; Gaspar da Silva *et al.* 2007; Tschirren *et al.* 2007). Early evidence for the effect of prior exposure to parasites influencing virulence again came from work by Stabler (1948). In an experimental study, Stabler (1948) inoculated rock doves with *T. gallinae* strains of varying virulence and found that no birds with prior exposure to less virulent strains succumbed to infection with the second, most severe strain, supporting the idea that previous infection acted as an immunization and protected individuals from future infection. However, this is very difficult to assess in wild birds, unless the same birds are recaptured and tested on a regular basis, providing information on rate of recovery from infection and a history of prior infection.

Primary transmission of *T. gallinae* is thought to be direct; passing both horizontally, between adults during courtship, and vertically, from parent to offspring during feeding of crop milk (Bunbury *et al.* 2007). An important alternative transmission route is the passage of infected agents between congregated birds, particularly at feeding stations and water sources, where overcrowding and cross-species contact is believed to facilitate the spread of infection (Altizer *et al.* 2006), as demonstrated by Thomas (2017), who found that both water sources and seed piles were able to carry live *T. galliane* parasites.

The use of garden bird feeders was considered to be a major contributor to the rapid spread of *T. gallinae* during the epidemic, as they encourage birds to

gather at high-densities to feed (Lawson *et al.* 2011a; Laaksonen and Lehikoinen 2013; Lennon *et al.* 2013). In the UK alone, it has been estimated that 15,000 tons of peanuts were fed annually to garden birds through the use of feeding stations (Daszak *et al.* 2000), with a more recent study estimating that almost half of households in the UK (48%) provide supplementary feed for birds in their garden (Davies *et al.* 2009). This encourages a diverse range of bird species to feed at high densities, increasing contact between individuals, both inter- and intra-specifically, and facilitating the horizontal transmission of disease between individuals that would have been unlikely to come into contact under normal foraging habits (Lawson *et al.* 2018). This has increased the prevalence of several avian diseases, including *Salmonella typhimurium* and *Escherichia coli* in British birds, and led to the rapid spread of *Mycoplasma gallisepticum* in the USA (Pennycott *et al.* 1998; Dhondt *et al.* 2007). Lennon *et al.* (2013), observed a higher prevalence of *T. gallinae* infection on farms with supplementary feeding for game birds than at those without, indicating that readily available food increases exposure of individuals to infection and facilitates the spread of disease (Lawson *et al.* 2012; Lennon *et al.* 2013). Similarly, when investigating infection of the purple finch (*Carpodacus purpureus*) and the American goldfinch (*Carduelis tristis*) in Canadian Maritime provinces, McBurney *et al.* (2015) identified *T. gallinae* isolates from an aggregate of bird seed collected from several bird feeders at a site with known cases of finch trichomonosis. Furthermore, a common factor observed in all geographic areas where trichomonosis is emerging, is the occurrence of mortality where birds gather at feeding and watering sites (Robinson *et al.* 2010; McBurney *et al.* 2015).

4.2.5 Parasite co-infection

Parasites live, feed and reproduce within their hosts, making each host individual an ecosystem in itself (Guégan and Hugueny, 1994). It is rare in nature to find hosts infected with a single parasite species only (Pedersen and Fenton, 2007, Knowles *et al*., 2013), rather, it is more common for hosts to experience concomitant infections, where a host's parasite fauna consists of multiple species and strains of parasite occurring within a single individual (Bush *et al*., 1997). Distribution of such co-occurring parasites amongst hosts

is unlikely to be independent of one another and community studies show that often, species co-occurrence deviates either positively or negatively from what would be expected if infections were entirely random (Poulin *et al*., 2000).

Such departure from randomness has numerous potential explanations, both extrinsic and intrinsic to the host (Behnke *et al*., 2005). Extrinsic factors, such as year, season and location, can have an important influence on parasite aggregation patterns, largely by influencing a host's exposure to parasites (Chappell, 1969, Montgomery and Montgomery, 1990, Behnke *et al.*, 2005, Stutz *et al*., 2014). Intrinsic factors, such as host age, sex and genetic predisposition, are also important. For example, age is important as many parasites are long-lived and cause sustained infection. This results in infections accumulating over time, with heavier and more diverse parasite burdens in older individuals (Behnke *et al*., 2001). On the contrary, parasites which cause host mortality would be negatively correlated with age as death of infected hosts would leave only healthier older individuals (Anderson and Gordon, 1982). There is also genetic variation in host resistance to and tolerance of parasite infections, which can impact the levels of co-infection within a host (Carius *et al*., 2001, Stutz *et al.*, 2014).

4.2.6 Identification of infection

Traditionally, *T. gallinae* has been identified via wet-mount microscopy, whereby a swab is taken from the inside of a bird's mouth, crop and oesophagus and suspended in solution on a slide (Cover *et al.* 1994; Bunbury *et al.* 2005; Anderson *et al.* 2009). However, this method had major drawbacks, including a relatively low sensitivity and variable rate of detection of infection (Nathan *et al.* 2015). This method also lacks the ability to separate different strains within the same species: a problem when trying to fully understand the ecology of *T. gallinae* infections, which are known to comprise several strains affecting wild birds.

Advances in molecular technology, combined with the need to detect *T. vaginalis* in humans, have driven the development of more robust diagnostic tools which are transferrable to the study of *T. gallinae*. In order to reliably diagnose subclinical infection of *T. gallinae* from turtle doves, a culture step is

required to allow parasite proliferation from swabs (Dunn *et al.* 2016). The InPouch culture kit was developed as a sensitive method to detect *T. vaginalis,* which is very portable and easy to use in field studies, as well as being highly effective for culturing *T. gallinae,* detecting more than twice as many positive infections than wet-mount microscopy during direct comparison (Bunbury *et al.* 2005). Following incubation of parasite swabs in culture medium, positive infections can be confirmed by polymerase chain reaction (PCR) using primers designed to bind to the ITS ribosomal region, typically utilised as a genetic marker in trichomonads. Subsequent to confirmation of the presence of trichomonads, DNA sequencing can be used to identify the strain of the parasite present.

The majority of studies investigating the strains present in *T. gallinae* infection use Sanger sequencing to identify strains present (Sansano-Maestre et al. 2009; Lawson, Cunningham, et al. 2011; Lawson et al. 2011b; McBurney et al. 2015). A major drawback of this method is it returns a single chromatogram, giving the sequence of just one parasite strain present in the sample. This method is therefore unable to detect co-infection with multiple strains of *T. gallinae* within a single host, resulting in this aspect of this parasite's ecology being largely overlooked, with only one other study assessing the rate of strain co-infection (Thomas *et al.* 2022). I will use high throughput sequencing (HTS) to identify if multiple parasite strains are occurring within a single host, in order to assess the rate of co-infection (Figure 4.1).

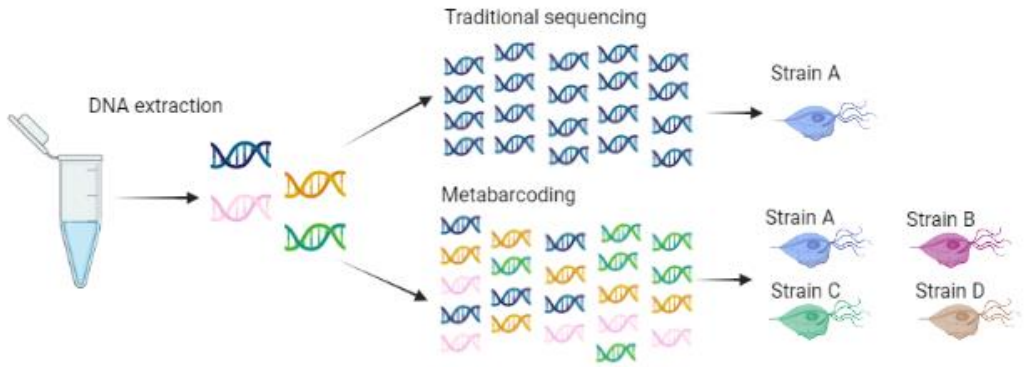

Figure 4.1: Graphic illustrating the process of detecting multiple parasite strains using HTS compared to traditional sequencing methods, such as Sanger sequencing.

Thanks to these advances in molecular technology, researchers are now able to gain much more reliable and detailed information regarding infections of *T. gallinae* in the wild. This approach will be used in this thesis to assess the prevalence of *T. gallinae* in turtle doves from both wintering grounds in Senegal, and breeding grounds in both western Europe (France) and eastern Europe (Hungary). Due to the potential for elevated transmission where individuals gather in high density at feed and water resources, I hypothesize that i) *T. gallinae* prevalence will be lower in areas with less intense farming and larger areas of naturally occurring fallow (Hungary), and ii) *T. gallinae* will be more prevalent in wintering grounds where scarce water forces congregation at watering holes. Furthermore, by sampling birds from a wide geographical range, I aim to answer questions regarding the distribution of *T. gallinae* strains across their range, testing the hypotheses iii) Strains composition of *T. gallinae* will differ between the western and central/eastern flyways iv) Turtle doves sampled in Senegal will have the most varied parasite strain composition, due to mixing of birds from locations across Europe.

4.3 Methods

4.3.1 Sample collection

Samples were collected from wild-caught turtle doves in the field locations detailed in Chapter 2 (2.3.1).

In order to obtain samples to test for *T. gallinae* infection, a sterile swab was used to collect surface tissue in the oral cavity, oesophagus and crop of turtle doves. Swabs were then used to inoculate individually labelled InPouch culture kits (BioMed, USA) which were incubated at 37°C for 7 days to allow parasite proliferation. After incubation, culture medium was removed from the pouch and transferred to a storage tube, where parasite samples were preserved with equal volume of culture medium and 100% ethanol, prior to DNA extraction. Samples were stored at room temperature whilst in the field and frozen at -20°C upon arrival in the UK. For samples collected in the breeding season, this will only have been a maximum of one week at ambient temperature, whereas, given the longer field season and a lack of available cold storage

space at the field site, samples from Senegal will have been stored for up to three months at ambient temperature. Due to the less than optimum conditions, some sample degradation may have occurred, particularly in samples from wintering grounds.

4.3.2 Parasite isolation

Protocols for parasite isolation are adapted from Riley *et al.* (1992) and are as follows: a volume of culture medium was centrifuged at 3200rpm for 5 minutes at 4°C and supernatant discarded. The remaining pellet was washed in 500µl phosphate-buffered saline (PBS) by centrifugation, at 3200 rpm for 5 minutes at 4°C and supernatant discarded. The rinsing stage was repeated to wash the pellet a second time. The pellet was re-suspended in 200µl PBS and stored at -20°C until DNA extraction was carried out.

4.3.3 DNA extraction

DNA was extracted from isolated parasites using two methods, either modified ammonium acetate method (Nicholls *et al.* 2000) or using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) depending on the date of extraction. For the ammonium acetate method, the pellet was obtained from 2.5ml of culture medium, for extraction using DNeasy Blood and Tissue kit, 200-400µl of culture medium was used. DNA extracted in 2018 used the modified ammonium acetate method, whereby isolated parasite samples were centrifuged at 3200rpm for 5 minutes, the supernatant discarded and the pellet retained. 250µl of Digsol buffer (20mM EDTA, 50mM Tris, 120mM NaCl, 1%SDS, pH8.0) and 20µl Proteinase K (Qiagen) were added and samples were incubated overnight at 37°C to allow digestion. Following digestion, 300µl of 4M ammonium acetate was added, mixed by vortexing, and incubated at room temperature for 30 minutes, vortexing every 10 minutes. Samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant transferred into a new tube, discarding the pellet. 1ml 100% ethanol was added to the supernatant to allow precipitation of the DNA. Each sample was vortexed and centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded and 500µl 70% ethanol was added to the DNA pellet before being spun at 13,000 rpm for 5 minutes to wash the pellet. The supernatant was discarded and the

pellet was left to air-dry until no ethanol remained, typically taking 1-2 hours. The DNA pellet was dissolved in 20-50µl of low TE buffer (0.1mM EDTA, 10mM Tris), depending on the size of the pellet, and incubated at 65°C until the DNA was fully resuspended. Extracted DNA was then stored at -20°C. For the Blood and Tissue kit, extraction was carried out following the manufacturer's instructions for extraction from tissue samples. The final elution was in 50µl TE buffer. Two extraction negatives were included for every extraction, whereby all reagents were added but no sample included, in order to detect contamination in extraction buffers.

4.3.4 PCR amplification

4.3.4.1 Confirmation of infection by PCR

Polymerase chain reaction was used to determine the infection status of each sample using the TFR1/TFR2 primer pair (Felleisen, 1997, Table S3.1) were used to amplify the 400bp length target region of the ITS1/5.8S/ITS2 ribosomal region of the genome (hereafter referred to as the ITS region). The PCR recipe used consisted of 1.8µl Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.225µl of 10µM forward and reverse primers (Merck KGaA, Darmstadt, Germany) and 2.25µl nuclease free water, giving a reaction volume of 4.5µl, to which 0.5µl DNA was added. A negative control of molecular grade water, and a positive control known to contain *T. gallinae* DNA was included in each PCR run.

The following touchdown PCR thermal cycling programme was performed using SimpliAmp^{TM} Thermal Cycler (ThermoFisher Scientific, Waltham, USA). The programme consisted of initial denaturing for 15 minutes at 95°C, 11 cycles of 1 minute at 94°C, 30 seconds at 66°C (decreasing by 1°C every cycle until 56°C), 1 minute at 72°C, followed by 24 cycles of 1 minutes at 94°C, 30 seconds at 55°C and 1 minute at 72°C, with a final elongation step of 10 minutes at 72°C. PCR products were electrophoresed though a 1% gel, stained using SYBR®Safe (Thermo Fisher Scientific, Paisley, UK) in 0.5X TBE buffer and visualised using UV light. Presence of *T. gallinae* infection was indicated by the occurrence of a band of the appropriate amplicon size when

visualised. In instances where no amplicon was observed, the sample was run through PCR a second time to confirm the absence of *T. gallinae*.

4.3.4.2 PCR amplification of Fe-hyd region for Sanger sequencing

For samples that had previously tested positive for *T. gallinae,* TrichHydFOR/REV primer pair (Lawson *et al.* 2011b, Table S3.1) was used to amplify the 1000bp region from the hydrogenosomal Fe-hydrogenase gene, (hereafter referred to as *Fe-hyd* region) for further differentiation of parasite strains into subtypes. 10µl reaction volumes consisted of 4µl Multiplex PCR Master Mix, 3.2µl nuclease free water, 0.4µl $MgCl_2^+$, 1µl of 10µM forward and reverse primers (Merck) and 0.4µl DNA, or nuclease free water for one PCR negative control per PCR. Using a SimpliAmp^{TM} Thermal Cycler, the thermal cycling profile was as follows: denaturing for 15 minutes at 95°C, 35 cycles of 45 seconds at 94°C, 30 seconds at 53°C 45 seconds at 72°C, with a final elongation step of 5 minutes at 72°C. PCR products were gel electrophoresed, as described previously to detect successful amplification. In instances where amplification of the *Fe-hyd* region failed, a PCR was run a second time, with the number of cycles increased from 35 to 40.

All samples where *Fe-hyd* was successfully amplified were purified following the manufacturer's instructions, using a QIAquick PCR purification kit (Qiagen). Purified PCR products were gel electrophoresed and sequenced in both directions using Sanger sequencing, by Eurofins Genomics (Eurofins, Ebersberg, Germany).

4.3.4.3 PCR amplification and library preparation of the ITS region for High Throughput Sequencing (HTS)

Each 25µl reaction volume consisted of 10µl Multiplex PCR Master Mix (Qiagen), 9µl nuclease free water, 2.5µl each of 3µM TFR1[f] and TFR2[r] primers (Eurofins), and 1µl template DNA. Both forward and reverse primers were labelled with MID-tags (Multiplex Identifiers) to create unique labels for individual DNA samples, with all samples having a unique MID-tag combination. Each PCR 96-well plate included 12 negative controls (11 extraction negatives and one PCR negative, containing 1µl nuclease free

water), arranged as described in Chapter 2 (2.3.2.1). Replicate samples were also incorporated for quality control purposes, with 10% of the total sample number being included as replicates. PCR amplification and confirmation of successful PCR reaction were carried out following the touchdown PCR and gel electrophoresis protocols described previously.

Each plate of MID-tagged PCR product was combined using a modification of the method described in Chapter 2 (2.3.2.1), based on the maximum DNA concentration per plate, using known concentrations determined by QIAxcel (Qiagen), to produce one equimolar pool of uniquely identifiable samples per 96-well PCR plate. Due to the wide range of concentrations of ITS amplicons (0.18ng/µl – 40.5ng/µl), the original method would have resulted in a large number of samples being excluded from the pool due to insufficient PCR product in samples with a concentration far lower than C_{max}.

For this reason, PCR products were electrophoresed and samples grouped together based on band brightness (faint, medium and strong). The average DNA concentration, per band brightness group, was calculated from the QIAxcel results, and the pooling calculation was carried out again, using the average concentration for strong bands as C_{max}. This gave the volumes to be added for all samples based on band brightness grouping. The average of all sample volumes added to the pool was calculated, and this was the volume added for all negative samples.

Cleaning of the pools and library preparation for Illumina sequencing was conducted as in Chapter 2 (2.3.3), with one modification: in the initial cleaning of the pools using SPRIselect beads (Beckman Coulter, Brea, USA), a ratio of 1:0.9 was used to remove fragments smaller than the desired 420bp. The concentration of DNA in the final pool following cleaning was again measured using a Qubit (24.7ng/µl). The final concentration of DNA from final Qubit was 10.8ng/µl and average library size from final TapeStation was (554bp).

4.3.5 Bioinformatic analysis

Two methods were used for analysis of the ITS region run through HTS. The first is the same as the approach used for metabarcoding and the second approach is more commonly used for analysis of genes with multiple copies,

such as the MHC family. The two methods have a common first step, but then diverge into two separate workflows. The metabarcoding approach is largely the same as that described for diet work in Chapter 2 (2.3.4), with some adjustments to parameters. In the quality-filtering step, the minimum sequence length was set to 380bp. During the demultiplexing stage, no primer mismatches were permitted, in order to prevent inflation in the number of OTUs generated and lead to the retention of higher quality reads, which are more likely to represent true variation (Thomas 2017). At the BLASTn stage, where ITS samples were assigned their closest match on GenBank, the minimum identity was set to 97% to remove poor quality matches.

4.3.6 Cleaning data set

Three criteria were used to clean the data in order to remove erroneous reads resulting from contamination, tag jumping, artefacts and possible polymorphic reads. The first two stages of data cleaning, removal of maximum contamination and artefacts created during PCR and sequencing, were completed as described in Chapter 2 (2.3.5).

In short, to remove maximum contamination, the highest read count detected in blanks (negative controls and unused combinations) for each taxon was assigned as maximum contamination, and only read counts exceeding this threshold were retained in the data set. To remove artefacts, whilst accounting for variation in read depth between samples (in this case, the read depth per sample range from 662-12,506), a proportional approach was adopted, looking at the relative abundance of zOTUs within a sample. To remove zOTUs with a low relative abundance, any zOTUs occurring at a proportion below 0.003 were excluded, as per Taberlet *et al.* (2018). One sample, following data cleaning, had a total of only 20 reads, so was excluded from further analysis.

The ITS region (ITS1/5.8s/ITS2) is the genetic marker typically used to identify trichomonads. The genomic region encodes the small 5.8S rRNA subunit (SSU) which is relatively conserved across genera, with only variation in a few nucleotides, whereas the flanking internal transcription spacers (ITS1 and ITS2) are non-coding, thus are more diverse (Katiyar *et al.* 1995). This marker has been widely used in studies of *Trichomonas* species (Gaspar da Silva *et*

al. 2007; Gerhold *et al.* 2008; Sansano-Maestre *et al.* 2009; Lawson, Robinson, *et al.* 2011; Marx *et al.* 2017), and has been shown to be capable of differentiating between *T. gallinae* strains. In all known animal genomes, rRNA occurs with multiple copies per cell, with some level of polymorphism between copies (Porazinska *et al.* 2010). Bearing in mind this polymorphism, slight variation in sequences produced by HTS may be produced by a different copy of an rRNA gene (Porazinska *et al.* 2010). In more traditional sequencing practices, these polymorphisms are not generally detected, as sequencing of the PCR product from a target locus typically results in a consensus sequence for each individual sample (Porazinska *et al.* 2010); however, when using HTS, all reads generated during sequencing are returned. Porazinska *et al.* (2010) investigated the repeatability of using HTS to analyse nematode mock communities, also targeting SSU rRNA. By using mock communities, all expected sequences were known, and about half of the reads generated in the dataset differed from the presumed consensus sequence by at least 1bp. Without extensive use of technical repeats, these polymorphisms cannot be distinguished from artefacts, but are likely to account in part to the high number of zOTUs in this run differing from known sequences.

After removing maximum contamination and low proportion reads from samples, there were still reads present in very low numbers, indicating the retention of some artefacts or polymorphic gene copies. In order to mitigate this, for the third clean up step, a cumulative depth curve was generated for each sample using the top 10 variants occurring in each sample (Lighten *et al.* 2014; Thomas 2017). Based on the assumption that artefacts occur in much lower numbers than genuine reads, the presence of a clear inflection point (as seen in Figure 4.2a) indicated the divide between a true sequence and an artefact, any reads occurring beyond this inflection point would be considered artefacts and not included in further analysis (Lighten *et al.* 2014; Thomas 2017). This was carried out for all samples to determine the percentage of reads accounted for beyond this inflection point. In some instances, determining an inflection point was more subjective (Figure 4.2b), where there was one stronger inflection point, followed by a less clear one. Finally, in a small number of cases, it was not possible to discern a clear inflection point (Figure 4.2c). In instances where no clear inflection point could be determined,

Lighten *et al.* (2014) discarded the sequence as poor quality. In this study, such instances may be a result of infection with multiple strains, thus the maximum percentage accounted for by a clear inflection point was used as the threshold for all samples to determine co-infection.

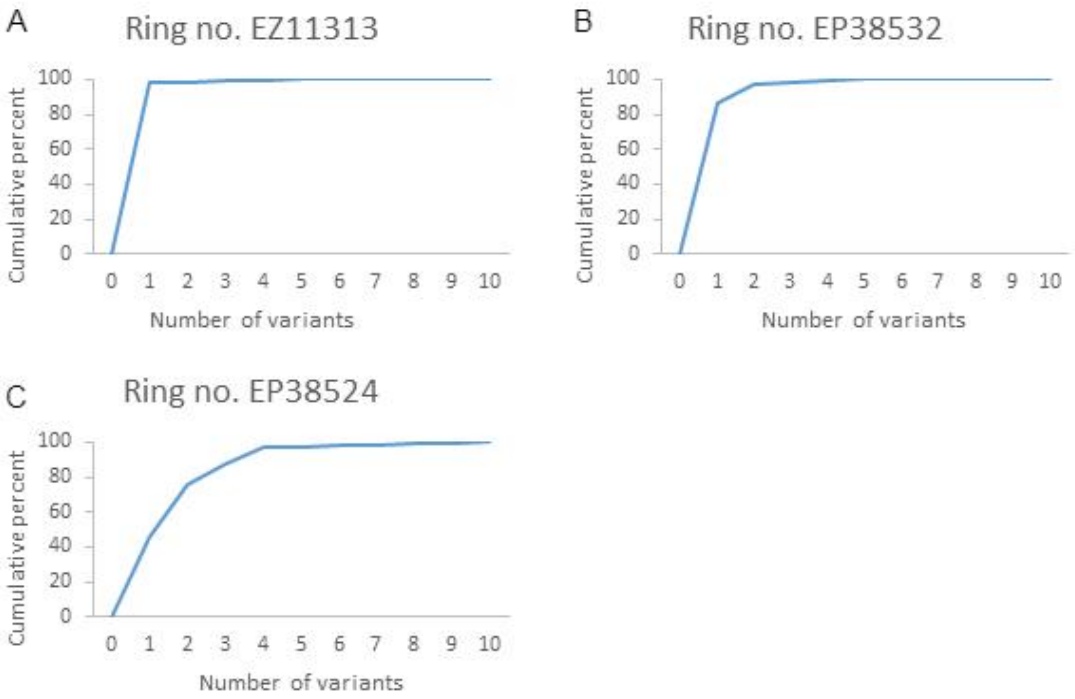

Figure 4.2: Examples of the cumulative proportion curves showing sequence depth accounted for by variants in a sample. A) 98.3% of sequences are from a single variant, creating clear inflection point so additional variants accounting for remaining 1.7% variation are removed as artefacts. B) two possible inflection points, so more subjective: if using point at 1 variant, 89.5% of variation accounted for by one variable, therefore threshold is 10.5%, if using point at 2 variants, threshold is 3%; c) cannot discern clear inflection point.

Different thresholds were tested for cleaning steps two and three (see Appendix 3, 3.3). Analyses were conducted on data sets generated using different thresholds to assess whether threshold values effected the ecological inferences taken from data. The final thresholds used were as follows: step 2) frequency of occurrence greater than 0.003 per sample; step 3) zOTUs accounting for a minimum of 7% of reads per sample.

For the MHC approach, the first step of the metabarcoding pipeline was followed to trim, align and quality check the data, using FastP (Chen *et al.* 2018). The desired sequence length was defined (380bp) and the resultant fasta file contained all operational taxonomic units (OTUs) identified in the sequencing run. By inputting the merged fasta file from this alignment step, as well as sequences for each primer pair, sequences were demultiplexed using jMHC (Stuglik *et al.* 2011). Barcodes and priming sequences were also removed and the output file contained variant read depths quantified among amplicons. As in the metabarcoding approach, data were cleaned by identifying the maximum amount of contamination occurring in the negatives and unused sequences for each variant, and subtracting this number across all samples for that variant.

Following the demultiplexing and cleaning of data, a custom Microsoft Excel macro was used to distinguish between biologically accurate sequences and artefacts created as a result of PCR and sequencing errors (Lighten *et al.* 2014). This distinction is based on sequencing depth, and made following the 'Degree of Change' approach. As with the third stage of HTS data cleaning, cumulative depth curves were used to identify artefacts to be removed from analysis.

4.3.7 Strain identification and phylogenetic analysis

For the ITS region, sequences were obtained from the output of the Unoise3 stage of bioinformatics analysis of HTS data. For the *Fe-hyd* region sequenced by Sanger sequencing, forward and reverse sequences were manually checked for errors against chromatograms, assembled to form one consensus sequence and trimmed using BioEdit version 7.0.5.3 (Hall 1999). To determine strains from both ITS and *Fe-hyd*, sequences were compared with NCBI Blast database. Currently, any deviation from known strains of *T. gallinae* are considered new strains, therefore only sequences with 100% identity to GenBank results could be assigned a strain. As nomenclature for *T. gallinae* varies between studies, nomenclature used was based on a table compiled of all strains available on GenBank at the tim of analysis (June 2020) (Thomas *et al.* 2022).

Phylogenetic trees were constructed in order to visualise evolutionary relationships of *T. galliane* strains. Representatives of all reported sequences for each region were downloaded from GenBank (Table 4.1, Table 4.2). Multiple alignment of ITS and *Fe-hyd* sequences was carried out with sequences from this study, plus and 28 and 37 sequences for ITS and *Fe-hyd*, respectively using clustalW in Molecular Evolutionary Genetics Analysis (*MEGA)* software*,* version 6 (Tamura *et al.* 2013). Following alignment, sequences were trimmed to 223bp (ITS) and 591bp (*Fe-hyd*). *T. vaginalis* was included as an outgroup in both trees, and *Tetratrichomonas gallinarum* was also included for ITS, as per (Thomas 2017).

The BEAST package, v1.10.4 (Suchard *et al.* 2018) was used to produce phylogenetic trees. The most appropriate nucleotide substitution models were chosen for ITS and *Fe-hyd* separately, using jModeltest (Posada 2008), based upon Baysian Information Criterion (BIC) scores. General Time Reversal plus Gamma was defined for ITS and equal-frequency Tamura-Nei plus gamma was defined for *Fe-hyd*. Priors were set in BEAUTi v1.10.4 using a strict clock and Yule speciation process (as per Quillfeldt *et al.* 2018; Thomas *et al.* 2022). Bayesian phylogenetic trees were constructed in BEAST v1.10.4 using Markov Chain Monte Carlo simulations. Chain length was set at 25,000,000, with a sampling frequency of 1000 (as per Thomas *et al.* 2022), and an effective sampling size >200 was confirmed using Tracer v1.7.1 (Rambaut *et al.* 2018). Consensus trees were constructed using TreeAnnotator v1.10.4, with a burn-in of 10%.

4.3.8 Statistical analysis

All statistical analyses were carried out in R, version 4.0.1 – “See Things Now” (R Core Team 2020).

4.3.8.1 Spatio-temporal strain variation

Generalised linear models (GLMs) were used to test for differences in the prevalence of *T. gallinae* strains between years and countries. As samples were collected from Senegal across three consecutive winters, the effect of year was investigated in the wintering grounds. Difference in overall

prevalence of *T. gallinae* across the three years was tested by GLM, with presence/absence of the *T. gallinae* infection was the dependent variable, modelled with a binomial distribution and logit link function, and year sampled included as the predictor variable. Tukey's post-hoc tests were used to evaluate pairwise differences between sampling years, using the *multcomp* package in R (Hothorn *et al.* 2008). To investigate trends in *T. gallinae* infection in greater detail, strains present within hosts were analysed, again using GLMs with binomial distribution and logit link function. The effect of year on the prevalence of individual strains of *T. gallinae* present in Senegal was examined. Each parasite strain was modelled separately, as the dependent variable, and year included as a predictor variable. Tukey's post-hoc tests were used, as above, to evaluate pairwise differences.

Due to sampling methods, with different study sites being sampled in different years, year and country are not independent, therefore could not be modelled as separate variables. They were therefore combined to make a single factor 'sampling season', consisting of year and country (Table 4.3). Further GLMs were run to incorporate all sampling locations. Four GLMs, with strain as the dependent variable, and sampling season as the single predictor variable, were constructed and pairwise comparisons conducted as above. The same was done for the four *Fe-hyd* subtypes occurring in more than one sample, with the modification of modelling *Fe-hyd* subtype as the dependent variable to assess the effect of season on subtype prevalence.

4.3.8.2 Effect of T. gallinae infection on host condition

The effect of infection with *T. gallinae* on host body condition was also considered, in relation to both the absence or presence of infection, and the strain(s) present. The body condition index was calculated is described in Chapter 2 (2.3.6.2), based on residual variation not explained by the correlation between weight and wing length, taking into account the time of day birds were caught. Considering all birds used in this study, regardless of infection status, condition was compared across seasons using an ANOVA, modelling condition as the dependent variable, and season the predictor

variable. Pairwise comparisons of mean condition from all seasons were conducted using Tukey's post-hoc test. Condition was incorporated into GLMs to determine the effect of the infection with *T. gallinae* on host condition using "infection status", reflecting the presence or absence of *T. gallinae*. A GLM was constructed, starting with the most complex model containing body condition as the dependent variable and infection status, sampling season and the interaction between the two as fixed variables, using Gaussian family and identity link function. Model simplification was carried out using the 'step' command in base R following stepwise removal of terms based on the Akaike Information Criterion (AIC).

The effect of infection with individual strains on host body condition was also considered. GLMs were produced for each of the four dominant strains individually. Models were constructed as above, but presence/absence of individual strains were included instead of 'infection status' as a fixed variable. Again, stepwise removal of terms based on AIC was used to identify the minimum sufficient model.

4.3.8.3 Co-infection

In order to analyse co-infection of individuals with multiple strains of *T. gallinae* (n=10 strains) the 'cooccur' function in the R package *cooccur* (Griffith *et al.* 2016). Using binomial data, cooccur tests whether co-occurrences of particular strains are correlated or random, and assesses the direction of correlation, based on a probabilistic model using the overall prevalence of each strain within the population (Griffith *et al.* 2016). The model assesses the expected co-occurrence of all possible strain pair combinations, and only those expected to co-occur more than once, are analysed, comparing observed co-occurrence with expected. To assess whether any patterns detected were an artefact of pooling multiple study sites, co-occur analysis was also carried out on a subset of just birds sampled in Senegal. The effect of multiple strain infection on the condition of individuals was tested using a GLM, modelling condition as the dependent variable and number of strains infecting the host as the predictor variable, using Gaussian family and identity link function.

Table 4.1: Accession codes of one representative of all reported strains of *T. gallinae* on GenBank, including host species, country of origin, sequence length and reference, used for assigning strains to sequences recovered from this study's phylogenetic analysis

Accession number	Host	Strain ID	Location	Length (bp)	Reference
KC215388	Band-tailed pigeon (*Patagioenas fasciata monilis*)	CA005639	USA	372	Girard *et al.* 2014
KX459511	European turtle dove (*Streptopelia turtur*)	GEO	Italy	346	Marx *et al.* 2017
MN587098	European turtle dove	GEO-TD	Senegal	323	Thomas *et al*.2022
KX266918	Unspecified Pigeon sp.	MR14	Iran	314	Rajabloo *et al*. unpubl.
KX844987	European turtle dove	Tcl-1	Malta	362	Marx *et al.* 2017
KT869152	Canary (*Serinus canaria*)	Tri-IR-13	Iran	372	Ghorbani *et al*. unpubl.
MT720718	European turtle dove	Ttl-TD	Senegal	214	Thomas *et al.* 2022
FN433474	Rock dove (*Columba livia*)	Type II	Austria	296	Grabensteiner *et al.* 2010
KX459514	European turtle dove	Type IIIa±	Italy	346	Marx *et al.* 2017
KX459484	European turtle dove	Type IIIb±	Spain	346	Marx *et al.* 2017

KX844989	European turtle dove	Type IIIc±	Malta	347	Marx *et al.* 2017
EU881911	Rock dove	Type A	Spain	375	Sansano-Maestre *et al.* 2009
EU215368	Broad-winged hawk (*Buteo platypterus*)	Type B	USA	337	Gerhold *et al.* 2008
EU215362	Rock dove	Type C	USA	357	Gerhold *et al.* 2008
KX459513	European turtle dove	Type C/V/N	Italy	359	Marx *et al.* 2017
EU215359	Common ground dove (*Columbina passerine*)	Type G	USA	376	Gerhold *et al.* 2008
EU215360	White-winged dove (*Zenaida asiatica*)	Type H	USA	351	Gerhold *et al.* 2008
EU215361	White-winged dove	Type I	USA	369	Gerhold *et al.* 2008
EU215365	Mourning dove (*Zenaida macroura*)	Type J	USA	345	Gerhold *et al.* 2008
EU215367	Band-tailed pigeon	Type K	USA	322	Gerhold *et al.* 2008

EU215366	Cooper's hawk (*Accipiter cooperii*)	Type L	USA	326	Gerhold et al., (2008)
KX459483	European turtle dove	Type P	Spain	360	Marx *et al.* 2017
KX459510	European turtle dove	Type Q	Italy	350	Marx *et al.* 2017
FN433478	Bearded vulture (*Gypaetus barbatus*)	Type VI	Austria	298	Grabensteiner *et al.* 2010
U86615	Human (*Homo sapien*)	*T. tenax*	USA	368	Felleisen (1997)
AY871048	Human	*T. vaginalis*	China	391	Xiao et al. 2006

Table 4.2: Accession codes of one representative of all reported strains of *T. gallinae* on GenBank, including host species, country of origin, sequence length and reference, used for assigning strains to sequences recovered from this study phylogenetic analysis. ± Sequences excluded from phylogenetic trees as too short to include in alignment

Accession number	Host	Strain ID	Location	Length (bp)	Reference
JF681137	Seychelles blue pigeon (*Alectroenas pulcherrimus*)	1	Seychelles	901	Lawson *et al.* 2011
JF681138	Seychelles blue pigeon	4	Seychelles	901	Lawson *et al.* 2011
JF681139	Seychelles blue pigeon	5	Seychelles	901	Lawson *et al.* 2011
JF681140	Seychelles blue pigeon	7	Seychelles	901	Lawson *et al.* 2011
JF681142	Zebra dove (*Geopelia striata*)	12	Seychelles	901	Lawson *et al.* 2011
KC529660	Eurasian sparrowhawk (*Accipiter nisus*)	A1.1	UK	881	Chi *et al.* 2013
KC962158	Wood pigeon (*Columba palumbus*)	A1.2	UK	803	Chi *et al.* 2013
KC529661	Wood pigeon	A1.3	UK	927	Chi *et al.* 2013
JF681141	Malagasy turtle dove (*Nesoenas picturatus*)	A2	Madagascar	901	Lawson *et al.* 2011

KC660127±	Band-tailed pigeon (*Patagioenas fasciata monilis*)	BTPI1	USA	570	Girard *et al.* 2014
AF446077	Rock dove (*Columba livia*)	C1	USA	1109	Voncken *et al.* 2002
KP900032	Booted eagle (*Aquila pennata*)	C2.1	Spain	932	Sansano-Maestre *et al.* 2016
KC529663	Eurasian collared dove (*Streoptopelia decaocto*)	C3	UK	591	Chi *et al.* 2013
KC529662	Wood pigeon	C4	UK	803	Chi *et al.* 2013
KP900040	Rock dove	C5	Spain	932	Sansano-Maestre *et al.* 2016
KP900041	Booted eagle	C6	Spain	932	Sansano-Maestre *et al.* 2016
KP900034	Eurasian collared dove	C7	Spain	932	Sansano-Maestre *et al.* 2016
KY569256	Rock dove	C8	UK	796	Alrefaei *et al.* unpubl.
MT418241	European turtle dove (*Streptopelia turtur*)	C8-TD	France	915	Thomas *et al* 2022

KY569258	Socorro dove (*Zenaida graysoni*)	C10	UK	796	Alrefaei *et al.* unpubl.
MT418239	European turtle dove	C11-TD	France	915	Thomas *et al* 2022
KC529663	Eurasian collared dove	C11-TD/C3§	UK	591	Chi *et al.* 2013
KY569260	Rock dove and Eurasian sparrowhawk	C12	UK	796	Alrefaei *et al.* unpubl.
KJ184168	Purple finch (*Carpodacus purpureus*)	Fe-hyd2	Canada	901	McBurney *et al.* 2015
KJ184171	Rock dove	Fe-hyd5	Canada	901	McBurney *et al.* 2015
KX894542	Rock dove	MR2	Iran	921	Rajabloo *et al*. unpubl.
KX894543	Rock dove	MR14	Iran	921	Rajabloo *et al*. unpubl.
KX894546	Rock dove	MR22	Iran	921	Rajabloo *et al*. unpubl.
KX894551	Rock dove	MR30	Iran	910	Rajabloo *et al*. unpubl.
KX894552	Rock dove	MR104	Iran	910	Rajabloo *et al*. unpubl.
MT418250	European turtle dove	T1-TD	Senegal	915	Thomas *et al* 2022
KY675299	European turtle dove	T2-TD	Malta	934	Marx *et al.* 2017

4.4 Results

4.4.1 Prevalence and DNA sequence identity

A total of 197 turtle doves were tested for *T. gallinae* infection across five sampling seasons, with T*. gallinae* isolates being recovered from 62.94% of birds sampled. Prevalence was calculated for each sampling season, ranging from 50% (France, spring 2018) to 82.5% (Senegal, winter 2017/2018) (Table 4.3), and all birds sampled were asymptomatic. The number of DNA sequences recovered from positive samples is detailed in Table 4.3.

A total of 112 *T. gallinae* isolates were successfully sequenced at the ITS region using HTS. Sequencing depth per sample ranged from 662 to 12,506 reads, and sequence length was 323-325bp. Genotype repeatability within the Illumina sequencing run was 100%. For strain identification, ITS sequences were compared to known sequences on GenBank. Six sequences identified in this study matched existing strains on GenBank, with 100% identity and query cover (Table 4.4, Figure 4.3: Maximum Clade Credibility Tree based on an alignment of the ITS ribosomal region of *Trichomonas gallinae* (256bp).). There were four dominant strains identified (Tcl-1, Type C, GEO and Type IIIc), which together accounted for 92.1% of strain identifications in this study. A further four sequences that were retained after data cleaning failed to match known ITS sequences, thus were considered new strains and named based on their closest genetic matches (Table 4.4). One sequence, hereafter GEO-TD2, exhibited a 96.3% similarity to GEO (GenBank accession KX459511). This result from HTS was confirmed by Sanger sequencing the sample from a bird carrying this strain. Whilst the sequence obtained from Sanger sequencing was shorter than that from HTS (242bp and 323bp, respectively), the Sanger sequence was a 100% match to the sequence obtained through HTS, supporting it as a potential new strain. The remaining sequences representing possible new strains were named Type IIIc-TD, which exhibited 99.69% similarity to Type IIIc (GenBank accession KX844989); Tcl-TD, which exhibited 98.77% similarity to type Tcl-1 (GenBanks accession KX844987) and GEO-TD3 which exhibited 99.38% similarity to type GEO (GenBanks accession KX459511). However, none of these were the dominant strain in their respective hosts, therefore could not be confirmed by Sanger sequencing.

Furthermore, when aligned and trimmed to produce phylogenetic trees, GEO-TD3 was 100% match to GEO (GenBank accession code KX459511). Of the strains identified, six only occurred in a very small number of birds (n≤5, Table 4.4), therefore only the dominant strains (GEO, Type C, Type IIIc and Tcl-1) were considered in all models.

The *Fe-hyd* region was sequenced using Sanger sequencing with varied success, with consensus sequences obtained ranging from 283bp – 1030bp. Due to poor sequencing quality, any sequences below 570bp in length were not included, resulting in a total of 40 sequences analysed. 570bp was selected as a cut-off as it is the minimum length of *Fe-hyd* sequence uploaded to GenBank (KC660127). When compared on GenBank, sequences identical to four previously reported subtypes were identified from this study, and seven potential new subtypes identified (Table 4.5, Figure 4.4). Of the new subtypes identified, three were within the Type C strain, hereby labelled C6-TD, C7-TD and C7.2-TD, one was within the strain Tcl-1, hereafter T3-TD and three were within the GEO strain, hereafter 12-TD, MR14/22-TD and MR104-TD.

C6-TD was the only subtype to occur in more than one individual sampled (n=6). Its occurrence in four birds from France and two from Senegal indicates that this is a reliable new sequence. All other new subtypes only occurred in a single bird sampled: C7-TD from Hungary, C7.2-TD from Senegal, T3-TD, 12-TD, MR22/14-TD and MR104-TD all from France. All of these samples were re-sequenced to confirm subtype identity, but ideally identification from further sampling would increase confidence in the subtype identity

Table 4.3: Number of birds sampled, percentage prevalence of *T. gallinae* and number of ITS and *Fe-hyd* sequences recovered per country and year sampled. Samples were collected from November to March in Senegal, hence two years per sampling season.

Country	Year	Sampling season	Total N sampled	N positive	% infected	N ITS sequences	N *Fe-hyd* sequences
Senegal	2015/2016	Winter 1 (W1)	19	10	52.63	10	0
Senegal	2016/2017	Winter 2 (W2)	36	20	55.56	17	7
Senegal	2017/2018	Winter 3 (W3)	63	52	82.54	46	12
France	2018	Spring 1 (S1)	66	33	50	30	20
Hungary	2019	Spring 2 (S2)	13	9	69.23	9	3
Total			**197**	**124**	**62.94**	**112**	**42**

Table 4.4: Accession codes of existing strains on GenBank sequenced at the ITS region assigned to sequences from HTS and potential new strains identified (marked with *). Sequences marked ± could not be included in phylogenetic trees as sequence variation occurred outside 256bp region used to formulate phylogenetic trees, therefore was lost when aligned trimmed.

Strain	Accession code	Country (in this study)	n samples	Reference
Tcl-1	KX844987	France, Hungary, Senegal	45	Marx *et al.* 2017
Type C	EU215362	France, Hungary, Senegal	30	Gerhold *et al.* 2008
GEO	KX459511	France, Hungary, Senegal	26	Marx *et al.* 2017
Type IIIc	KX844989	France, Hungary, Senegal	27	Marx *et al.* 2017
GEO-TD	MN587098	Hungary, Senegal	5	Thomas *et al.* 2022
ESWD	KY767988	Senegal	1	Peters (*unpublished*)
GEO-TD2*	XXXXXX	Senegal	2	This study
Type IIIc-TD*	XXXXXX	Senegal	1	This study
GEO-TD3*±	XXXXXX	Senegal	1	This study
Tcl-TD*	XXXXXX	Senegal	1	This study

Table 4.5: Accession codes of existing subtypes on GenBank sequenced at the *Fe-hyd* region, assigned to sequences from Sanger sequencing and potential new subtypes identified (marked with *). Sequences marked ± could not be included in phylogenetic trees as sequences were too short when aligned.

Strain	Accession code	Country (this study)	n samples	Reference
C8-TD	MT418241	France, Senegal	11	Thomas *et al.* 2022
C9-LD	*Unpublished*	Senegal	1	Thomas *et al.* 2022
T1-TD	MT418250	France, Senegal	8	Thomas *et al.* 2022
T2-TD	KY675299	France, Hungary, Senegal	8	Marx *et al* 2017
T3-TD*	XXXXXX	France	1	This study
C6-TD*	XXXXXX	France, Senegal	6	This study
C7-TD*	XXXXXX	Hungary	1	This study
C7.2-TD*	XXXXXX	Senegal	1	This study
12-TD*	XXXXXX	France	1	This study
MR22/MR14-TD*±	XXXXXX	France	1	This study
MR104-TD*±	XXXXXX	France	1	This study

4.4.2 *Trichomonas gallinae* phylogeny

The phylogenetic tree for the ITS region was comprised of four distinct clades with high bootstrap support (Figure 4.3). The first clade contains the Type A (lethal strain), B, C, C/V/N, D and E, as well as two uncharacterised strains, Tri-IR-13 and CA005639. Of these strains, only Type C occurred in the study. The next group contained a number of similar strains, occurring in different columbid species: GEO, GEO-TD, GEO-TD2, GEO-LD. These are sister taxa to Type P and again a number of similar strains occurring in different columbids: Tcl-I, Tcl-BBWD, Tcl-TD, Tcl-LD. Within this clade, seven strains were identified in this study (Figure 4.3, Table 4.4). The third group consisted of *T. vaginalis*, and Types G, H, I, J, K and L, none of which occurred in this study. The final group consists of three Type III strains, one of which was identified in this study, WQR-Env, an uncharacterised strain isolated from a water source in Senegal and MR14. Type Q was not included in any of the clades, appearing as an outgroup, separate to *Tetratrichomonas gallinarium.*

The *Fe-hyd* phylogenetic tree had three groups with high bootstrap values. The first group contained four unclassified subtypes, MR 2, 14, 22 and 104, one subtype isolated from a zebra dove (*Geopelia striata*) in the Seychelles, 12, and a subtype identified in this study, in Senegal, with 12 as its closest match on GenBank, 12-TD. The second group consists of four subtypes, T1,2 and 3-TD, NT1-BBWD, three of which occurred in this study, with one being newly identified (Figure 4.4, Table 4.5) . The final group is the largest, containing predominantly subtypes A and C. There is strong support (bootstrap value 0.99) for Type A forming a separate sub-clade, but support for Type C as a separate sub-clade is weaker (bootstrap value 0.41).

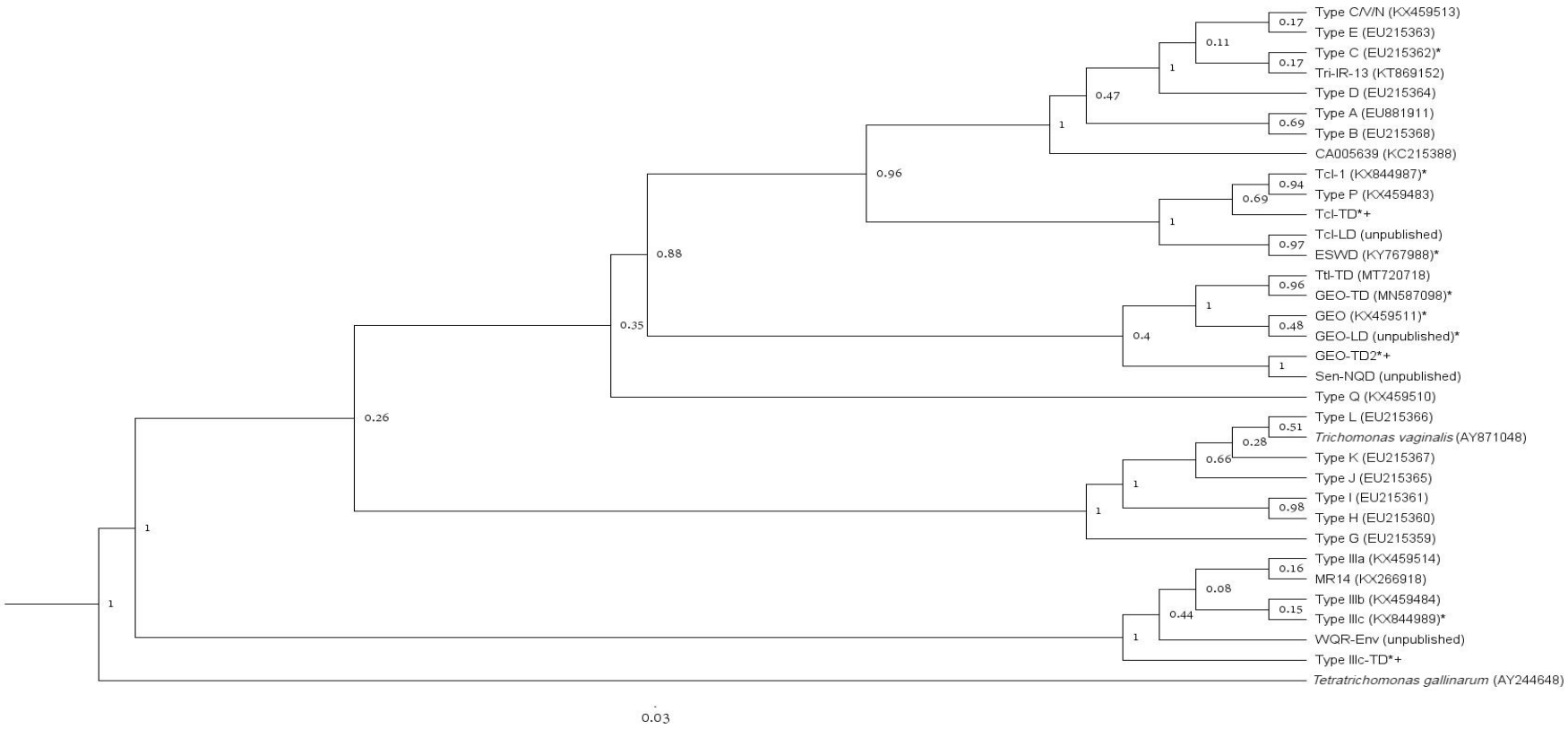

Figure 4.3: Maximum Clade Credibility Tree based on an alignment of the ITS ribosomal region of *Trichomonas gallinae* (256bp). Constructed using Markov Chain Monte Carlo using BEAST v1.10.4. Support of branches is calculated using a bootstrap of 1000 repeats and is presented as a proportion. Representatives of all sequences, of sufficient length, from GenBank are included and labelled with accession number.

* Previously reported sequences identified in this study; + Newly reported sequences from this study.

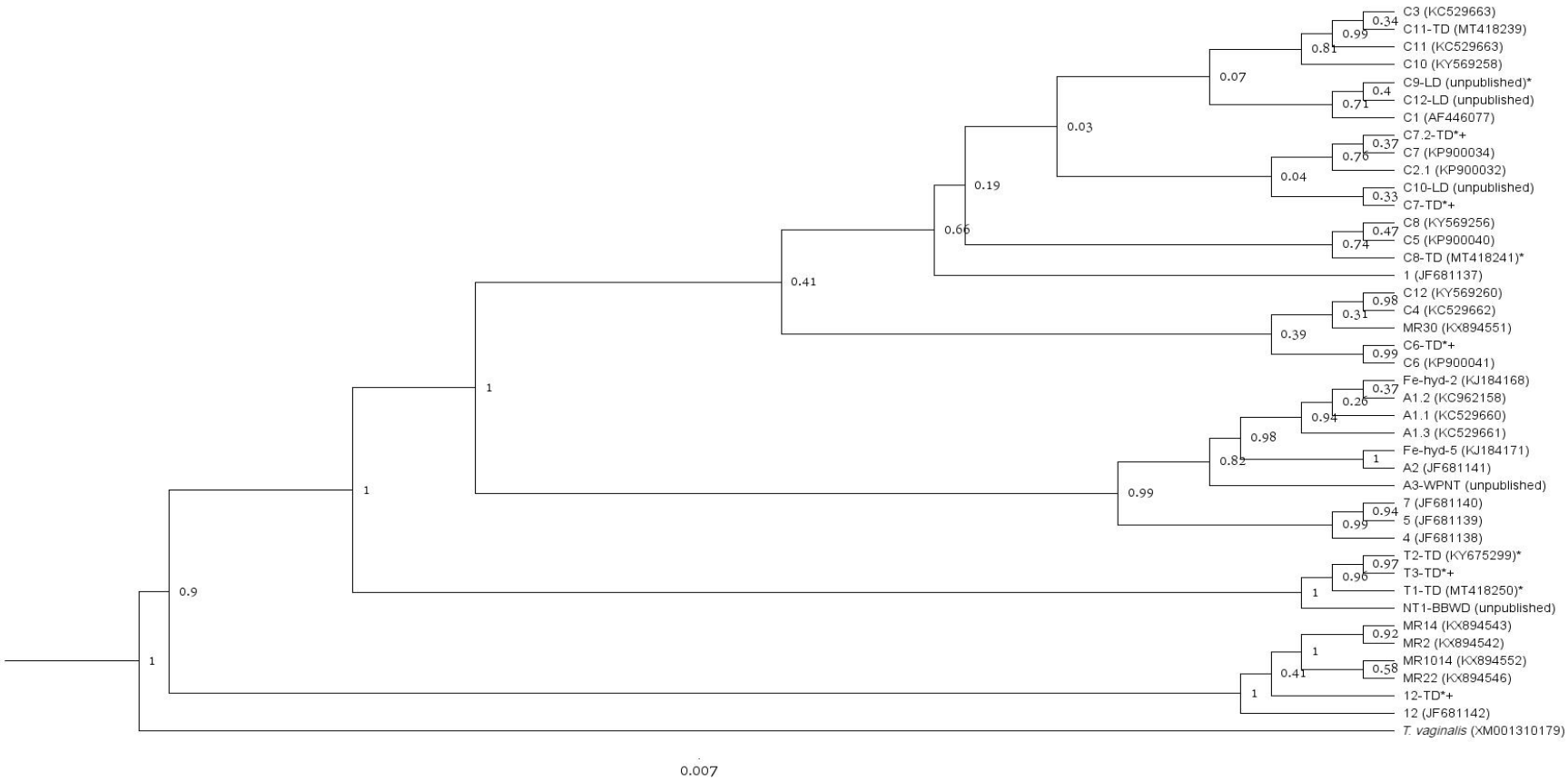

Figure 4.4: Maximum Clade Credibility Tree based on an alignment of the *Fe-hyd* ribosomal region of *Trichomonas gallinae* (591bp). Constructed using Markov Chain Monte Carlo using BEAST v1.10.4. Support of branches is calculated using a bootstrap of 1000 repeats and is presented as a proportion. Representatives of all sequences, of sufficient length, from GenBank are included and labelled with accession number.

* Previously reported sequences identified in this study; + Newly reported sequences from this study.

4.4.3 Spatiotemporal variation in *Trichomonas gallinae* strain prevalence

Temporal variation in *T. gallinae* strain community composition was assessed across three consecutive winters in Senegal. There was a significant difference in overall prevalence of *T. gallinae* across years (GLM: LRT_2 = 11.066, p = 0.004), with rate of infection being significantly higher in 2017/2018 than in either 2015/16 or 2016/17 (Figure 4.5). Prevalence of strains was also not consistent across years, and level of variation differed between ITS haplotypes (Figure 4.6). Within the dominant strains, year had a significant effect on the prevalence of GEO (p=0.007) and Type IIIc (p<0.001), but no significant effect of year was observed in Tcl-1 or Type C (Table 4.6). In the rarer strains, year had no significant effect on GEO-TD.

GEO was at observed at a relatively low prevalence in the winters of 2015/16 (N=9) and 2016/17 (N=17) (11% and 6%, respectively), increasing to a prevalence of 39% in the winter of 2017/18 (N=46). A similar trend was observed in Type IIIc, with 11% prevalence in 2015/16, complete absence of detection in 2016/17 and 37% prevalence in 2017/18. The prevalence of Type C declined across the years, but not significantly. Tcl-1 was the dominant strain in both 2016/17 (65% prevalence) and 2017/18 (43% prevalence). Despite an overall significant effect in two strains (GEO and Type IIIc), there were no significant differences detected in pairwise comparisons between sampling years within each strain.

When looking at all three countries sampled, year and country were combined to make a single independent variable ‘sample season’. Sample season had a significant effect on the prevalence of GEO (p<0.05), Type C (p<0.05) and Type IIIc (p<0.01), but not on Tcl-1 (Table 4.7). Sampling season did not have a significant effect on any of the rare strains identified. When looking at pairwise comparisons between seasons, the only significant result was the comparison between Senegal 2017/18 (W3) and France 2018 (S1) for Type C (W3-S1, coefficient = -1.63, p<0.05), where infection with Type C was significantly higher in France than Senegal. These two years represent the highest (France 2018, 43%, N=30) and lowest (Senegal 2017/18 13%, N=46) prevalence of Type C recorded in this study (Figure 4.7, Figure 4.8).

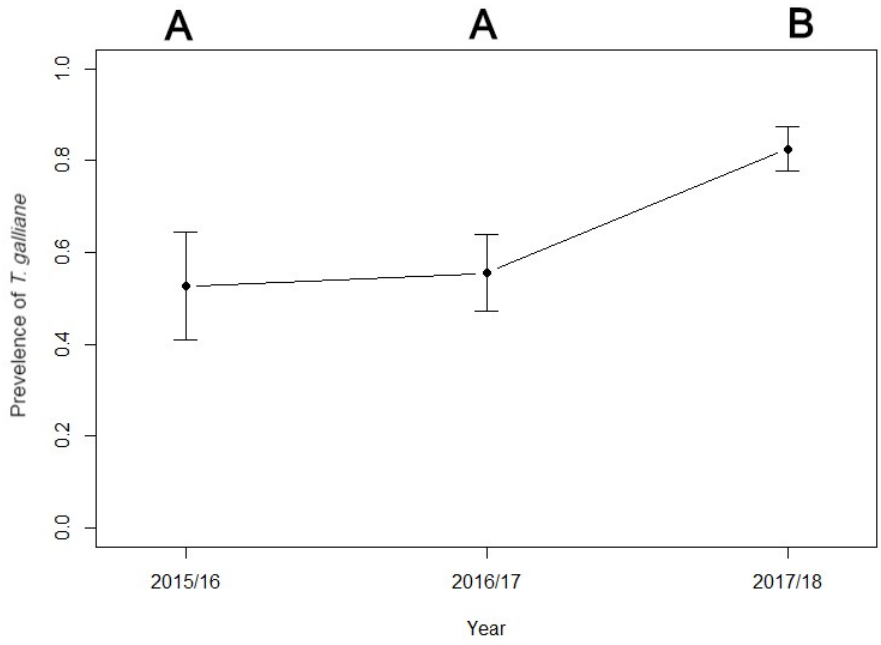

Figure 4.5: Difference in prevalence of *T. gallinae* across sampling years in Senegal. Dots represent prevalence of infection, ± S.E. Letters above indicate where significant differences lie when pairwise comparisons tested with Tukey's post-hoc. N=118

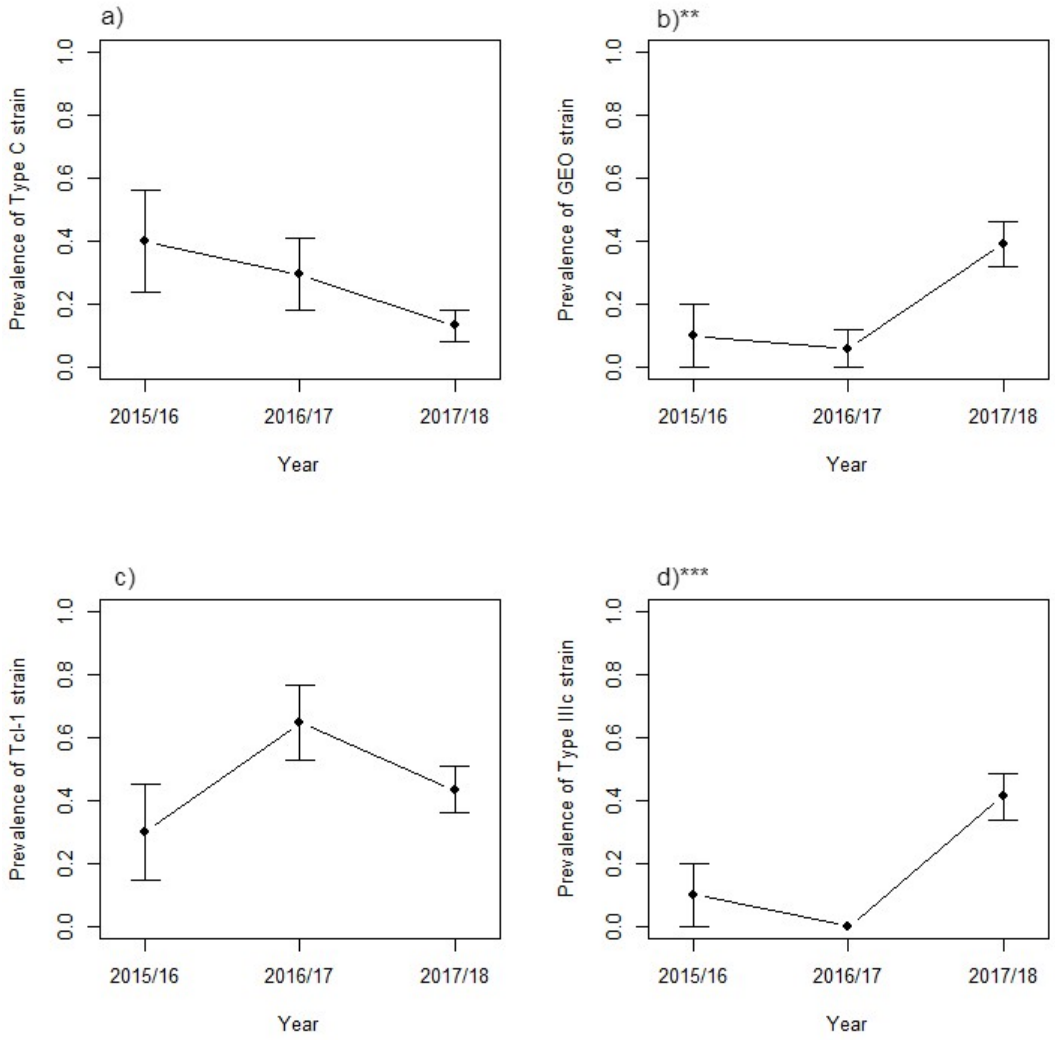

Figure 4.6: Variation in mean prevalence ±S.E. of different strains detected in different years in Senegal for a) Type C b) GEO** c) Tcl-1 and d) Type IIIc***.

Strains marked with * showed significant overall difference between years. Probability values associated with graphs: ***=p<0.001, **=p≤0.01, *=p≤0.05, N = 73. No significant pairwise differences detected between sampling years within each strain when pairwise comparisons were tested with Tukey's post-hoc test.

Table 4.6: Results of binomial GLM, logit link function, modelling prevalence of the four dominant *T. gallinae* strains against year captured in Senegal for three consecutive winters. Year associated with 2 degrees of freedom. Dev = deviance, N = 73.

	Type C		GEO		Type IIIc		Tcl-1	
	Dev	*p*	Dev	*p*	Dev	*p*	Dev	*p*
Year	5.105	0.078	9.618	**0.008**	14.095	**<0.001**	3.075	0.215

Table 4.7: Results of binomial GLM, logit link function, modelling prevalence of the four dominant *T. gallinae* strains against sampling season. Sampling season associated with 4 degrees of freedom. Dev = deviance, N = 112.

	Type C		GEO		Type IIIc		Tcl-1	
	Dev	*p*	Dev	*p*	Dev	*p*	Dev	*p*
Season	9.900	**0.042**	12.594	**0.013**	18.542	**<0.001**	8.677	0.070

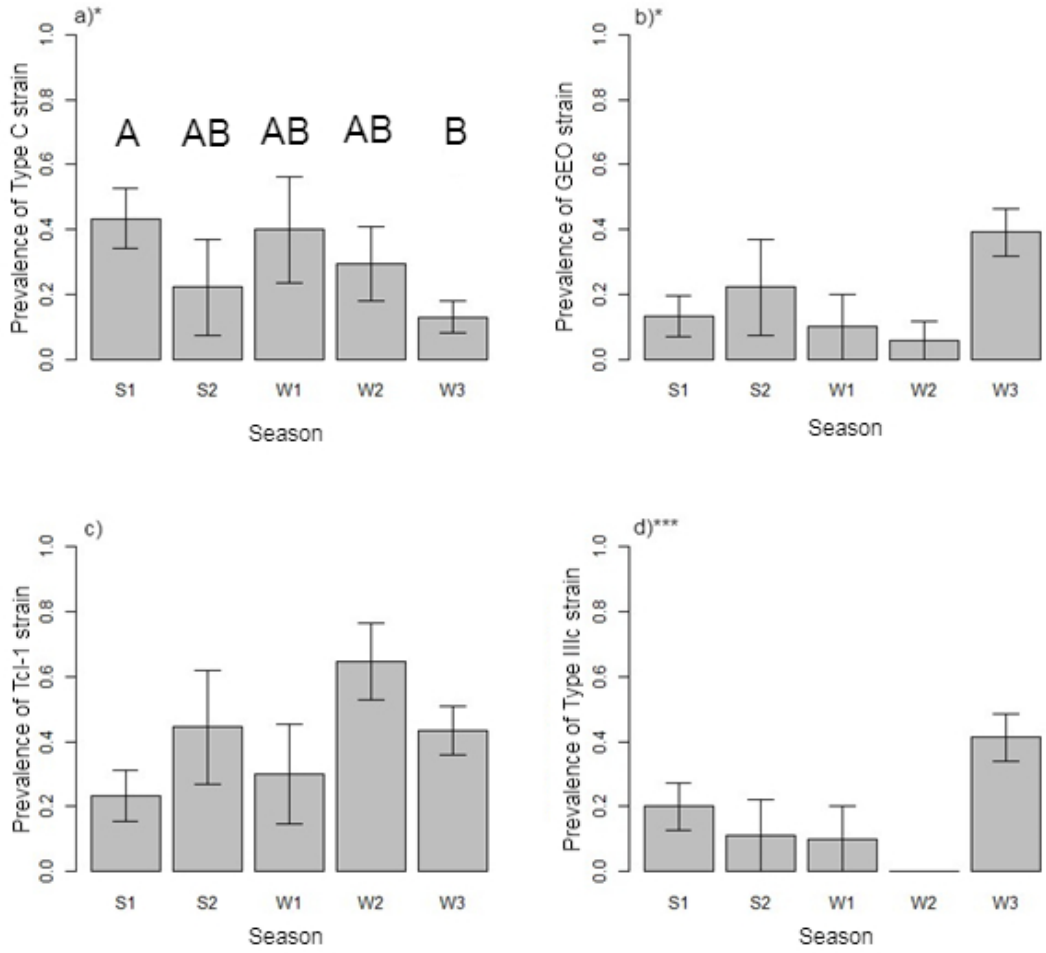

Figure 4.7: Bar graphs showing variation in mean prevalence ±S.E. of different strains detected in different sampling seasons (W=winter, S=spring) for a) Type C* b) GEO* c) Tcl-1 and d) Type IIIc***.

Strains marked with * showed significant overall difference between seasons. Probability values associated with graphs: ***=p<0.001, **=p≤0.01, *=p≤0.05, N = 112. Only one significant pairwise difference was identified following tested with Tukey's post-hoc test, as indicated on the graph for Type C.

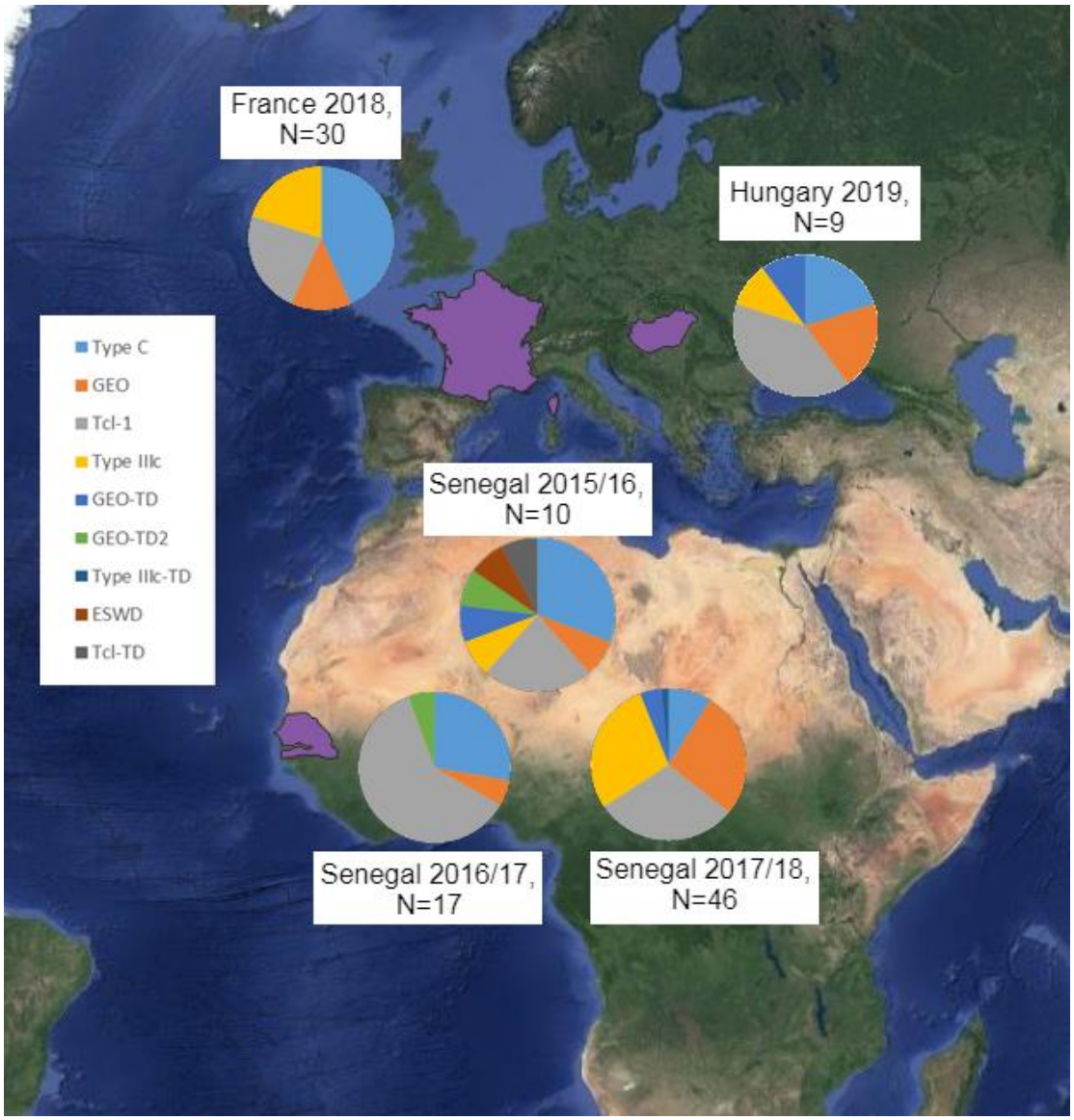

Figure 4.8: Pie charts showing the proportion of different strains, based on the ITS sequencing region, detected in sampling seasons

Four of the *Fe-hyd* subtypes identified occurred in more than one bird. Type C subtype C8-TD was the most prevalent subtype, occurring in 33.3% of birds sampled (N=42) and detected in three sampling seasons, in France (n=8) and Senegal (W2, n=2; W3, n=4). Three other subtypes were detected in Senegal, including two Type C subtypes newly identified in this study, C6-TD, which was detected in Senegal in 2017/18 (W3, n=2) and C7.2-TD, detected in 2016/17 (W2, n=1). Two Tcl-1 subtypes, T1-TD and T2-TD, were detected in 6 (W2, n=3; W3, n=3) and 4 (W2 = W3=3) birds, respectively. France had the greatest diversity observed for the *Fe-hyd* region, with nine subtypes being identified. These consisted of three from Type C (C8-TD, n=8; C9-LD, n=1; C6-TD, n=4),

three from Tcl-1 (T1-TD, n=1; T2-TD, n=2; T3-TD, n=1) and three from GEO (12-TD, n=1; MR22/14-TD, n=1; MR104-TD, n=1). Hungary had two subtypes of T2-TD (n=2) and the new subtype C7-TD (n=1). Season did not significantly affect the occurrence of *Fe-hyd* subtypes.

4.4.4 Effect of infection status on body condition

Mean condition varied significantly between seasons, as determined by one way ANOVA ($F_{4,184}$=26.67, p <0.001). Birds sampled in France (S1) were in the best condition (Figure 4.9), and mean condition score was significantly higher in S1 than all other seasons, based on pairwise comparisons using Tukey's post-hoc test (Table S3.8). The only other significant pairwise comparison showed birds sampled in W3 to be in significantly better condition than those samples in W2 (Figure 4.9, Table S3.8).

Based on AIC, infection status, season and infection status x season interaction were all included in the final model, however, only season had a statistically significant effect on body condition (Table 4.8). When the effect of individual strains on host condition was considered, based on AIC, GEO was the only strain to be retained in the model assessing the effect of strain and season on body condition, but presence of this strain did not have a significant effect on body condition.

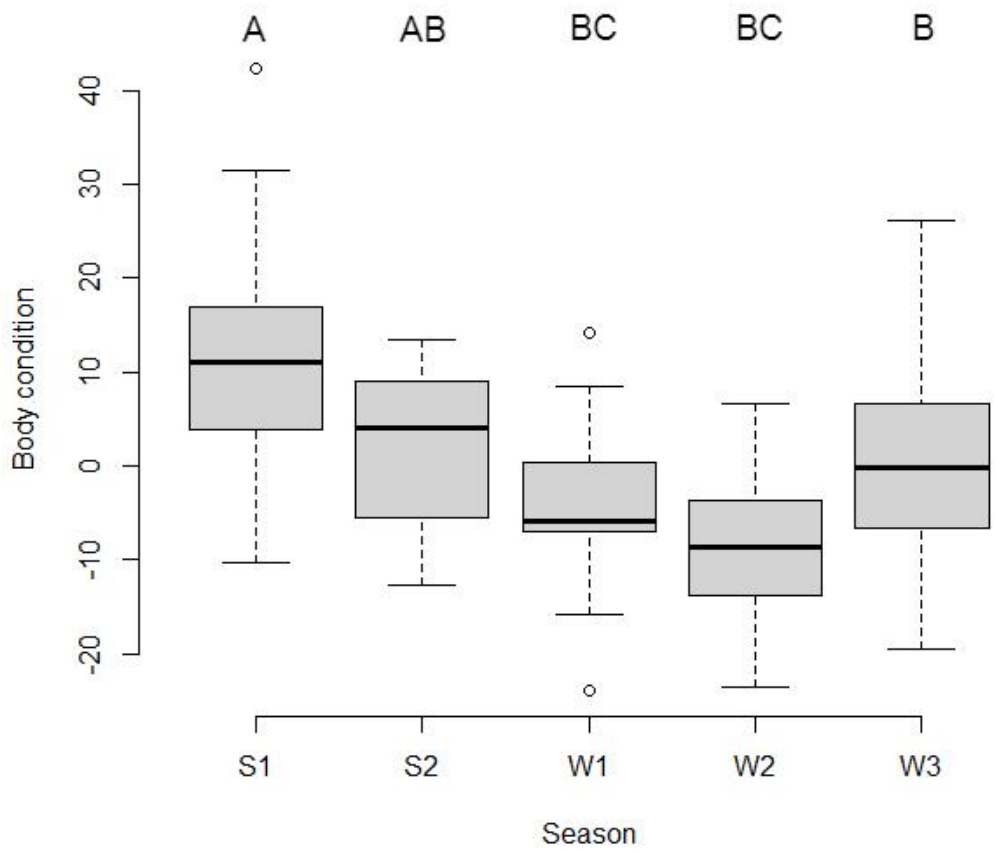

Figure 4.9: Boxplot comparing the condition of all turtle doves sampled across five seasons sampled. Significant pairwise differences in mean body condition determined by Tukey's post-hoc test and are indicated by letters. For full breakdown of p-values associated with plot pairwise differences see Table S3.8.

Table 4.8: Results of Gaussian GLM, identity link function, modelling the effect of infection with *T. gallinae* and season on host body condition, based upon AIC comparison for most parsimonious model. N=189.

	Infection status		Season		Infection status x Season	
	Dev_{df}	*p*	Dev_{df}	*p*	Dev_{df}	*p*
Body condition	197.9_{187}	0.140	9633.1_{183}	**<0.001**	744.8_{179}	0.087

4.4.5 Co-infection with multiple strains of *T. gallinae*

All birds from which ITS sequences were obtained by HTS were examined for co-infection (N=112). Overall, infection with multiple strains of *T. gallinae* was detected in 16.96% of birds sampled (Table 4.9). Rates of co-infection within a sampling season were generally low, but the highest was recorded in Senegal 2017/2018, where 32.6% of birds sampled harboured more than one strain of *T. gallinae.* The average number of strains infecting an individual within a sampling season ranged from 1.0 (France, 2018) to 1.5 (Senegal, 2018/18) (Table 4.9).

The co-occurrence analysis on the full data set revealed that of the 45 potential strain combinations, 35 pairs (77.8%) were expected to co-occur in <1 instance, thus were not included for further analysis. Ten pairs were analysed, revealing four significant associations, all of which were negative (Table 4.10, Figure 4.10). This means that strain pairs occurred significantly less frequently than would be expected if infection with different strains occurred at random. The number of strains infecting an individual did not affect body condition. When the assessment was run on only samples from Senegal, there were 36 of the 45 pair combinations (80%) were expected to occur in <1 instance, so nine pairs were analysed, of which there were three significant negative associations. These three were consistent with those that were detected as being negatively associated in the full data set, with just one species pai (Tcl-1 and Type IIIc) no longer having a significant effect when samples from Senegal were assessed in isolation (Table 4.10).

Table 4.9: Summary of the levels of co-infection of different strains of *T. gallinae* within populations sampled. No significant difference between mean number of strains per individual when tested using a GLM with Poisson distribution and a logarithmic link function

Country	Year	Season	% individuals with coinfection	Max. strains per individual	Mean strains per individual
Senegal	2015/16	W1	11.1	4	1.11
Senegal	2016/17	W2	5.88	2	1.06
Senegal	2017/18	W3	32.61	4	1.46
France	2018	S1	0	1	1.00
Hungary	2019	S2	11.1	2	1.11
Overall			**16.96**	**4**	**1.24**

Table 4.10: Significant results from co-occur showing the observed number of birds infected with each strain of a pairwise comparison, and co-infected with both; the probability of co-occurrence of strain pairs based on occurrence of each strain within the population; and expected number of individuals co-infected with both strains. *P* Lt and Gt represent the probabilities of the two strains co-occurring at a frequency less than, or greater than observed, respectively. Pair marked with * was not significantly correlated when only samples from Senegal were assessed. Effect size refers to the standardised difference between the expected and observed frequency of co-occurrence. For full table including non-significant pairs, see Table S3.9

Strain 1	Strain 2	Strain 1 #infected	Strain 2 #infected	Observed #infected	Probability of co-occurrence	Expected number co-infected	*p* Lt	*p* Gt	Effect size
Tcl-1	Type C	45	30	1	0.108	12.1	<0.001	1	-0.099
Tcl-1*	Type IIIc*	45	27	6	0.097	10.8	0.023	0.993	-0.043
GEO	Type C	26	30	0	0.062	7	<0.001	1	-0.063
Type C	Type IIIc	30	27	0	0.065	7.2	<0.001	1	-0.064

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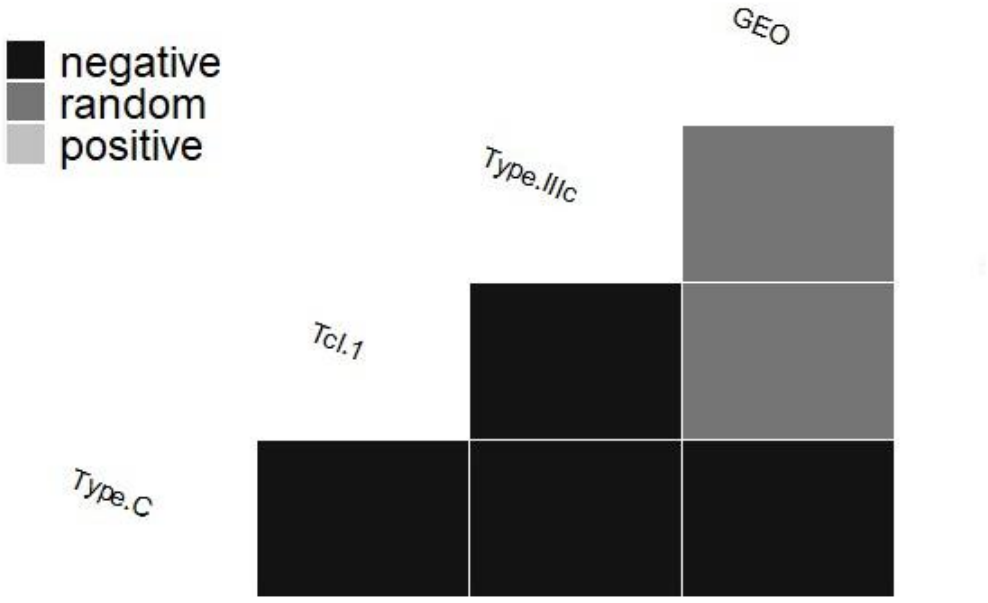

Figure 4.10: Heat plot for *T. gallinae* strains showing non-random associations between four strains

When assessing co-infection using jMHC, it was more difficult to identify artefacts using cumulative depth curves, as a much greater proportion of samples had no clear inflection point following processing using jMHC (23.4%) than using the bioinformatics pipeline (BP) (5.6%). Of the 92 samples which did have a clear inflection point under both jMHC and BP, results were relatively consistent, with only one sample giving results of differing co-infection levels (one strain present in jMHC results, two strains present in BP results) and one sample identified as a different strain present. However, the main drawback of the jMHC method was that following processing through the Excel macro, the resulting read counts were vastly lower than when using the BP (Table S3.10). This variation in read counts was not consistently proportional between jMHC and the BP throughout all samples, with the proportion of reads per sample obtained using the BP compared to jMHC ranging from 31.5 to 165.5. Finally, based on previous use of jMHC to analyse co-infection of *T. gallinae* strains, any samples with a read count lower than 50 were excluded from analysis (Thomas 2017). In this data set, this threshold

resulted in 33% of samples being removed from analysis. For these reasons, only results from the BP are reported in this thesis.

4.4.6 Effects of filtering thresholds applied

Selecting appropriate filtering thresholds is key due to the potential of over or under-filtered data to alter ecological inferences. Due to a lack of standardised protocols for using HTS to analyse parasite co-infection, all analyses were carried out using data sets generated using three different filtering thresholds at stage 3 of data cleaning: 7%, 10.5% and 11.7%. For further information on threshold testing, see Appendix 3, 3.5.

Due to some strains occurring in very few birds, most statistical analysis was carried out on the four dominant strains. When analysing both the effect across years for birds sampled in Senegal, and the effect of season, on the presence of dominant strains, results were largely the same at all filtering thresholds. There was no change in which variables had a significant effect and no change in the significance of pairwise interactions. The only difference regarding significance of results was that at 10.5% filtering threshold, Type IIIc had a significance level $p \leq 0.01$, whereas with 7% and 11.7% filtering, it was $p < 0.001$. When considering the effects of different strains on host body condition, no strains had a significant effect on condition at any filtering threshold.

4.5 Discussion

4.5.1 Prevalence

Due to the propensity of *T. gallinae* to infect columbiforms, several studies have been carried out on a range of species and sites, identifying much variation in the prevalence of this parasite, as well as host susceptibility (Stabler 1948; Höfle *et al.* 2004; Amin *et al.* 2014). Reported prevalence ranges from 5.6% in mourning doves (*Zenaida macroura*) (Schulz *et al.* 2005) to 95% in white-winged doves (*Zenaida asiatica*) (Conti and Forrester 1981) and 96-100% in European turtle doves (Stockdale *et al.* 2015; Thomas 2017). It is important to take into account the method of sampling when assessing prevalence of *T. gallinae* within populations, as this can have major

implications for the chances of successfully detecting infections. For example, Dunn *et al.*(2016) demonstrated the importance of culturing swab samples prior to parasite isolation for reliable detection of infection. In addition, often samples are taken from hunter-shot birds (Schulz *et al.* 2005; Marx *et al.* 2017), which, depending on how soon birds are sampled after shooting, and how they are stored, may impact results (Marx *et al.* 2017). Sampling method is therefore considered when discussing my results in comparison with previous studies.

T. gallinae was present in all populations sampled during the course of this study, with the prevalence ranging from 50% to 82%. This observation is supported by several studies of columbids previously reporting moderate to high prevalence (50-100%) (Conti and Forrester 1981; Lennon *et al.* 2013; Stockdale *et al.* 2015; Marx *et al.* 2017; Thomas 2017). Investigation of *T. gallinae* infection in Mauritius, detected a moderate rate of infection in the Madagascan turtle dove (*Streptopelia picturata*), with 47% prevalence, and an infection rate of 59% prevalence, in the zebra dove (*Geopelia striata*) (Bunbury *et al.* 2007). In the same region, an average prevalence of 50.3% of *T. gallinae* was detected amongst the endangered pink pigeon (*Columba mayeri*) over a two year sampling period (Bunbury *et al.* 2008a).

When investigating infection rate of columbids in the UK, Lennon *et al.* (2013) reported moderate levels of infection in woodpigeons (47%) and stock doves (40%), however, a higher rate of infection was observed in turtle doves (86%). Whilst this elevated prevalence in turtle doves reported by Lennon *et al.* (2013) is higher than was observed in this study for all sampling seasons, it is only slightly greater than levels observed in Senegal 2017/18 (82%) or Hungary (69%).

However, when compared to other recent studies investigating the prevalence of *T. gallinae* in turtle doves, the prevalence reported in this study is lower than expected (Stockdale *et al.* 2015; Marx *et al.* 2017; Thomas 2017). There is evidence of high infection rates of turtle doves with *T. gallinae*, with 93% prevalence detected from cultured swab samples collected in Italy, Spain, Germany and Malta (Marx *et al.* 2017) and 100% in the UK (Stockdale *et al.*

2015). More surprising is direct comparisons which can be made where identical sampling sites were visited previously. Two of the three sites in France visited during this study were sampled in 2014, reporting 100% infection rate (Thomas 2017), a markedly higher result than the 50% prevalence reported in this study. Similarly, the same site in Senegal was visited during the two winters prior to this study, with samples being collected from January to March, and 100% prevalence being reported both years (Thomas 2017).

The differences between Thomas (2017) and the current study could be due to differences in *T. gallinae* prevalence through time or be methodological artefacts. The latter is unlikely because the field and laboratory protocols used in these two studies were consistent, from sample collection and culturing, preservation and storage, and throughout lab work with DNA extraction and PCR protocols. Initially, DNA extraction was carried out using a modified ammonium acetate method, but upon observing a lower than expected prevalence of *T. gallinae,* the more sensitive DNeasy blood and tissue kit was used to confirm absence of parasite in these samples, as was used for all subsequent extractions. All samples were screened with both regular and MID-tagged PCR primers, to confirm absence of *T. gallinae* and rule out false PCR negatives. It is possible that storage may have caused samples collected in Senegal to degrade as, due to insufficient facilities, it was not possible the freeze, or even refrigerate cultured samples as would be recommended. Instead they were preserved in absolute ethanol and stored at room temperature prior to import into the UK, at which point they were frozen as soon as possible. The time between sample collection and freezing ranged from two weeks to three months. However, samples collected by Thomas *et al.* (2017) were stored in similar conditions in Senegal, with the only difference being that parasites were isolated from culture medium in the field site, before preservation with 100% ethanol, thus this would not necessarily explain the difference from these results. Furthermore, this possible underestimate due to storage would not affect European samples, as following culture period, samples in culture medium with 100% ethanol were kept refrigerated for a maximum of two weeks prior to import into the UK, at which point they were

immediately frozen. I therefore conclude that this is an accurate result of infection rate of birds sampled in this study being lower than previously reported.

This study is the first to investigate the prevalence of *T. gallinae* in Eastern Europe, with a 67% prevalence reported from birds sampled in Hungary. I hypothesized that prevalence would be lower in Hungary as a result of less intensive farming and therefore an increase in use of natural resources rather than over-crowding at artificial feed sites. This was not observed, as one of the highest prevalence recorded in this study was for birds sampled in Hungary. However, the sample size for this location was small, in part due to this being a new study site, and therefore sampling effort being impacted by the need to scope out the best locations for capturing birds. It would be beneficial to increase sampling effort across eastern Europe in order to gain a fuller insight into the distribution and community structure of *T. gallinae* within this area.

4.5.2 Spatio-temporal repeatability

Within the field of parasitology, there has been much uncertainty about the extent to which parasite communities are structured, as well as whether observed patterns are transient or sustained (Poulin 2007). Many studies of parasite ecology look at a single population or year, thus providing only a snapshot of parasite prevalence and community composition; observations which are rarely upheld when spatially or temporally replicated communities are observed (Gonzalez and Poulin, 2005). By sampling the same wintering site across three consecutive winters, it was possible to assess the temporal variation in parasite prevalence and strains community composition across years.

Year had a significant effect on strain community composition, indicating a temporal variation in strain composition of *T. gallinae*. Whilst this is the first study to assess repeatability of *T. gallinae* in turtle doves in a wintering site, there is support for these findings from a similar study conducted on UK breeding habitat. Incorporating samples from turtle doves in the UK over a period of five years, from 2011 to 2015, Thomas *et al.* (2022) also identified significant variation in strain composition across years. Furthermore, whilst it

was using a sampling period of every two months, as opposed to a yearly, Bunbury *et al.* (2008a) identified significant variation in the prevalence of *T. gallinae* in the pink pigeon between sampling periods, illustrating the high potential for fluctuations in prevalence of this parasite species.

Temporal variability is common in parasite infection, and is potentially due to several factors, both intrinsic and extrinsic to the host (Behnke 2008). The lack of temporal repeatability in prevalence observed in this study may be a result of climatic variation between years altering either the parasite's persistence in the environment, or the likelihood of transmission (Behnke 2008). Climate variation has been shown to affect the presence of *T. gallinae* in Madagascan turtle doves in Mauritius, with warmer temperatures and lower rainfall being associated with increased prevalence (Bunbury *et al.* 2007). Temperature was shown to have a greater effect than rainfall on the presence of *T. gallinae*, leading to the hypothesis that this parasite is better adapted to persist at warmer temperatures (Bunbury *et al.* 2007). It is possible that fluctuations in yearly temperature and rainfall may have contributed to the temporal variation at the wintering site. Although in depth analysis of climate is beyond the scope of the present study, weather station information from Dakar, Senegal, was used to assess variation in temperature across the sampling period (November-March) (World Weather Online 2022). Whilst Bunbury *et al.* (2007) found temperature to have a greater effect, climate information showed that temperatures remained relatively consistent across years sampled in Senegal (World Weather Online 2022).

As well as host persistence in the environment, climate has the potential to impact parasite transmission, with horizontal transmission between hosts at shared water resources being a potential source of *T. gallinae* infection (Altizer *et al.* 2006; Thomas 2017). Water is suggested as an important factor in the transmission of *T.* gallinae in poultry (Stabler 1954), and has since been suggested as important in transmission in wild columbids (Bunbury *et al.* 2007; Thomas 2017). Bunbury *et al.* (2007) detected the presence of *T.gallinae* in water resources used by pink pigeons in Mauritius, but only in relatively low numbers (two water sources tested positive, out of 15 samples). Thomas

(2017) tested water at sites around Britain in two consecutive years. Whilst only a small number were tested in 2013 (N=6), *T. gallinae* isolates were detected in 67% of water sources. Increased sampling the following year showed that 71% of water sources sampled (N=21) tested positive for *T. gallinae* at least once during the season, and 48% tested positive on repeated samplings, supporting the suggestion that this parasite may be transmitted through shared water resources. Thomas (2017) detected a higher rate of *T. gallinae* presence in water resources sampled than food resources sampled, with a higher prevalence of *T. gallinae* predicted with increased temperature, highlighting this as a potential route of transmission, particularly in warmer climates where water is scarcer.

Climate data were also used to compare rainfall in the months preceding the wintering seasons (August-October) in each year sampled. Winter 2015/16 had by far the most rain with an average of 17 days and 108.41mm of rain across the three months, compared to 7.7 days and 42.45mm in 2016/17 and 6 days and 20.34mm in 2017/18 (World Weather Online 2022). This very low amount of rainfall in the months prior to the sampling season in 2017/2018 would have resulted in depleted water resources, and more arid conditions than in previous years, which was observed during the field season (Chris Orsman, RSPB, *personal communication*). It is therefore possible that the higher infection rate observed in 2017/18 was a result of increased horizontal transmission due to large intraspecific gatherings at watering holes, exacerbated by low rainfall reducing water availability that winter.

Whilst little is known about the turnover rate of infection within individual birds, early work conducted by Stabler (1948) investigating the impact of infection with sub-lethal strains upon subsequent infection gives some indication of the recovery rate. Eight squabs were orally inoculated with a severe strain, and developed oral caseation, but the six which did not succumb to the disease exhibited no visible lesions 1-2 months after infection. Of squabs given a milder strain, only one exhibited mild symptoms (N=8), which were gone within 12 days. Furthermore, this study demonstrated individuals which have recovered from a prior infection were protected, at least to some degree, against

subsequent infection, as none of the birds tested exhibited any symptoms following inoculation with a normally lethal strain following prior exposure. It is possible that reasonably rapid strain turnover, resulting from a combination of individuals being able to recover, and potentially prior exposure increasing individual's capacity to fight infection, may limit repeatability of infection rates within a population.

4.5.3 Parasite strain composition

The four dominant strains identified in this study are consistent with previous works which have found high levels of infection with these strains in columbids (Martínez-Herrero *et al.* 2014; Marx *et al.* 2017; Thomas 2017; Quillfeldt *et al.* 2018). When comparing the findings of this study to work previously carried out at the same sampling sites in France and Senegal, there is some support for previously reported strain compositions (Thomas 2017). For example, in France, Type C was the dominant strain in both studies. GEO was the dominant strain in Senegal in both years according to (Thomas 2017), whist I only found it to be the dominant strain in one of the three years samples, it was a consistently highly occurring strain. GEO-TD was previously identified as a potential new strain after detection from two birds in Senegal, a turtle dove and a laughing dove *Streptopelia senegalensis* (Thomas 2017). This study detected GEO-TD in four samples from Senegal and one from Hungary, providing support for the differentiation of this sequence as a new strain. A second strain (ESWD) initially identified in an emerald spotted wood dove by Peters (*unpublished*), was detected in one bird from Senegal in this study. One strain that has been consistently detected in columbids is Type A (Sansano-Maestre *et al.* 2009; Martínez-Herrero *et al.* 2014; McBurney *et al.* 2015; Marx *et al.* 2017), however, this strain did not appear in this study. This is potentially, at least in part, due to the sites sampled during this study. Previous sampling in Senegal did not detect any infections in turtle doves with Type A (Thomas 2017), supporting its absence in Senegal in this study.

Another possible reason for the absence of detection of Type A is that Type A is the lethal strain. It is a clonal strain of that which was determined to cause mass mortality in finches (Lawson, Cunningham, *et al.* 2011) and has been

linked to turtle dove mortality (Stockdale *et al.* 2015). It is therefore possible that it was not detected in this study because individuals infected with this strain experienced pathology and were either in too poor condition to fly and thus be captured, or had succumbed to the disease.

4.5.4 Co-infection

Co-infection with multiple parasites is almost ubiquitous in nature (Knowles *et al.* 2013) but there is less evidence as to whether this extends to the presence of multiple strains of the same parasite. Whilst there are still limited studies using genetic analysis to assess co-infection with multiple strains, HTS is being used for analysis of parasite strain co-infection, for example, in the protozoan parasite *Trypanosoma cruzi* (Dumonteil *et al.* 2020; Pronovost *et al.* 2020). Whilst the literature provides minimal support for the co-infection of multiple strains of *T. gallinae* within a single host, it is possible that such co-infection is widely underreported as a result of detection methods. This study is only the second to use HTS to identify *T. gallinae* strains present in infected birds. As Sanger sequencing returns one chromatogram per sample, if multiple strains are present within one sample, this method does not have the capacity to identify them separately. Whilst clonal culturing is a viable method used to identify multiple strains within one host, it is both time consuming and costly, making it less appropriate for use in large scale monitoring activities (Grabensteiner *et al.* 2010). The use of HTS makes it possible to ascertain the presence of a multiple strains and by its nature of highly paralleled processing, it is more efficient than previous methods (Taberlet *et al.* 2012; Clarke *et al.* 2014).

To my knowledge, Thomas *et al.* (2022) is the only other example of using HTS to analyse *T. gallinae* strains community composition, a study also carried out on turtle doves. The present study identified a higher rate of co-infection than Thomas *et al.* (2022) with multiple strains being detected in 19 birds (17% of total sampled, N= 112), compared to just one instance of co-infection (N=107) (Thomas *et al.* 2022). Higher rates of co-infection have been identified using HTS to assess parasite strain co-infection in other study systems, with a rate of 38% co-infection between multiple strains of the protozoan parasite

Trypanosoma cruzi being previously detected in dogs screened in the USA (Dumonteil *et al.* 2020).

When considering the number of strains detected in each host, Thomas *et al.* (2022) only identified the presence of two strains infecting one bird, whereas this study found 3 examples of 3 or 4 strains infecting a single host. This may be a result of the different methods of data processing used in the two studies as Thomas *et al.* (2022) used jMHC. However, whilst when I trialled this method in this thesis I found the results to be of poorer quality than those obtained with the bioinformatics pipeline developed by Drake *et al.* (2021), levels of co-infection detected were consistent in all but one sample, therefore methodology may not be the sole reason for the observed differences.

When assessing co-infection, four significant associations were found, all of which were negative, indicating that strains were co-occurring less than would be expected if infections were random. In a previous assessment of the correlation of strains present in turtle doves, all correlations were detected were again negative, supporting these results (Thomas *et al.* 2022). Of the three negative correlations previously detected, two cannot be compared with this study, as they involved Type A, which was not detected in this study. However, the third negative correlation detected, between Type C and GEO was consistent with the findings of this study. This correlation was supported both by the full data set across all sites, and when the analysis included just birds from Senegal, indicating that it was not a result of pooling sampling locations for analysis. The other negative three correlations detected in this study, did not show any significant correlation when previously assessed (Thomas *et al.* 2022). Despite a higher proportion of individuals sampled exhibiting co-infection than previously observed, these negative correlations show that certain strains are still co-occurring within a single host less commonly than would be expected if infection were random, suggesting there is a mechanism acting to reduce the presence of multiple strains.

There are several possible causes for the observed negative correlation, but due to the nature of this study using a correlational approach for analysis of co-infection, determining the underlying cause will remain largely conjectural

(Johnson and Hoverman 2012). One potential cause is a lack of exposure, however, this explanation is unlikely to be appropriate in this study, as the occurrence of several different strains within this study confirms that there are multiple strains of *T. gallinae* circulating in all populations sampled. Furthermore, Thomas (2017) showed that multiple strains could be present within a single environmental resource, therefore it is possible for hosts to be exposed to multiple strains at the same time. An alternative explanation is competition for resources within the host. As all strains of this parasite will occupy the same niche, it may be the case that certain strains are dominant, resulting in an antagonistic effect and the outcompeting of alternative strains, reducing the likelihood of co-infection occurring (Palinauskas *et al.* 2011). Thirdly, it is possible that infection with multiple parasite strains increased the pathogenic effect, resulting in co-infected individuals being more likely to suffer mortality as a result of *T. gallinae*, thus removing them from the sampling populations. Such effects would be consistent with those observed in alternative study systems, including tilapia (*Oreochromis niloticus*) and poultry flocks, whereby co-infection with multiple pathogens increased host mortality (Xu *et al.* 2009; Sid *et al.* 2015).

4.5.5 Methods

As previously described, HTS offers the opportunity to detect multiple parasites or strains within a single sample which is not possible using traditional methods, such as Sanger sequencing. It is also highly parallel, making it more efficient and cost effective for use on large numbers of samples. However, there are drawbacks; unlike Sanger sequencing, chromatograms are not produced, removing the opportunity for manual checking of sequences. However, in this study, where *Fe-hyd* was assessed using Sanger sequencing, these results were compared to the ITS results obtained from the same bird. Out of a total of 41 samples (including repeated samples) which had data for both *Fe-hyd* and ITS, the strain sub-type was found to be consistent with the strain identified in all but one instance supporting the accuracy of using HTS. Where inconsistency was observed, both the ITS haplotype (GEO) and the *Fe-hyd* subtype (C8-TD) were confirmed with Sanger sequencing. This result was unexpected, but may be caused by of horizontal gene transfer, which has been

observed to occur in other *Trichomonas* species (Alsmark *et al.* 2009), in an individual that has been infected with both GEO and Type C strains concurrently, or hybridization of strains (Alrefaei *et al.* 2019). It has previously been observed in *T. gallinae* that *Fe-hyd* subtype sequences from Type G and Type H were more similar to Type A , and that in *Trichomonas stableri Fe-hyd* genes sequences from the ITS strain Type K were more akin to the Type L strain (Alrefaei *et al.* 2019).

Another drawback of using HTS to analyse parasite data is it is a relatively novel approach, resulting in a lack of established protocols for cleaning of data. Whilst I did find examples of previous studies using HTS to identify co-infection with multiple parasites, or strains of parasites, there was commonly little, or no description of the data cleaning process, and there is no general consensus of which approach to use (Avramenko *et al.* 2017; Huggins *et al.* 2019; Dumonteil *et al.* 2020; Pronovost *et al.* 2020). Two approaches were tested on the data presented in this study. The method used to obtain the data set used in analysis presented in this chapter was an adapted diet metabarcoding pipeline. An extra step was added to the data cleaning process to make this method more applicable to use with parasite community data. This cleaning step was adapted from that used by Lighten *et al.* (2014) to remove artefacts from MHC data when using the programme jMHC. Use of jMHC was the second method testing for producing a data set for analysis in this study, having been previously successfully adopted for the investigation of *T. gallinae* strains (Thomas 2017)

When data generated following the two methods were compared, overall results obtained were very similar, with both methods producing consistent results for the absence or presence of co-infection for most individuals sampled. The metabarcoding approach was adopted in this study for two reasons; firstly, it has a greater capacity for manually changing parameters, and these could be edited at every stage of the bioinformatics process, as it was all carried out using customisable scripts (Drake *et al.* 2021). Secondly, when using the jMHC method, read counts per sample produced following demultiplexing are considerably lower than the outputs following the metabarcoding approach. Previous use of this method states that read counts

below 50 should be excluded from analysis due to variable sequence quality. If this approach was followed, 33% of samples would be excluded from analysis, despite them exhibiting high read counts using the metabarcoding pipeline (read counts ranging from 662 to 4333 removed, average read count of removed samples = 2278).

4.5.6 Conclusion

This study detected a lower prevalence of *T. gallinae* than has previously been observed in turtle doves, but this parasite still occurred in at least 50% of the individuals caught in each sampling season. Four previously determined strains were dominant in the birds sampled in this study, but the lethal strain, Type A, was not detected. One strain which had previously been reported in only two birds was detected in this study, supporting the classification of GEO-TD as a strain of *T. gallinae*, and a further four novel strains were detected.

The observed lack of temporal repeatability of parasite prevalence highlights the importance of consistent monitoring of infection in order to establish the health of populations. Furthermore, the variation in strain composition observed indicates an area for further study in order to assess not only the rate of infection turnover, but also whether prior exposure appears to incur some level of protection against subsequent infection. If this is the case, it indicates a vaccination programme may be feasible to protect highly vulnerable species against the most virulent strains of *T. gallinae.* This study has been the first to assess infection rate in eastern Europe, and further study in this area on a wider range of locations and greater sampling size would be beneficial to enhance the understanding of the distribution of this parasite across the turtle dove's range.

4.6 Acknowledgements

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Chapter 5 - Investigating the association between diet and infection with *Trichomonas gallinae* in the European turtle dove

Photograph by Rebecca Young

An additional data set was used in this chapter to compare the findings of this work with similar a similar dietary study conducted in the UK. These data were provided by Rebeca Thomas and Jenny Dunn who completed sample collection, lab work and bioinformatic processing to produce this data set.

5.1 Abstract

Trichomonas gallinae is a protozoan parasite infecting a wide range of birds, and has been a cause for conservation concern since its recognition as an emerging infectious disease. It has had a notable impact on several avian species, including causing widespread mortality in greenfinches and chaffinches, and hindering the recovery of the endangered pink pigeon. An important route of transmission of this parasite is horizontal transmission between birds congregating at feeding sites. I therefore consider the prevalence of this parasite in the declining European turtle dove, in relation to the diet of individuals, in the first study to analyse these two important factors in the decline of this species together. By using birds sampled from both the breeding and wintering grounds, the dietary composition of individuals, as well

as the proportion of diet accounted for by wild and cultivated seeds, was compared to the presence of *T. gallinae* infection, as well as to the absence or presence of specific *T. gallinae* strains. Dietary variation was summarised into two axes using detrended correspondence analysis, but neither of these affected the presence of *T. gallinae,* or any specific strains. In addition, the proportion of diet accounted for by cultivated seeds did not affect parasite presence, despite the spread of this parasite being associated with supplementary feeding. Significant dietary overlap was observed between infected and uninfected individuals in all sites, with almost complete dietary overlap being observed in France. The level of dietary overlap between individuals infected with specific strains fluctuated between sampling seasons, indicating no consistent relationship between diet and infection status.

5.2 Introduction

5.2.1 Importance of disease as a conservation issue

For a long time, the role of infectious diseases and parasitism in driving host population dynamics was underappreciated, until work by Anderson and May highlighted the importance of parasites in ecological systems (Anderson and May 1979; May and Anderson 1979). In recent decades, Emerging Infectious Diseases (EIDs) have increasingly become recognised as an important conservation issue, contributing to the decline of species across a wide range of taxa worldwide (McCallum and Dobson 1995; Daszak *et al.* 2000). From the amphibian population crashes caused by chytridiomycosis, to the extinction of endemic Hawaiian birds, EIDs have the potential to spread rapidly amongst populations, resulting in mass mortality, local extinctions and contributing to global species decline (Warner 1968; Berger *et al.* 1998; Cunningham and Daszak 1998; McCallum 2012).

High mortality rates and local bat population crashes have been observed as a result of the rapid spread of white-nose syndrome, a condition caused by the fungal pathogen *Geomyces destructans,* in bats across North America. First recognised in New York state in 2006, white-nose syndrome has rapidly spread across North America, occurring throughout the north east and mid-Atlantic regions of the USA, as well as Ontario and Québec provinces of

Canada (Blehert *et al.* 2009; Frick *et al.* 2010). White-nose syndrome has been linked to mass mortality in hibernating bat colonies. For example, this disease has been associated with local colony collapse of the previously abundant and widespread little brown bat (*Myotis lucifugs*), with decrease in bat numbers at infected sites ranging from 30% to 99% (Frick *et al.* 2010). Disease also has the potential to hinder population recovery of endangered species, such as the golden lion tamarin (*Leontopithecus rosalia*). Endemic to Brazil's Atlantic forest, this species suffered severe population declines as a result of extensive deforestation, resulting in habitat fragmentation, with the population falling to just a few hundred individuals in the early 1970's (Dietz *et al.* 2019). Extensive conservation efforts over two decades, including the release of captive bred tamarins boosted the population to around 3700. However, in 2016, southeast Brazil experienced the worst outbreak of yellow fever in 80 years, which has been implicated in a 32% decline in the number of free ranging golden lion tamarins between 2014 and 2018 (Dietz *et al.* 2019).

A current example of a disease at risk of driving a population to extinction can be seen in the Australian indigenous marsupial the Tasmanian devil (*Sarocophilus harrisi*), which has suffered severe population declines due to the rapid spread of the Facial Tumour Disease since it was first recognised in 1996 (McCallum and Jones 2006). This infectious cancer manifests as highly contagious facial tumours, spread via biting, in both aggressive and sexual interactions (McCallum 2008). In 2006, this disease was reported to have caused population crashes of up to 90% in just one decade and is predicted to drive the species to extinction highlighting just how important a role disease can play in population dynamics, and thus its importance as a consideration in species conservation (Jones *et al.* 2007).

5.2.2 Disease transmission routes and feeding behaviour

Transmission route is an important factor when assessing the impacts or disease on a population, and understanding both inter and intraspecific transmission of disease is vital in species management (Herrera and Nunn 2019). Numerous factors can have an impact on disease transmission and its consequences for wild populations, including the presence of a reservoir host,

ecological changes, such as introduced species and population fragmentation, and host behaviour (De Castro and Bolker 2005; Herrera and Nunn 2019).

The introduction of novel species can affect disease transmission by exposing naïve hosts to new pathogens, or by facilitating the spread of disease by introducing previously absent vectors. The native red squirrel (*Sciurus vulgaris*) was previously widespread across the British Isles, however, following the introduction of the grey squirrel (*Sciurus carolinensis*) in the 1890's, severe declines were observed in the native red squirrel population (Middleton 1930). These declines have been largely attributed to competition between red and grey squirrels for resources and the spread of squirrelpox virus, a pathogen for which grey squirrels act as a reservoir host, but to which naïve red squirrels are highly susceptible (Sainsbury *et al.* 2000; Chantrey *et al.* 2019). Likewise, drastic changes occurred in endemic bird populations in Hawaii following the introduction of the principal transmission vector for avian malaria, the mosquito *Culex quinquefasciatus* (Warner 1968; Lapointe 2008). Prior to this event, whilst migrant avian species known to be carriers of *Plasmodium*, causing avian malaria, would use Hawaii either as a stop-over location, or wintering ground, endemic Hawaiian species were not infected due to the absence of a transmission vector (Warner 1968). However, following the accidental introduction of *C. quinquefasciatus*, avian malaria rapidly spread among multiple endemic Hawaiian species and is believed to have driven several species to extinction (Warner 1968).

Animal behaviour is another important factor in disease transmission, with certain behaviours increasing or decreasing the risk of disease transmission (Herrera and Nunn 2019). One factor that has been attributed to an increase in the spread of disease is the congregation of animals. For example, gorillas living in groups exhibited a higher mortality rate from Ebola virus than solitary individuals (Caillaud *et al.* 2006), and a higher rate of the blood parasite *Haemoproteus* was detected in Columbia in birds found in mixed-species flocks than those not found in such flocks (González *et al.* 2014).

5.2.3 Disease transmission and supplementary feeding

It is common for wildlife to aggregate in small spatial areas as a response to resource availability, in particular, food (Sorensen *et al.* 2014). The provision of supplementary food for wildlife by humans has become a common activity, with many ecological and social benefits, including supplementing shortfalls in natural food resources (Weidman and Litvaitis 2011), increasing reproductive success (Robb *et al.* 2008a) and eco-tourism (Orams 2002). Supplementary feeding to account for shortfalls in the availability of wild food resources is commonplace in agri-environment schemes, with active schemes in the UK for both winter and summer supplementary feeding for farmland birds (UK Government 2021a). As well as intentional supplemental feeding, wildlife may gather at other anthropogenic sites, such as landfills (Tortosa *et al.* 2002), livestock feed troughs (Atwood *et al.* 2009), harvested crops and spilt grain (Browne and Aebischer 2003a) in order to benefit from the abundance of food available. Provision of food resources can have positive outcomes, for example less severe declines being observed in target species of over-winter supplementary feeding programmes (Siriwardena *et al.* 2007), and increased breeding success as a result of earlier laying dates and increased fledging success being observed in blue tits (*Cyanistes caeruleus*) following the provision of peanuts (Robb *et al.* 2008b). However, there are important negative implications arising as a result of larger inter and intraspecific aggregations at feeding sites, such as increased predation and disease transmission (Sorensen *et al.* 2014).

Higher densities of individuals in an area increase the probability of interaction and close contact, thus elevating the risk of direct disease transmission (Sorensen *et al.* 2014). Close contact in the form of aggressive behaviours has been associated with animal aggregation at feeding sites in the banded mongoose (*Mungos mungo*) (Flint *et al.* 2016). Infection with *Mycobacterium tuberculosis* was positively correlated with resulting injuries, as bacteria can spread through open wounds (Flint *et al.* 2016). Increased animal-to-animal contact when herds were corralled into areas for supplementary feeding, along with higher stress and poor hygiene, has also been linked to the simultaneous

outbreak of three infectious diseases in a herd of semi-domesticated reindeer (*Rangifer tarandus tarandus*) in Sweden (Tryland *et al.* 2019).

Indirect pathogen transmission is also increased when animals congregate at feeding sites as a result of infective stages being shed in the environment from infected hosts, via routes such as faeces and saliva (Murray *et al.* 2016). The length of time such stages can persist in the environment varies greatly between organisms and with environmental factors (Walther and Ewald 2004; Turner *et al.* 2021), but higher densities of wildlife at food sources will increase the likelihood of these transmissible stages being deposited and accumulating, facilitating an increase in indirect disease transmission (Murray *et al.* 2016). Furthermore, the abundance of food at a single site reduces the need to move to forage, encouraging sedentary behaviour which contributes both to indirect transmission, via a build-up of infective agents in the environment, and increased chance of direct transmission through contact between individuals as animals are not dispersing, thus increasing exposure time (Murray *et al.* 2016).

A range of diseases have been associated with congregations of wildlife at feeding sites, highlighting the importance of considering this factor when investigating disease transmission (Sorensen *et al.* 2014). The use of supplementary cattle feed troughs has been identified in badgers (*Meles meles*) and racoons (*Procyon lotor*), two reservoir hosts for bovine Tuberculosis (bTB), as potentially facilitating disease bTB transmission (Atwood *et al.* 2009; Payne *et al.* 2016). Supplemental feeding of white-tailed deer (*Odocoileus virginianus*) has also been associated with increased rates of bTB within the free-ranging deer population (Miller *et al.* 2003).

A common source of supplementary food is garden bird feeders, with millions of households provisioning food in this way every year (Robb *et al.* 2008a). The use of such feeders results in much higher rates of inter and intra-specific congregation of birds than would occur under natural foraging behaviours (Lawson *et al.* 2018). In the UK, increased occurrences of *Salmonella typhimurium* and *Escherichia coli* have been associated with aggregation at feeding sites (Pennycott *et al.* 1998), and congregation at garden bird feeders

has been linked to the spread of intestinal coccidians (*Isospora* sp.) and canarypox virus (Avipoxvirus) in house finches (*Haemorhous mexicanus*) in the USA (Giraudeau *et al.* 2014). The spread of *Trichomonas gallinae* in chaffinches (*Fringilla coelebs*) and greenfinches (*Chloris chloris*), beginning in western England and Wales, and spreading across large areas of mainland Europe (McBurney *et al.* 2015), caused significant declines in the British population of greenfinches and chaffinches, resulting in the highest recorded impact of an infectious disease on a European wild bird population (Lawson *et al.* 2018). This parasite is known to affect a wide range of avian orders, includin Passeriformes, Columbiformes and Falconiformes (Bunbury *et al.* 2008a; Sansano-Maestre *et al.* 2009; Amin *et al.* 2014), with at least 23 strains of the parasite, of varying virulence identified (Clark *et al.* 2016). The congregation of birds at artificial feeding sites is believed to have been a key contributor to the rapid spread of *T, gallinae*, via horizontal transmission through conaminated food and water resources at these sites (Robinson *et al.* 2010; Gerhold *et al.* 2013; McBurney *et al.* 2015).

As well as being commonplace in residential gardens, supplementary feeding is an important tool used in the management of vulnerable species, particularly when habitat degradation, and the concurrent loss of food resources, has been a driver in a species' decline (Edmunds *et al.* 2008; López-Bao *et al.* 2010; Ewen *et al.* 2015). It is therefore crucial to consider this route of disease transmission so as not to introduce or increase the risk of disease as a threat to already declining species (Sorensen *et al.* 2014). Psittaciformes (parrots) are one of the avian orders most vulnerable to extinction, with 59% of species declining (IUCN 2022). Since its emergence in the 1970s, Psittacine beak and feather disease has been a major driver in species decline, and one of the most common infections occurring in parrots (Kundu *et al.* 2012). This viral disease is widespread and not species-specific, and has been implicated in the decline of several wild parrot populations, including the Mauritius echo parakeet (*Psittacula eques*) (Kundu *et al.* 2012). The endemic echo parakeet was one of the world's rarest parrots, with the population declining to fewer than 20 individuals during the 1980's, before intensive species management, including the provision of nest boxes and supplemental feed to support them

in their degraded habitat, brought the population to over 500 (Richards *et al.* 2010; Raisin *et al.* 2012). However, population recovery was impacted by the emergence of Psittacine beak and feather disease in 2005 and 2006, potentially as a result of a large population of rose-ringed parakeets becoming established on Mauritius, and acting as reservoir hosts (Kundu *et al.* 2012). This virus is highly transmissible (Ritchie *et al.* 2000), therefore the management strategies vital in supporting this recovering population, including supplementary feeding, facilitate the spread of this disease via horizontal transmission when birds congregate at feeding stations (Raisin *et al.* 2012; Fogell *et al.* 2019).

Supplementary feeding is also commonly used in relation to turtle doves, both a management strategy to increase turtle dove population, and as a means to facilitate the capture of birds for the purpose of research and population monitoring (Dunn *et al.* 2015; Thomas 2017). Given the mounting evidence of disease transmission at supplemental feeding sites, as well as the association of *T. gallinae* with birds congregated at supplementary feed, this is an important consideration in plans to mitigate the decline of this species. I therefore bring together two key elements of this thesis to address the interaction between the diet of European turtle doves, and infection with *T. gallinae.* By using data obtained through high-throughput sequencing on the diet and infection with *T. gallinae* in turtle doves, I aim to address the following hypotheses: i) The proportion of the diet consisting of cultivated crop seeds will be positively correlated with presence of *T. gallinae;* ii) There will be an association between diet consumed and strain of *T. gallinae* detected.

5.3 Methods

5.3.1 Sample collection, DNA extraction and PCR amplification

Faecal and parasite samples were collected from Senegal, France and Hungary as described in Chapter 2 (2.3.1), and DNA extraction, PCR amplification and sequencing was conducted as described in Chapter 2 (2.3.2) and Chapter 4 (4.3.3).

An additional data set, collected for another study in the UK was analysed in order to further assess the interactions between diet and strain composition

(Dunn *et al.* 2018; Thomas *et al.* 2022). These data were included in the analysis in order to incorporate the lethal strain, Type A, which was detected in the previous study in the UK, but did not occur in the samples collected in this study. Data were collected in the UK, from 12 farmland sites across Essex, Suffolk, Cambridgeshire and Norfolk in 2011-2014. Sampling was conducted following the same protocols used in this study, and full details of sampling are given in Dunn *et al.* (2015). Methods for seed DNA extraction were consistent across the two studies, and extraction of DNA from *T. gallinae* was done using the salting out method, as described in Chapter 4 (4.3.3). Due to the application of different bioinformatic processing pipelines and data cleaning in the two data sets, UK data were analysed separately from data collected on the breeding grounds in this study.

5.3.2 Dietary variation and infection status

To assess the relationship between diet and infection status, detrended correspondence analysis (DCA) was first carried out in order to summarise dietary data, based on the presence or absence of dietary taxa within each bird sampled. DCA is a dimension reduction technique, capturing the sources of dietary variation in a small number of variables, which can be included as independent variables in a Generalised Linear Model (GLM). Unlike principal component analysis, DCA does not assume a linear relationship between variables and the gradient, therefore is more appropriate for categorical data, such as ecological occurrence data. Analysis was carried out using the 'decorana' function in the R package *vegan* (Oksanen *et al.* 2020) and visualised using functions from the *ggplot2* (Wickham 2016) package.

As a high proportion of dietary items occur in a very low number of samples, this may mask co-occurrence patterns of plant taxa in dietary samples, therefore DCA was conducted on a subset of data, containing only frequently consumed taxa. In order to determine which dietary items should be classified as such, four different thresholds representing how frequently items occurred in the diet were tested, to determine the most appropriate threshold for defining 'frequently occurring taxa' for this analysis. The four data subsets included taxa occurring in the diet of at least 3%, 5%, 7% and 10% of birds sampled (Table 5.1).

Table 5.1: The number of different genera consumed and the sample size of resulting data subsets at each of four thresholds

Threshold	Number of genera in diet	Number of individuals
3%	15	182
5%	13	179
7%	9	168
10%	6	155

DCA was conducted on each subset and the scores of samples inspected to assess for outliers within the sample. These scores represent the number of standard deviations a row/column is from the central point within the biplot (barycentre). According to Bendixen (2003), points should be considered as potential outliers if their co-ordinates indicate that they are greater than one standard deviation from the barycentre. When there are outliers within the contingency tables, these can dominate the interpretation of axes of variation in DCA, resulting in clustering of other points, and thus potentially obscuring the variation which may be occurring (Bendixen 2003). The optimum threshold for DCA was determined based on the absence of outliers in the data.

Following selection of the appropriate threshold, the number of dimensions to be included as independent variables in the GLM was determined using a scree plot, illustrating the variation explained by the DCA dimensions. A GLM was fitted with the presence or absence of *T. gallinae* infection as the dependent variable, using the binomial family and logit link function. Independent variables included in the model were season, body condition, and the coordinate scores for DCA1 and DCA2. This model was repeated to assess individual strains with the presence or absence of GEO, Tcl-1, Type C and Type IIIc being the dependent variable in four respective models. A reduced data set containing 153 samples with genetic information for strain of *T. galliane* present was used for GLMs on specific strains. Model simplification was performed on all GLMs using the ‘step’ function.

5.3.3 Dietary overlap

Dietary overlap between infected and uninfected birds, and birds infected with different strains of *T. gallinae,* was visualised using non-metric multidimensional scaling (NMDS). For the purpose of NMDS analysis, genera occurring in a single dietary sample were removed, as this analysis is liable to outliers skewing the results. The command 'metaMDS', from the *vegan* package was used to estimate differences between the presence of genera in individual samples, using Jaccard distance. 'Ordispider' was used to visualise results from nMDS via plotting with *ggplot2*.

Pianka's measure of overlap (Pianka, 1986) was used to assess the extent to which diet overlapped for uninfected and infected birds, and in birds infected with different strains. This measure is given as a value from 0, indicating no dietary overlap, to 1, indicating complete dietary overlap. Two matrices were created, including plant genera present in the diet, against either infection status (presence or absence of *T. gallinae* infection) or the presence or absence of each *T. gallinae* strain, recording counts of the number of times each genus was present in the diet of infected/uninfected birds, or birds infected with a specific strain present. Due to the segregation of dietary items between Europe and Africa, Pianka's overlap statistic was calculated for the breeding and wintering grounds separately. Average pairwise niche overlap was calculated using the 'niche_null_model' command in the R package *EcoSimR* (Gotelli & Ellison, 2013) with 9999 repeats. Pairwise overlap was also assessed between specific strains.

Pianka's niche overlap was repeated for both infection status and *T. gallinae* strains present in individual sampling seasons to assess whether patterns were consistent across years (Senegal) and between countries (Europe). Two sampling seasons, W2 and S2, were excluded from this analysis due to small sample sizes (N = 11 and 10, respectively), as small sample size increases the likelihood of under-estimating overlap (Linton *et al.* 1981).

5.3.4 Source of food and *T. gallinae* infection

The effect of the proportion of wild and cultivated seeds in the diet upon the infection probability by *T. gallinae* was assessed using binomial GLM. The

absence or presence of *T. gallinae* infection was the dependent variable, and the independent variables were season and proportion of the diet accounted for by wild seeds. This model was repeated to assess individual strains, modelling the presence/absence of GEO, Tcl-1, Type C and Type IIIc in turn as the dependent variable.

The presence of *T. gallinae* was also considered in the context the dominant food type occurring in the diet. Diet was classified as either dominated by cultivated or wild seed, where the majority of taxonomic units detected fell within these categories, or 'even', in instances where the number of taxonomic units detected were classes as wild or cultivated was the same.

5.4 Results

5.4.1 Dietary variation and infection status

A total of 190 birds had molecular information for both diet and parasite infection, thus this subset was considered in this analysis. Five strains of *T. gallinae* were very rare within this study, occurring in just one (Tcl-TD, GEO-TD3, Type IIIc-TD, ESWD), two (GEO-TD2), three (Ttl-TD) or four birds (GEO-TD), reflecting 0.6%, 1.1%, 1.7% and 2.3% of the total number of birds sampled, respectively. Due to their low occurrence, these strains were not included in strain specific analysis.

Four different thresholds were tested for removing uncommon taxa from the analysis. When accounting for outliers in the dataset, the threshold for the total proportion of samples within which a taxa had occurred was set at 7%, as this was the lowest threshold which did not introduce outliers, potentially skewing the results. All reported results are based on this data subset.

DCA1 accounted for the geographical separation of samples, with the majority of samples from Senegal having positive values on dimension 1, whilst birds sampled from France largely had negative values (Figure 5.1). Following the removal of outliers, only one sample from Hungary was retained in analysis. When looking at the distribution of taxa consumed (Figure 5.2), there was an evident division between Europe and Africa. All seeds consumed in Africa, apart from *Sorghum* and *Helianthus* had positive scores, and all seeds

occurring only in Europe, but absent in Africa occurring in the negative pole of DCA1. Sorghum, which was commonly detected in both French and Senegalese samples, was close to 0 for DCA1, and *Helianthus*, which was consumed in Senegal, but less commonly, had a more negative DCA1 score. There was less of a clear pattern to be observed in DCA2, but it does illustrate less variation in the diet of birds samples from France, with points being grouped more closely together, with positive values on dimension 2. Birds sampled in Senegal were distributed more widely across the poles, with greater dissimilarity between the points indicating more varied diet.

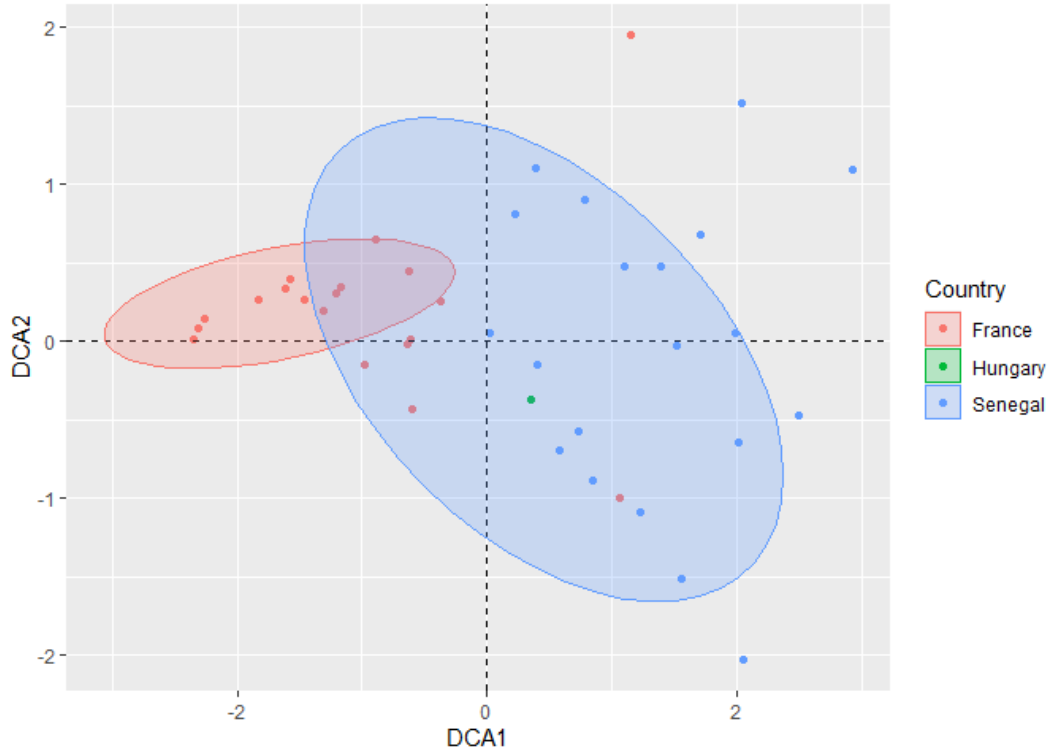


Figure 5.1: Biplot illustrating the variance accounted for by DCA1 and DCA2, with the associated Eigenvalues of 0.827 and 0.568, respectively. Shaded ellipses represent 95% confidence interval

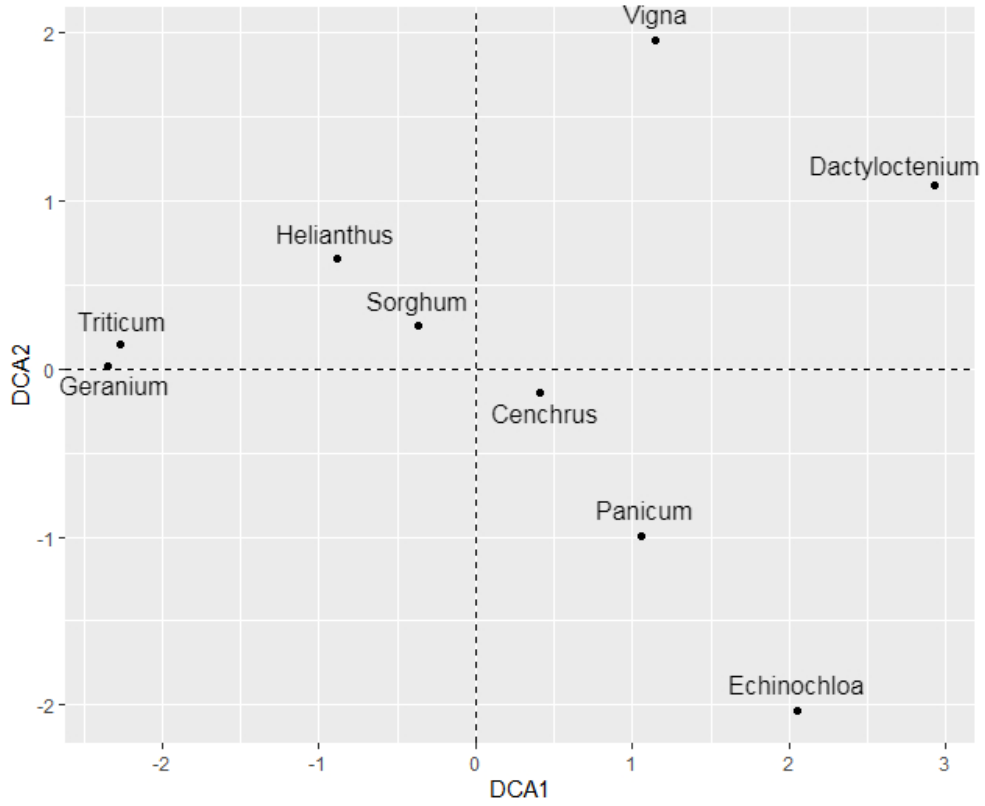


Figure 5.2: Biplot showing the distribution of dietary taxa across DCA1 and DCA2

DCA1 and DCA2 explained a high proportion of the variation observed in this data set, as illustrated by the elbow observed in the scree plot at DCA3, indicating a smaller amount of variation in the data was explained in DCA3 and DCA4. Therefore, DCA1 and DCA2 only were included as explanatory variables in GLM analysis (Figure 5.3).

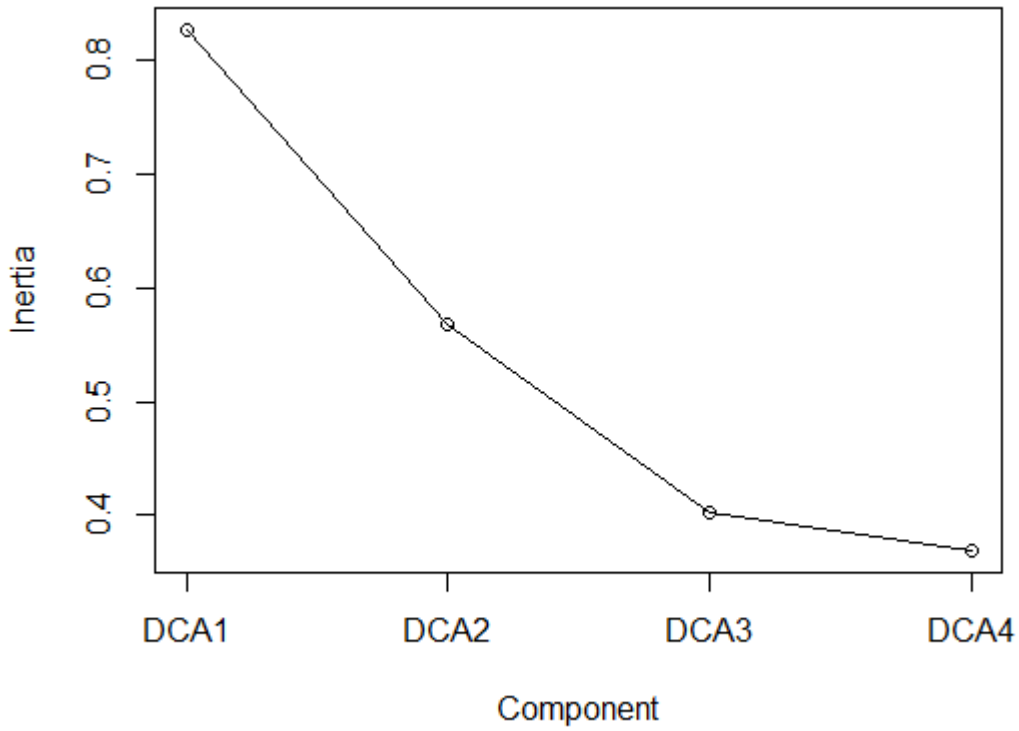


Figure 5.3: Scree value illustrating the Eigenvalues of the four DCA dimensions. DCA1 and DCA2 were included in GLM models.

The selected data set contained only one sample from Hungary. This sample was removed for GLM analysis following model assumption testing, as this point had leverage equal to zero, potentially disrupting the model. When assessing the relationship between infection status, season and diet, season was the only variable to affect presence of *T. gallinae* (GLM: LRT = 33.494_4, *p<0.001*). When considering individual strains, no variables had a significant effect on the presence of Tcl-1 or Type C, but season had a significant effect on the presence of GEO (GLM: LRT = 48.809_4, *p<0.001*) and Type IIIc (GLM: LRT = 13.5121_4, *p = 0.009*). Seasonal variation of *T. gallinae* strains is discussed in more detail in Chapter 4 (4.4.3). The variables explaining dietary variation (DCA1 and DCA2) did not affect the presence of any strain of *T. gallinae*.

5.4.2 Dietary overlap

Pianka's niche overlap suggests significant dietary overlap between infected and uninfected individuals on the wintering and breeding grounds sampled in

this study. The lowest overlap was observed on the wintering grounds (Pianka = 0.577, *p≤0.001*, SES = 4.632), with almost complete overlap identified on the breeding grounds (Pianka = 0.943, *p≤0.001,* SES = 8.592). When visualising dietary segregation in using NMDS spider plots, centroids are close together, supporting this result of little difference in the diet between infected and uninfected individuals (Figure 5.4, Figure 5.5).

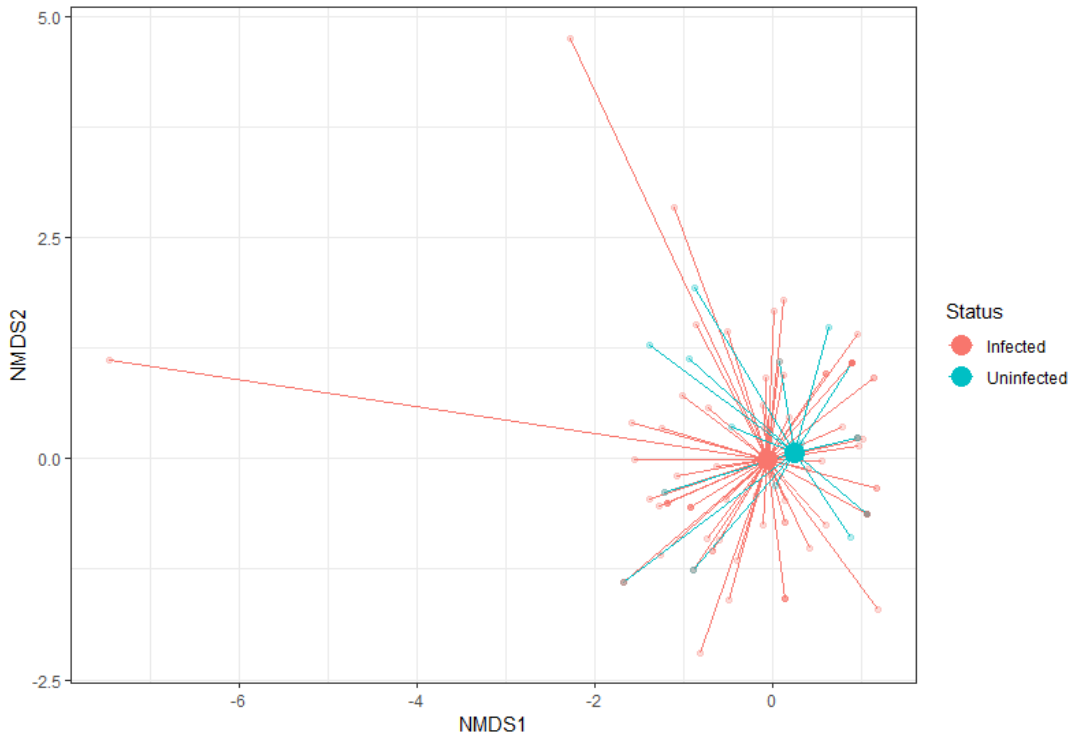


Figure 5.4: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves in the wintering grounds, differentiated by status of infection with *T. gallinae.* Stress = 0.07, N = 122. Ordination of multidimensional data condenses information into two-dimensional axes, representing variation in the diet. Each small point is representative of the diet of a single turtle dove sampled, and the distance between the points is representative of the similarity of diet composition between individual (a shorter distance between points indicates greater similarity between diets). The larger points forming the centroid of each group (season) represent the mean co-ordinates per group.

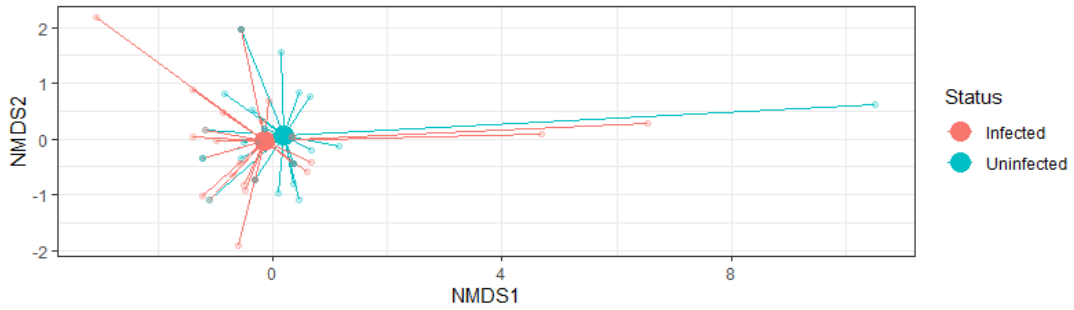


Figure 5.5: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves in the breeding grounds (this study), differentiated by status of infection with *T. gallinae.* Stress = 0.07, N = 71.

When considering sampling seasons separately, the winter of 2015/16 (W2) and spring 2018 samples from Hungary (S2) had small sample sizes, and all individuals sampled in winter 2014/15 (W1) were infected, thus Pianka's niche overlap between infected and uninfected individuals could not be performed on these sampling seasons. However, when looking at years sampled in Senegal individually, a higher level of dietary overlap was detected than when looking at the data as a whole, with a relatively consistent level of dietary overlap between infected and uninfected individuals across the winters of 2016/17 (W3) and 2017/18 (W4) (W3: Pianka = 0.874, $p \leq 0.001$, SES = 7.898; W4: Pianka = 0.824, $p \leq 0.001$, SES = 7.602). The dietary overlap was highest in France (S1), where almost complete dietary overlap was observed between infected and uninfected individuals (S1: Pianka = 0.957, $p \leq 0.001$, SES = 8.635). The lowest dietary overlap between infected and uninfected individuals was observed in data collected from the UK (Pianka = 0.770, $p \leq 0.00$, SES = 5.479) (Dunn *et al.* 2018; Thomas *et al.* 2022). The close proximity of centroids to each other when visualising overlap support the high level of dietary overlap between infected and uninfected individuals (Figure 5.6).

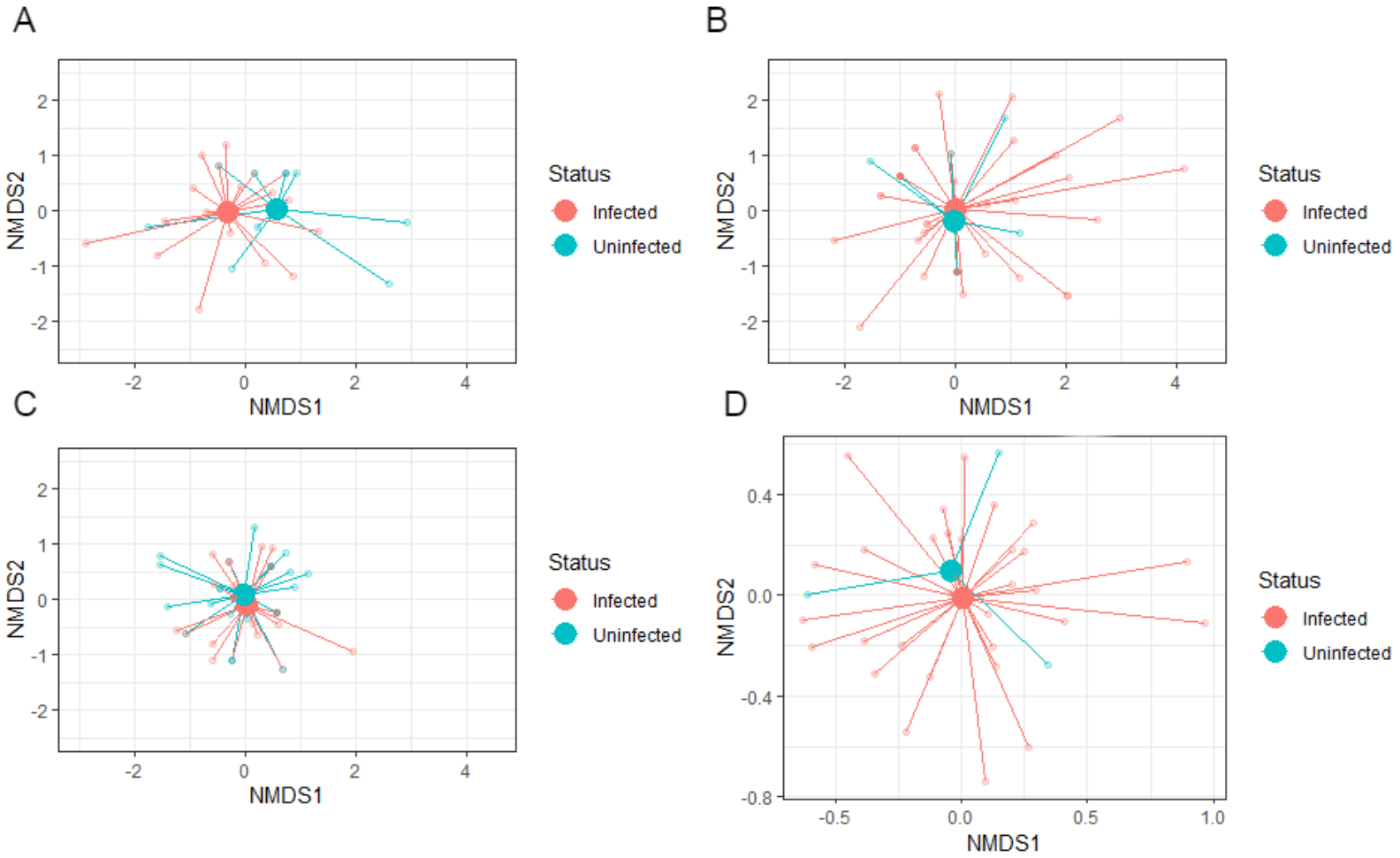


Figure 5.6: Spider plots derived from non-metric multidimensional scaling of genera consumed by turtle doves in different sampling season, differentiating dietary composition based on bird's infection status with *T. gallinae.* A) Winter 2016/17 (W3), stress = 0.07, N =

25. B) Winter 2017/18 (W4), stress = 0.05, N = 67. C) Spring, France (S1), stress = 0.13, N = 56. D) Spring, UK (Dunn et al. 2018; Thomas et al. 2022), stress = 0.25, N = 34.

Dietary composition was investigated in relation to strains present, considering the four dominant strains infecting turtle doves: GEO, Type C, Type IIIc and Tcl-1, as well as uninfected individuals. The average pairwise overlap was greater in the breeding grounds (Pianka = 0.807, *p≤0.001,* SES = 22.218) than in the wintering grounds (Pianka = 0.765, *p≤0.001,* SES = 21.265), but both exhibited fairly strong overlap of dietary items consumed by individuals infected with different strains of *T. gallinae*. When visualising dietary overlap, centroids representing different strains in both the breeding and wintering grounds are grouped together, supporting the high level of dietary overlap detected by Pianka's niche overlap (Figure 5.7, Figure 5.8). This being said, in the breeding grounds, the strain GEO is segregated from the other points, suggesting that there may be some dietary segregation in this strain (Figure 5.8).

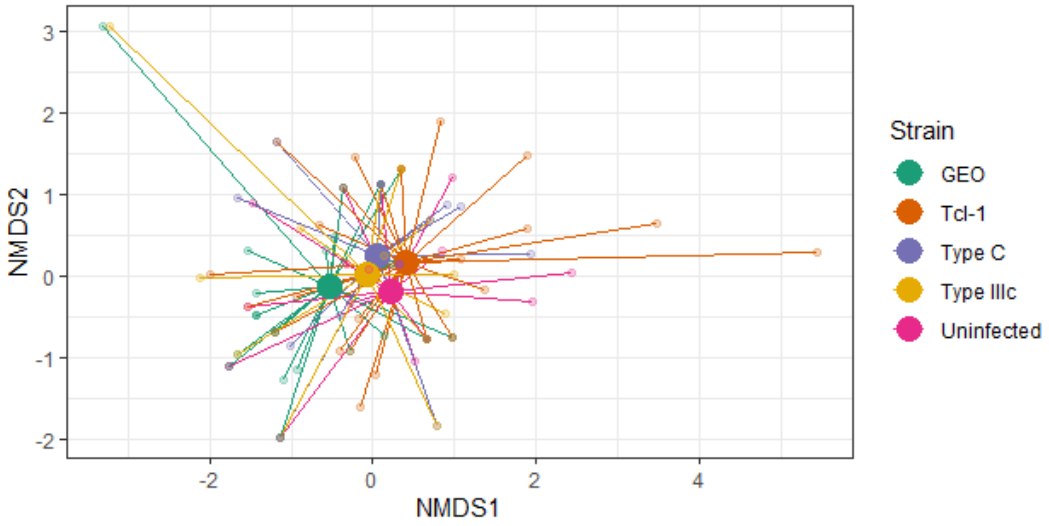


Figure 5.7: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle across birds sampled in the wintering grounds, differentiated by the strains of *T. gallinae* present. Stress = 0.066, N = 110.

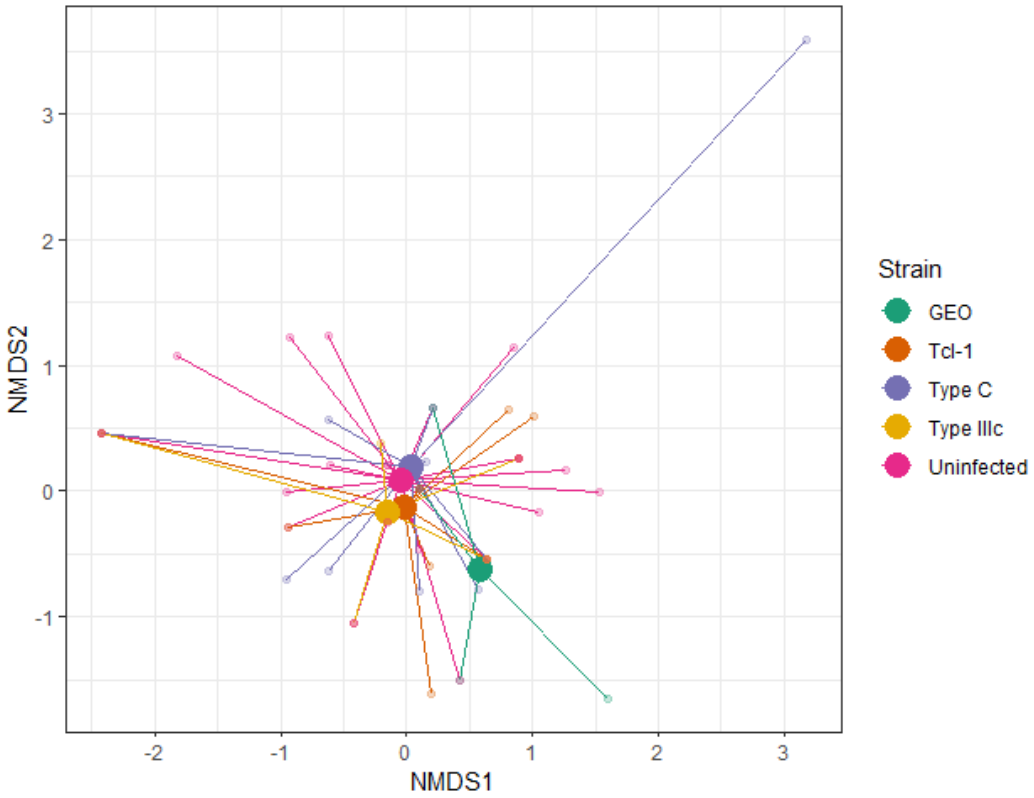


Figure 5.8: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle across birds sampled in the breeding grounds, differentiated by the strains of *T. gallinae* present. Stress = 0.086, N = 63.

When assessing Pianka's niche overlap on individual sampling seasons, low dietary overlap was detected in W1 and W3 (W1: Pianka = 0.418, *p≤0.001*, SES = 6.459; W3: Pianka = 0.460, *p≤0.001*, SES = 9.464), which is supported by the distribution of centroids in Figure 5.9A and Figure 5.9B. Much higher dietary overlap between birds infected with different strains was observed in W4 and S1 (W4: Pianka = 0.785, *p≤0.001*, SES = 22.325; W3: Pianka = 0.835, *p≤0.001*, SES = 23.83) (Figure 5.9C, Figure 5.9D).

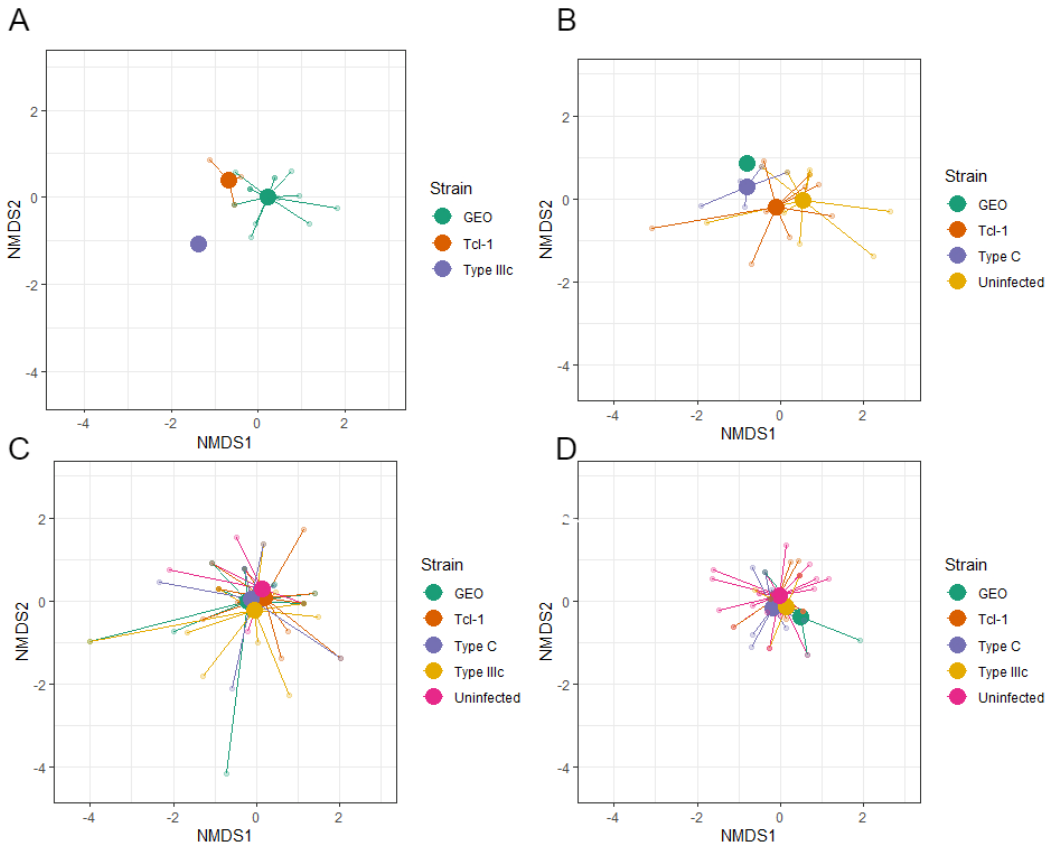


Figure 5.9: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle across birds sampled in the breeding grounds, differentiated by the strains of *T. gallinae* present. A) Winter 2014/15 (W1), B) Winter 2016/17 (W3), C) Winter 2017/18 (W4), D) Spring, France (S1)

When analysing data collected from turtle doves sampled in the UK, strains assessed were GEO, Type A, Type C and Tcl-1. Two strains, Type IIIc and WQR-Env were only detected in one bird from this population, thus were removed from analysis as a rare strain, detected in 2.8% of the population sampled. Results for niche overlap in the UK were quite high, and a similar level to those observed in W4 and S1 (Pianka = 0.770, $p < 0.001$, SES = 5.479). When visualising these data, the overlap of centroids observed in UK data is not as strong as that of samples from W4 and S1 (Figure 5.10).

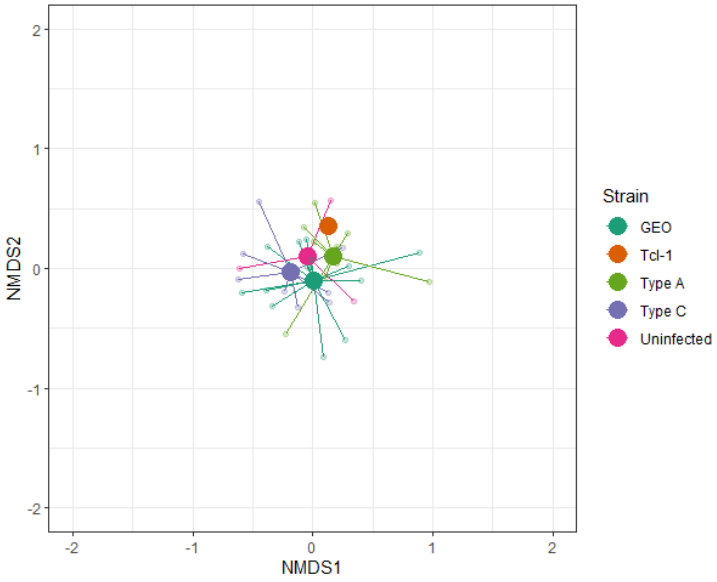


Figure 5.10: Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle across birds sampled in the UK, differentiated by the strains of *T. gallinae* present. Stress = 0.246, N = 34.

5.4.3 Source of food and *T. gallinae* infection

The proportion of diet accounted for by wild and cultivated seeds was considered in relation to infection with *T. gallinae* (N=190). Season was significantly associated with the presence of *T. gallinae* infection (GLM: Dev_5 = 33.608, *p≤0.001),* as well as strain specific associations for GEO (GLM: Dev_5 =46.65, *p≤0.001*) and Type IIIc (GLM: Dev_5 =22.71, *p≤0.001*), with seasonal variation occurring as discussed in Chapter 2 (4.4.3). The proportion of wild seeds consumed did not have an effect on the presence of *T. gallinae* infection, or on the presence of any individual strain of *T. gallinae.*

Overall, a higher proportion of individuals with a diet dominated by cultivated seeds were not infected with *T. gallinae*, whereas for both individuals with a diet dominated by wild seeds, and those with an even balance of wild and cultivated genera in the diet, a higher proportion of birds were infected with *T. gallinae* (Figure 5.11).

When considering countries in isolation, in France, the cultivated seeds were important in the diet of both infected and uninfected individuals, with 75% and 62% of uninfected and infected birds, respectively, having a diet dominated by cultivated seeds (Figure 5.12, Figure 5.13). In Hungary all uninfected birds and

the majority of infected birds (86%) had a diet dominated by wild seeds (Figure 5.12). Results were more variable in Senegal, where the greatest proportion of uninfected birds (58%) had a diet dominated by cultivated seeds, whilst the dominant food resource was more similar in infected individuals, with 40% of infected birds having a diet dominated by cultivated seeds and 39% of infected birds having a diet dominated by wild seeds (Figure 5.12, Figure 5.14).

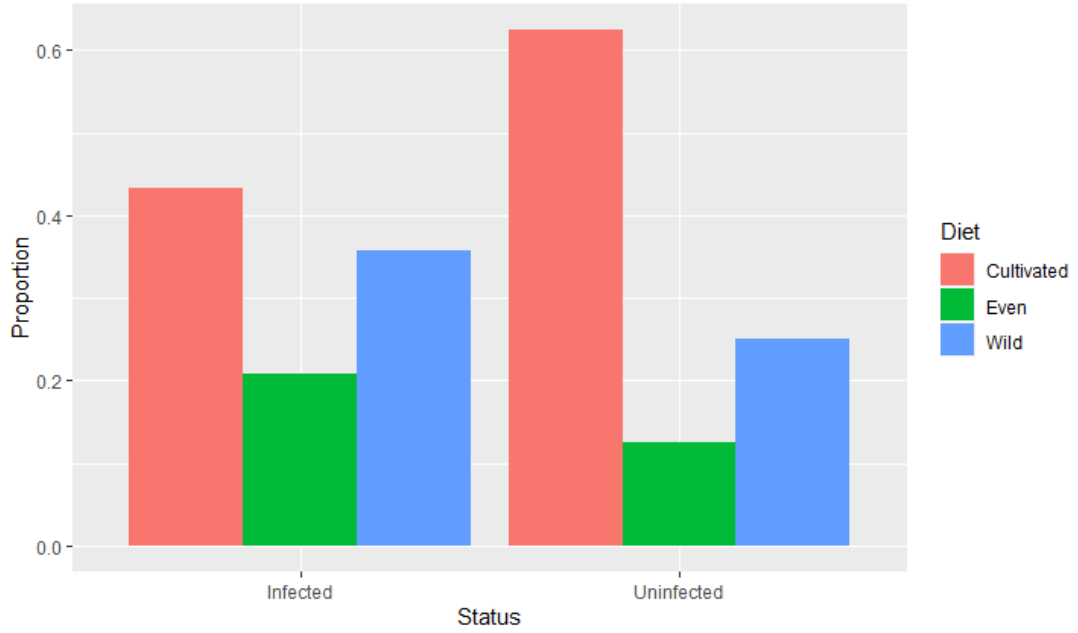


Figure 5.11: Proportion of total birds (N=190) infected or uninfected with *T. gallinae,* where the taxonomic units detected in the diet were dominated by wild or cultivated seeds, or with an even split of wild and cultivated food sources. Calculated based on the proportion of dietary items consumed by individuals accounted for by wild seeds. Infected birds, n=134, uninfected birds, n=56

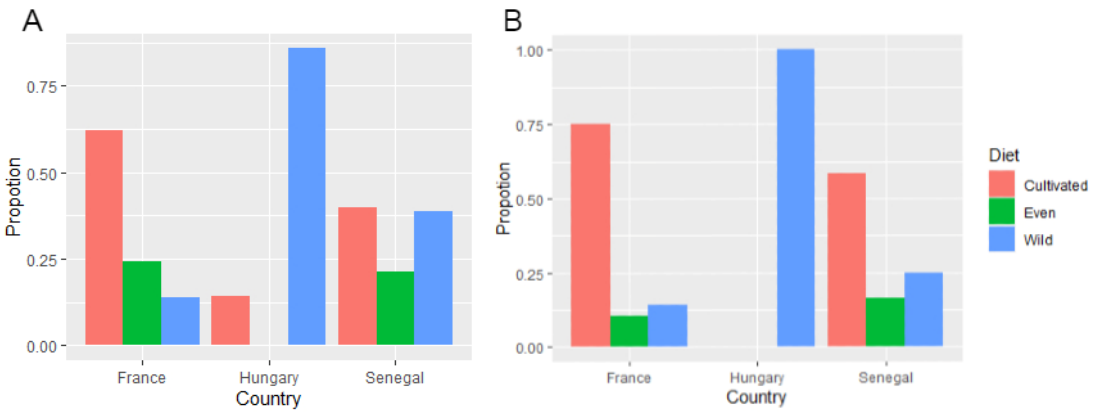


Figure 5.12: Proportion of birds A) infected (n=134) or B) uninfected (n=56) with *T. gallinae,* where the taxonomic units detected in the diet were dominated by wild or cultivated seeds, or with an even split of wild and cultivated food sources, separated by sampling location. Calculated based on the proportion of dietary items consumed by individuals accounted for by wild seeds.

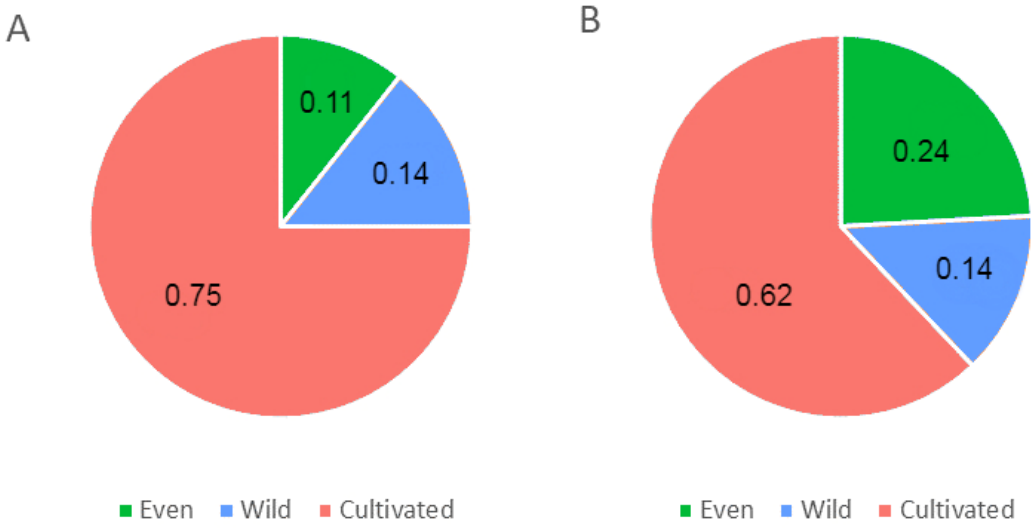


Figure 5.13: The proportion of individual birds sampled with a diet dominated by wild seed, cultivated seed, or a diet consisting of an even number of wild and cultivated genera in a) uninfected (n=28), and b) infected (n=29) individuals from France

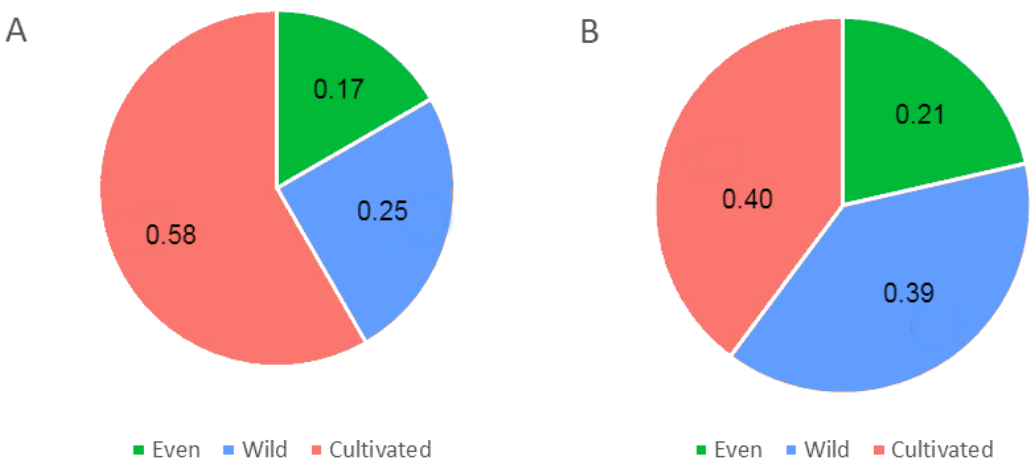


Figure 5.14: The proportion of individual birds sampled with a diet dominated by wild seed, cultivated seed, or a diet consisting of an even number of wild and cultivated genera in a) uninfected (n=24), and b) infected (n=98) individuals from Senegal

5.5 Discussion

Whilst there is a large body of research reporting the increased transmission of disease at supplementary food sites, this is one of only a small number of studies specifically investigating the relationship between diet and infection status of individuals. Very few significant associations were found between infection with *T. gallinae* and diet. Dietary variation was not found to have an effect on the probability of *T. gallinae* infection, and significant dietary overlap was observed between infected and uninfected individuals. When considering specific strains, there was again no significant dietary segregation identified.

5.5.1 The relationship between rate of infection and consumption of cultivated seed

Based on the findings of numerous previous studies implicating individuals congregating at supplementary feeding sites in the increased spread of disease (Pennycott *et al.* 1998; Dhondt *et al.* 2007; McBurney *et al.* 2015; Fogell *et al.* 2019), I hypothesised that infection with *T. gallinae* would be positively associated with the proportion of non-wild seeds in the diet. This is because a higher proportion of non-wild seed was expected to indicate increased use of supplementary food resources, thus increased chance of

contact with other individuals. However, I did not find evidence to support this hypothesis, with no association being detected between diet and overall infection status, and high levels of dietary overlap being observed when comparing the diet of infected and uninfected individuals using Pianka's niche overlap.

When looking at countries individually, 75% of uninfected birds in France had a diet dominated by cultivated seeds. These findings may be related to the method of supplementary food delivery. The rationale for my hypothesis that birds feeding on a greater proportion of cultivated seeds would be more likely to be infected with *T. gallinae* is based on previously published associations between this parasite and congregation of birds at food and water sources (Robinson *et al.* 2010; Gerhold *et al.* 2013; McBurney *et al.* 2015; Thomas 2017). However, it is possible that due to the nature of supplementary food delivery in this study, this was not the case, as it did not result in the congregation of animals. Bait was used to attract individuals into potter traps, whereby once they entered the trap and triggered the door, solitary individuals were shut in traps until they were extracted for processing. This contrasts with previously reported incidences of transmission at supplementary feeding stations, as these encourage the congregation of large numbers of individuals, facilitating disease transmission, and birds were not seen to congregate around potter traps *T. gallinae* has been shown to persist on seed resources for over 48 hours in laboratory conditions (McBurney *et al.* 2017), and has been detected in swab samples from seeds at a range of supplementary food sources (Thomas 2017). This persistence demonstrated in environmental supplementary seed means that risk of disease transmission may still have been elevated by the potential for multiple birds to attend the same seed pile within a day. However, large aggregations of individuals at a single site did not occur as a result of supplementary feeding in this study.

Similarly, in Senegal, cultivated seed dominated the diet in a higher proportion of uninfected (58%) than infected (40%) individuals. Due to the high level of agriculture around the capture site in Senegal and the abundance of cultivated seeds in the environment, such as sorghum and millet, which dominated turtle dove diet in the wintering grounds (Chapter 2, 2.4.2), it is most likely that birds

were obtaining this food resource from harvested fields. Whilst birds do feed in groups in harvested fields, these are spread over a wide area of arable land, rather than dense congregations observed at single bird feeding stations (*personal observation*). Foraging group sizes varied from two or three birds, to over 10, and whilst they were often all turtle doves, other African columbids such as the mourning dove (*Streptopelia decipiens*) and Namaqua dove (*Oena capensis*) would also be seen foraging in the area (*personal observation*). It has been previously shown that *T. gallinae* was less likely to be detected from low density supplementary feeding sites, such as sown seed plots, than high density feed sites, like seed piles (Thomas 2017). Therefore, it is likely that whilst birds may come into contact at a higher rate than if they were feeding on wild resources, large inter and intra-specific groups of birds would not congregate at feeding sites where cultivated seed is available to the extent that they do at bird feeders, reducing the risk of disease transmission in this instance.

As well as this potential behavioural explanation, it is possible that birds in France eating supplementary food were in better condition as a result of utilising this abundant food resource, and therefore more capable of fighting off infection, as associations have been shown between immune system functioning and body condition (Møller *et al.* 1998; Chandra 1999; Becker and Hall 2014). My previous dietary analysis detected a positive correlation between body condition and proportion of diet accounted for by supplementary seed (2.4.3), and birds captured in France, where such resources were commonly consumed, were in the best body condition. Furthermore, some commonly fed seeds, such as sunflower, which was included in bait in France, have a high calorific value (Hullar *et al.* 1999), which may help improve body condition by alleviating nutritional stress (Becker and Hall 2014).

In a study by Wilcoxen *et al.* (2015), forested sites where supplemental feed was provided were compared to control sites without supplemental food. They reported improvement of health metrics of birds at sites provided with supplementary food, including increased antioxidant levels, more rapid feather growth, reduced stress indicators, improved body condition index and improved innate immune defence (Wilcoxen *et al.* 2015). Positive effects were

also observed in the fitness measure of fat score for silvereyes (*Zosterops lateralis*) assessed by Galbraith *et al.* (2016) when investigating the effects of supplemental feeding. These findings support the suggestion that use of this supplementary food may improve body condition, and therefore boost immune function, reducing host susceptibility to *T. gallinae*. However, it is important to note that, whilst these studies did demonstrate positive impacts of supplementary feeding on hosts, they also both showed significant increase in parasite and disease transmission associated with supplementary feeding. Whilst this was not supported in the current study, it is still a key consideration when providing supplementary food (Wilcoxen *et al.* 2015; Galbraith *et al.* 2016).

Within natural systems, host-parasite interactions are very complex, and there is variability in the link between body condition and infection. We most commonly consider the negative impacts of parasite infection on host condition, with an array of negative consequences that can result from infection (Sánchez *et al.* 2018). For example, infection may reduce host activity, thus limiting foraging ability and food intake (Gegear *et al.* 2006) or infection may result in energetic costs, both as a direct impact of the parasite, such as ticks consuming host blood (Botzler and Brown, 2014), and through hosts mounting energetically costly immune responses against infection (Verant *et al.* 2014). However, there are also instances whereby host behaviour may incur positive condition-infection relationships (Sánchez *et al.* 2018). Foraging commonly leads to ingestion of parasite transmission stages, as discussed for the spread of *T. gallaine* at supplementary feeding sites (Altizer *et al.* 2006; Lawson *et al.* 2011a; Laaksonen and Lehikoinen 2013; Lennon *et al.* 2013). In instances where hosts eat more, they may simultaneously be in better condition, due to higher nutritional intake, and have a higher parasite burden, due to ingestion of more parasites (Hall *et al.* 2007, Sánchez *et al.* 2018). In such instances, the higher parasites burdens do not necessarily have negative implications for the host, for example, it has been observed in African buffalo that individuals in good body condition appear more able to tolerate higher parasite loads, as no fitness cost was detected when hosts infected with *Cooperia fuelleborni* were in otherwise good condition

(Budischak *et al.* 2018). It is possible that this may be a occurring in individuals asymptomatically infected with *T. gallinae.* Birds may have a high body condition as a result of good nutrition, which may protect them from clinical infection with trichomonosis, despite potential exposure to the parasite at feeding sites.

The prevalence of infection in France was low compared to other sites in the present study, and to previously published work on *T. gallinae* prevalence (Stockdale *et al.* 2015; Marx *et al.* 2017; Thomas 2017). As discussed above, this may be related to the body condition of individuals sampled at this site. Alternatively, it could be a result of mild exposure to parasites, as has been observed in house finches in response to the pathogen *Mycoplasma gallisepticum* (MG) (Moyers *et al.* 2018). The rapid spread of this bacterial pathogen has been linked to use of garden bird feeders, with transmission via fomites deposited at bird feeders by infected individuals (Dhondt *et al.* 2007; Moyers *et al.* 2018). It has been identified in two studies that exposure to MG at feeding sites may be linked to a level of immune protection against more severe symptoms (Dhondt *et al.* 2007; Moyers *et al.* 2018). Dhondt *et al.* (2007) provided the first empirical evidence of transmission of this pathogen indirectly between birds, via pathogens deposited at feeding stations. This study identified that finches infected via this route developed milder cases of the disease, from which they were quicker to recover than when birds were directly exposed to MG via infectious swabs, indicating that indirect transmission from pathogens deposited at supplementary feeding sites can result in lower levels of infection (Dhondt *et al.* 2007). Moyers *et al.* (2018) investigated the relationship between bird feeder density and infection with MG, hypothesising that a higher density of bird feeders would result in individuals staying at feed sites for longer, increasing pathogen transmission. Their results supported this, reporting higher disease transmission in treatments with higher feeder densities, but also finding that treatments with a lower density of feeders available resulted in birds having higher concentrations of MG-specific antibodies than those fed at high densities, but with very low rates of detectable infection (Moyers *et al.* 2018). This suggests that birds in the low feeder density

treatments may have been exposed to subclinical doses of MG, leading to them acquiring an immune response against this pathogen.

Previous work on *T. gallinae* has shown that prior infection with a less virulent strain of the parasite can infer protection against subsequent infection (Stabler 1948). It is therefore possible that birds feeding at potter traps in France, which are low density feeding stations, spread throughout the forest, may have been exposed to low levels of *T. gallinae* when feeding here, potentially resulting in some level of immune protection. Similarly, birds feeding on cultivated seeds in Senegal do forage in groups, but in lower densities than at supplemental feeders as seeds are dropped in harvested fields, again leading to the possibility of low-level exposure to *T. gallinae*.

I have discussed a range of potential mechanisms through which dietary choice and foraging behaviour may be influencing parasite infection, however, it is important to consider that this relationship may go in both directions: infection status may influence foraging behaviour, making it more difficult to discern associations in either direction. Parasite infection may affect a host's foraging behaviour in a number of ways. Negative clinical impacts on a host as a result of parasite infection may prevent it travelling as far to forage, alteration of a host's nutritional state through competition for nutrients from digested food, and behavioural manipulation of the host by parasites to facilitate their own transmission, are all examples of how infection status may influence host foraging behaviour (Barber and Huntingford, 1995; Levri and Lively, 1996; Ghai *et al.* 2015). With this in mind, it is important to consider how infection with *T. gallinae* may influence turtle dove behaviour. Clinical symptoms of trichmonosis includes difficulty swallowing, resulting in regurgitation of food which could impact the seeds infected individuals are able to consume (McBurney *et al.* 2015). In addition, the presence of a high occurrence of fault bars in the tail feathers of wild greenfinches (*Chloris chloris*) that died from trichomonosis are potential markers for a lack of food availability during feather growth, possibly indicating impaired foraging as a result of disease (Männiste and Hõrak , 2017, Quillfeldt, *et al.* 2018).

5.5.2 *Trichomonas gallinae* strains in relation to diet

As both the *T. gallinae* strain composition, and the dietary composition differed between the sites sampled in this work (Chapter 2, Chapter 3), it was hypothesised that this may be caused by an association between certain strains and food types. Previous work identified four strains of *T. gallinae* (Type A, Type C, GEO and Tcl-1) detected at supplementary food sites from the UK (Thomas 2017). Type A was the strain most commonly detected in food resources, followed by Tcl-1, indicating a possible link with these strains and survival on supplementary food resources. However, when considering the association of specific strains of *T. gallinae* with dietary items, I again found little support for any association between dietary items and specific strains, with significant dietary overlap being observed between individuals infected and uninfected with specific strains. In addition, when investigating the dietary overlap of birds infected with different strains per sampling season, no consistent patterns were observed across season, with lower dietary overlap being observed in two years in Senegal, and high dietary overlap occurring in one year in Senegal, and in France. This lack of support for dietary items being linked to specific strains suggests variation in the strains previously detected in supplementary food resources may reflect another unknown environmental factor, or simply reflected variation in the parasite community composition, rather than an association with dietary items. In addition to investigating niche overlap, when modelling the effects of dietary variation on the presence of *T. gallinae* infection, and specific strains of the parasite, no association was found between the diet and the presence of the parasite.

5.5.3 Conclusion

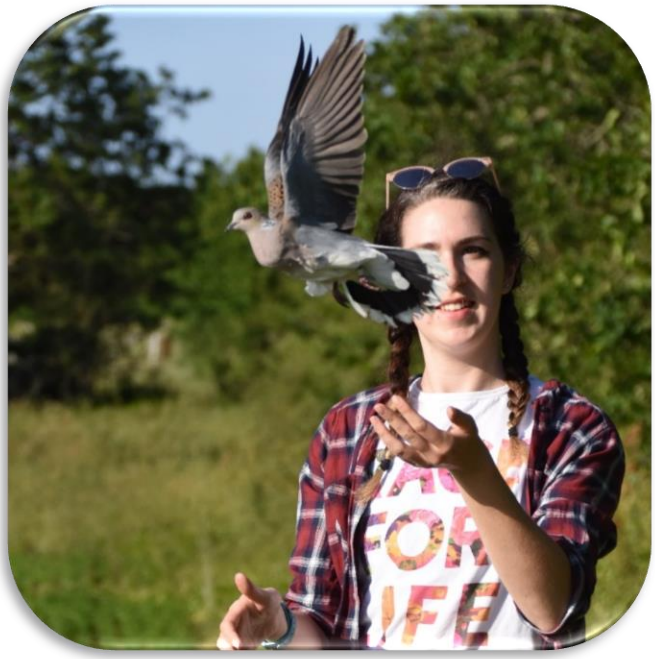
Whilst I was not able to find evidence to support my hypotheses, the results of this study potentially provide support for advice on supplementary feeding. As discussed, there are potential benefits of alleviating nutritional stress through supplementary feeding, which may improve body condition, such that improved immune function can fight off disease. Whilst it is important not to overlook the trade-off between potential disease transmission and nutritional benefits, it is possible that the mode of delivery of supplementary feed is important in reducing the impact of disease transmission. For example,

distributing supplementary seed over a wide area, similar to dropped grain in harvested fields in Senegal, may reduce the risk of disease transmission at bait sites.

5.6 Acknowledgements

This work was funded by the Natural Environment Research Council in the form of a GW4+ doctoral training partnership and the Royal Society for the Protection of Birds. I would like to thank my supervisors for support in bringing the elements of this project together in the final analysis, in particular Jenny Dunn and Ian Vaughan for their advice and insights into methods of analysing dietary overlap and correspondence analysis. I also thank Jenny Dunn and Rebecca Thomas for the inclusion of their data on diet and parasite infection of turtle doves in the UK.

Chapter 6 - General discussion



Photograph by Nicolas Viacrroze

6.1 Thesis summary

This thesis investigates two possible drivers of the decline of the European turtle dove: their diet, and infection by the protozoan parasite *Trichomonas gallinae*. Whilst these drivers are considered in the context of a single declining species in this study, both have wide scale relevance. A loss of natural food resources is contributing to the decline of species from a wide range of taxa across the world (Gibbons *et al.* 2000; Donald *et al.* 2001; Benton *et al.* 2003; Hester and Harrison 2007; Goulson *et al.* 2015). Most dietary studies of migratory birds focus solely on the breeding grounds (Browne and Aebischer 2003a; Rocha and Quillfeldt 2015; Gutiérrez-Galán and Alonso 2016; Dunn *et al.* 2018; Mansouri *et al.* 2019). However, survival and subsequent breeding success has been shown to be strongly related to winter food availability, in turtle doves and other species (Robb *et al.* 2008b; Eraud *et al.* 2009). This study is novel in its assessment of diet on both the breeding and wintering grounds, and the comparison of the two.

Trichomonas gallinae is a multi-host pathogen, with a vast geographical range, therefore having implications for many bird species and populations

(Bunbury*et al.* 2008a; Sansano-Maestre *et al.* 2009; Amin *et al.* 2014). High throughput sequencing (HTS) was used to detect dietary items from faecal samples, and *T. gallinae* infection and strain identity from oral swabs. This is only the second study, to my knowledge, to apply the use of HTS to identification of infection and co-infection of *T. gallinae* in wild bird populations, and the first to combine it with a detailed study of diet.

Metabarcoding and HTS have been widely used in dietary studies for a number of years, with the use of universal primers making it possible to detect a wide range of dietary items from a single sequencing run (Pompanon *et al.* 2012; Clare 2014; Piñol *et al.* 2018). Within this growing field, the development of new primers continues to improve the breadth of dietary items that can be detected, such as the developments of universal ITS2 primers to detect a wide range of plants (Moorhouse-Gann *et al.* 2018). Whilst it is still rarely used in the research area, HTS has great potential in the field of parasite ecology (Bourret *et al.* 2021), and is used in this study to detect co-infection, because it can simultaneously detect multiple parasite strains within a single sample. In addition to being able to obtain a greater level of information than using traditional sequencing methods, such as Sanger sequencing, HTS speeds up the process of analysing a large number of samples by pooling for sequencing runs, making it an efficient tool in ecological analysis. This study is unique in its approach of analysing both the diet and infection status of each individual sampled, offering a novel opportunity to investigate these two drivers of decline in conjunction, and to determine whether there is an interaction between the two.

All of these elements are important for conservation management. With high levels of anthropogenic changes in land use, a reduction in food availability is a notable driver in the decline of a wide range of species (Gibbons *et al.* 2000; Donald *et al.* 2001; Benton *et al.* 2003; Hester and Harrison 2007; Goulson *et al.* 2015). A common method of conservation management is provisioning of supplementary food resources to help make up for the shortfall in naturally available resources, or providing safe food, free of environmental contaminants, such as diclofenac-free food for vultures (Gilbert *et al.* 2007; Murray *et al.* 2016). This may be a necessary intervention in order to support

recovering populations in degraded habitats, where there are insufficient natural resources. However, consideration should be made to the most appropriate methods of food delivery, particularly in light of extensive evidence that the spread of a wide range of infectious diseases and parasites can be exacerbated by inter and intra-specific congregation at supplementary feeding sites (Lawson *et al.* 2018). *Trichomonas gallinae* is of particular note, having been the cause of widespread avian mortality following the outbreak of the trichomonosis epidemic in finches in the UK and Europe (Lawson *et al.* 2006; Robinson *et al.* 2010; Lawson, Robinson, *et al.* 2011).

This chapter will examine the findings of this thesis, discussing them in the context of conservation interventions, and considering how these findings can be applied to species management and conservation. The use of metabarcoding in analysis of diet and disease will be discussed, followed by the limitations of this study and areas for future work.

6.2 Ecological implications

6.2.1 The positive effects of supplementary feeding

One of the main findings of this study was the high level of cultivated seed in the diet of birds sampled on both the breeding and wintering grounds. This is consistent with the findings of other recent dietary studies, and supports the dietary switch from wild to cultivated seed described for this species (Browne and Aebischer 2003a; Dunn *et al.* 2018). I also identified a positive correlation between the presence of cultivated seeds in the diet and body condition, and observed that birds sampled in France exhibited the highest average body condition score, and the greatest proportion of cultivated seeds in their diet. This positive association between consumption of cultivated seeds and body condition has been previously observed in adult turtle doves caught in the UK (Dunn *et al.* 2018), and several other specific positive responses to supplementary food provisioning have been observed in birds. For example, reduction in the abundance of parasitic nest flies (*Protocalliphora sialia),* attributed to improved innate immune responses in the eastern bluebird (*Sialia sialis*)*,* rearing of larger broods in the Mauritius kestrel (*Falco punctuatus*) and improved winter survival in three species of crane (*Grus japonensis; G.*

monacha; G. vipio) have all been linked to supplementary feeding (Jones 2004; Knutie 2020). Whilst in the present study, supplementary food in the form of bait was only consistently provided for birds in France, birds were still accessing and consuming such cultivated resources elsewhere, potentially from garden bird feeders, and certainly from spilt grain in Senegal. Whilst the route of accessing the resources may be different, birds sampled in this study still showed a high rate of cultivates seeds in the diet, such as those generally provided in supplementary food.

Providing supplementary food to birds and other wildlife around the home is a popular method for people to connect with and support nature, practised by over 52.8 million Americans, and over 50% of UK households (Davies *et al.* 2012; Wilcoxen *et al.* 2015). It is also a commonly used intervention in the management of declining species, as food supply can have a large influence on species survival, and a paucity of natural food resource availability can be a key limiting factor for population sizes (Newton 1998; Jones 2004; Newton 2004a). In New Zealand, supplementary food is provided for the critically endangered Kakapo (*Strigops habroptilus*) to increase productivity, both by increasing the frequency at which birds breed, and the survival of offspring (Elliott *et al.* 2001). The pink pigeon (*Columba mayeri*) is endemic to Mauritius, and declined to around 10-12 birds in 1991 in a single remnant population, with one of the main drivers being habitat loss, due to extensive deforestation (Bunbury *et al.* 2008a). Despite the success of captive breeding and reindroduction of this species, this population must still be managed, as there are insufficient natural food resources to maintain the population, resulting in a need for supplementary feeding (Edmunds *et al.* 2008). Species management through supplementary feeding has also been found to improve the fledging success of Spanish imperial eagle (*Aquila adalberti)*, following a period of poor breeding success, which was attributed to a limitation of available prey, caused by disease outbreak (González *et al.* 2006).

As demonstrated by these examples, there is a strong argument for supplementary feeding in the management of declining species. A number of agri-environmental schemes have been put in place, designed to combat the loss of natural food resources resulting from extensive agricultural

intensification across Europe, in particular in the UK (Newton 2004b; UK Government 2021a). Such schemes can be very effective, particularly when very targeted, for example, in the case of the Cirl bunting (*Emberiza cirlus*) (Peach *et al.* 2001). By concentrating on a single population, in a very restricted region, numbers increased by around four-fold over a 10 year period in response to food provisioning (Peach *et al.* 2001). This programme promoted weedy spring-sown barley stubble over autumn sown crops, which increases availability of crucial food resources over winter and early in the breeding season (Evans and Smith 1994; Peach *et al.* 2001). Specific supplementary feeding schemes have been designed for both winter and summer support and are available specifically for turtle doves, with currently used supplementary seed mix containing oilseed rape, millet, canary seed and sunflower hearts, with the addition of wheat in summer and linseed in winter (UK Government 2021a; UK Government 2021b). However,

6.2.2 Negative impacts of supplementary feeding

Given the popularity of supplementary feeding, it has been the focus of many studies which have found that, as well as the positive effects previously discussed, there is an array of potential negative implications (Murray *et al.* 2016; Shutt and Lees 2021). Reported negative effects on individuals include protein deficiencies and a reduction in sperm quality, and wider ecological implications include increased disease transmission and an increase in interspecific competition as species benefit disproportionately to food supplementation (Murray *et al.* 2016; Lawson *et al.* 2018; Støstad *et al.* 2019; Shutt and Lees 2021). Many of the grains provided in supplementary feed are the cultivated seeds which have come to dominate the diet of turtle doves.

The dietary switch identified in turtle doves, from a diet dominated by wild seed resources to one heavily consisting of cultivated seeds as a result of agricultural intensification, occurred concurrently with a reduction in breeding output, suggesting a link between the two (Browne and Aebischer 2003a). Dunn *et al.* (2018) found that, in contrast to the findings in adult birds, nestling turtle doves were in better condition when fed on a higher proportion of wild seeds, suggesting the importance of these resources in early development. Furthermore, it has been shown that nestlings in better condition at seven days

old were more likely to survive for 30 days post-fledging, demonstrating a link between nestling survival and the availability of wild food resources (Dunn *et al.* 2017). Similarly, lower productivity per nesting attempt has been attributed to a lack of natural food resources in white-winged doves (*Zenaida asiatica*) fed only agricultural seeds compared to those fed a mix of agricultural and native seeds, and when investigating passerine birds living in urban environments (Pruitt *et al.* 2008; Chamberlain *et al.* 2009). These findings highlight that, whilst it may have benefits particularly to breeding adults, provisioning of supplementary food resources does not necessarily have positive effects on breeding success, and is not sufficient to halt and reverse species decline.

6.2.3 Considerations for supplementary feeding management plans

Turtle doves can produce two or three broods in a season, but this is becoming increasingly uncommon, with fewer nesting attempts later in the season, and the average number of nesting attempts undertaken by each pair per year being almost halved (Browne and Aebischer 2004; Browne *et al.* 2005). This is believed to be caused in part by autumn migration commencing earlier, and has been cited as a potential driver in the decline of this species, with simulation models indicating that the observed reduction in productivity has the potential to cause a 17% population decline per annum (Browne and Aebischer 2003b; Browne and Aebischer 2004). The dietary switch, resulting from a reduction in food availability is believed to be one underlying cause of this reduction in breeding performance (Browne *et al.* 2005). In England, early agri-environmental schemes focussed on providing turtle doves with nesting habitat, and schemes which would promote the establishment of semi-natural seed food resources, such as uncropped field margins, were more limited and less popular with landowners (Dunn *et al.* 2015). However, provisioning of food has become a higher priority, with work being conducted with farmers in key turtle dove breeding areas in England to increase feeding habitat availability and provide food resources early in the breeding season to enable birds to recover from their spring migration (RSPB 2022).

In turtle doves, the findings of Dunn *et al.* (2018) suggest that a reliance on supplementary resources could have negative implications for nestling

survival, due to the positive correlation between wild food and body condition. In addition, foraging habitat analysis in Senegal in the present study shows grassland to be favoured over both arable land and areas with a mosaic of natural and agricultural land. This indicates the importance of wild seeds as resources, as they are selected even in an environment where cultivated seeds are readily available. Therefore, I suggest that an integrated approach would be appropriate when managing this species. Currently available schemes are heavily orientated around provisioning of supplementary cultivated seed, which, as this study and others have shown, can have positive effects on adult body condition (Dunn *et al.* 2018). This would be particularly beneficial early in the breeding season, in supporting adults in preparing to breed following migration. However, due to the potential negative effects of reliance on cultivated seed (Browne and Aebischer 2003a; Dunn *et al.* 2017; Dunn *et al.* 2018), this supplementary feeding should be combined with the establishment of uncultivated areas in farmland in order to increase access to high quality wild seed resources for developing nestlings. Once established, these areas will naturally regenerate and continue to provide food resources to wildlife, and have been found to have positive effects on other taxa, including pollinators (Dunn *et al.* 2013). The aim of this combined provisioning is that initial supplementary feed would encourage earlier egg laying, increasing the chance of a second and third brood and greater yearly population recruitment, while access to wild resources would increase the post-fledging survival rate.

It is important to note that these interventions are not a ‘one size fits all’ solution, and an animal’s feeding ecology can play a key role in how well they work. Turtle doves are ground- feeding granivores, which do not forage in dense vegetation, rather they favour a sparser habitat, with easy access to dropped seeds. It has been shown by Dunn *et al.* (2015), by comparing experimental plots, that turtle dove foraging was more likely to occur in managed plots with lower density vegetation. Management techniques used included scarification and topping to prevent sward growing at too high a density for turtle doves to forage (Dunn *et al.* 2015). This is important for farmers wishing to attract this species to their land and support the declining population, however does come with the drawback of increased effort to

manage the land appropriately. It has also been observed that soil type can contribute to the density and structure of field margins, with sandy or chalky soil resulting in lower vegetation density, more suited to turtle dove foraging (Dunn *et al.* 2015). This is consistent with the study site in Senegal, where soil is very sandy, and dry during the wintering season, resulting in relatively sparse grassland vegetation, which was selected for foraging. In addition, under these soil conditions, appropriate turtle dove foraging habitat is more likely to become established when land is left unfarmed and allowed to grow naturally, as often the weed seeds may be present in soil seed banks. It may therefore be beneficial to target farmers with these soil types in turtle dove breeding regions to improve their food availability, without the need for as much input from farmers. This may increase uptake due to less need to manage the land to ensure the sward does not become too dense for turtle dove foraging.

6.2.4 Diet on the wintering grounds in Senegal

The present study, unlike a lot of previous work, also considers the diet on the wintering grounds, and how this may be contributing to the decline of this species. The importance of wild food resources on the wintering ground is indicated by the selection of grassland as foraging habitat in Senegal. Despite the abundance of cultivated seed in the diet, and the high availability of farmed areas within the landscape, the most highly selected habitat type was grassland, with areas of land use comprising cultivated food resources, such as natural and agricultural mosaic and arable land, being ranked lower for habitat selection.

When considering the diet in relation to body condition of birds sampled in Senegal, an increase in body condition occurred as the season progressed, concurrent with an increase in the proportion of diet accounted for by wild seeds. It is likely that body condition improved as birds invested more resources later in the season in building up muscle and fat reserves in preparation for the energy demanding migration (Cooper *et al.* 2015). From the present study and literature currently available, it is not possible to determine what drove this increase in wild seed consumption later in the wintering season. It is possible that wild seeds are selected later in the season

for their nutritional value, to prepare the body for migration. Alternatively, as discussed in Chapter 3, the increase in wild seeds later in the wintering season may be a result of crop seeds being depleted following harvest from November to January. Whilst I cannot identify the cause, these findings of an increase in consumption in wild resources as the season progresses provide evidence of the importance of these weed seeds in the wintering grounds. Whether they are consumed for their nutritional value, or as an alternative due to a lack of the cultivated resources, which are abundant early in the wintering season, it is crucial that these wild resources are available to migratory birds in order to gain the energy reserves they need to make their long journeys.

Whilst schemes such as those agri-environment schemes operating within Europe are uncommon in African countries, the findings of this study highlight the importance of preserving natural grassland in this region, and provides a strong backing for re-greening initiatives. Global initiatives have been brought about to try to combat desertification in Africa, with goals aimed at promoting the sustainable use of terrestrial ecosystems to halt and reverse land degradation and the loss of biodiversity associated with it (Thomas *et al.* 2020). It is crucial to promote the preservation of grasslands and natural areas within farmland across the Sahel and Sub-Saharan Africa, regions upon which so many migrant species rely.

6.2.5 The risk of disease transmission

Another potential, and widely reported negative effect of supplementary feeding is the increased spread of disease (Pennycott *et al.* 1998; Giraudeau *et al.* 2014; Sorensen *et al.* 2014; McBurney *et al.* 2015; Lawson *et al.* 2018). There is much evidence of birds congregating at feeding sites causing increased disease transmission, in particular in relation to *T. gallinae* (McBurney *et al.* 2015; Lawson *et al.* 2018). Whilst infection of turtle doves by *T. gallinae* was detected in all sites in this study, the prevalence was lower than has been reported in previous work (Stockdale *et al.* 2015; Marx *et al.* 2017; Thomas 2017). All detected infections were, as far as could be detected from examination of birds in the field, asymptomatic, and no negative correlation between infection status and body condition was observed.

Contrary to my hypothesis that increased consumption of cultivated seed would be positively correlated with infection with *T. gallinae*, I did not detect an association between the abundance of cultivated seed in the diet and prevalence of *T. gallinae*. I hypothesised that this may be, in part, due to the fact that much of the supplementary food consumed in this study was not from bait sites or bird feeders where birds congregate. Rather it was dropped seed found in cultivated fields in Senegal, or bait piles in traps which would only hold a single bird at a time in France. This assumption was supported by a meta-analysis conducted by Becker *et al.* (2015), who found that whilst intentional supplementary feeding sites generally increased the risk of disease transmission, accidental feeding, which included in agricultural fields, generally had a small, or negative effect on infection measures. This suggests that delivering supplementary feed in such a way that limits congregation at feeding sites, such as spreading it over a wider area, does not increase the rate of *T. gallinae* transmission, thus is preferable to feeding stations which encourage congregations of individuals, and should be considered in the planning of food provisioning interventions.

Based on the findings of this study of a lower prevalence of *T. gallinae* than previously reported in turtle doves, a lack of clinical symptoms and a lack of evidence for increased transmission at supplementary feeding sites, I suggest that the transmission of *T. gallinae* is not a strong driving factor in the decline of this species, rather the lack of appropriate food is a more important contributor to population decline. Whilst mortality in turtle doves has been associated with *T. gallinae* infection (Stockdale *et al.* 2015), columbids are the natural host of *T. gallinae,* and asymptomatic parasite infection is common (Stabler, 1948). The most significant declines caused by trichamonosis have occurred in passerines, which were novel hosts (Lawson *et al.* 2011), thus I suggest that turtle doves are not as likely to suffer clinical symptoms and mortality following infection with *T. gallaine*, potentially due to a longer history of co-evolution.

6.3 Methodology

Metabarcoding and HTS are commonly used for dietary analysis across a range of taxa (Pompanon *et al.* 2012; Cuff, Drake, *et al.* 2021; Hacker *et al.*

2021; Shutt *et al.* 2021), although surprisingly few studies have been on columbid diets. This method is being used increasingly to describe biological communities in a number of other contexts, including studying gut microbiomes, characterising microbial communities in soil and aquatic systems, and using environmental DNA for conducting community surveys (Tedersoo *et al.* 2014; Deiner *et al.* 2017; Pollock *et al.* 2018). There is also an increasing number of examples of the use of metabarcoding to study disease, such as assessing the gastrointestinal nematodes in cattle, detecting amphibian pathogens in environmental samples and characterising ecto- and endo-parasites of fish (Scheifler *et al.* 2019; Sachs *et al.* 2020; Wang *et al.* 2020). However, it is not yet widely applied within the field of parasitology, but holds great potential for surveying parasite communities (Bourret *et al.* 2021).

One aspect that holds back the use of metabarcoding to describe parasite communities is the diversity of parasites: when considering avian parasites alone, these are documented from four kingdoms and 11 phyla (Bourret *et al.* 2021). This makes development of universal parasite primers challenging, but for studies of different strains within a single parasite, this method is available and ready to be applied, as it has been for the study of *Trypanosoma cruzii* in a range of hosts, including rodents, bats and non-human primates (Dario *et al.* 2017; Herrera *et al.* 2019; Pronovost *et al.* 2020). To my knowledge, only one other study has used metabarcoding to assess the communities of *T. gallinae* within avian populations (Thomas 2017). Wider application of this methodology would allow a greater understanding of parasite disease dynamics.

The present study detected six previously reported strains of *T. gallinae*, differentiated by the ITS region, including two which had not been commonly reported (Chapter 4, Table 4.4). GEO-TD was previously reported from one European turtle dove caught in Senegal, and in this study was detected in five birds, from Senegal and Hungary. The strain ESWD, detected in one bird from Senegal in this study, has been reported from an Emerald spotted wood dove (*Turtur chalcospilos*). This study also increased the reported diversity of this parasite with four new strains being detected, all from birds sampled in Senegal. Two were most genetically similar to GEO, one to Type IIIc and one to Tcl-1. As well as new strains, seven new sub-types were detected in this

work, increasing the known diversity of Type C in particular, with four new C sub-types being detected, one of which (C6-TD) was the fourth most commonly detected sub-type in this study, present in birds caught in both France and Hungary.

The current study is the second to analyse the rate of co-infection of multiple strains of *T. gallinae* within a single host, and detected a higher rate of co-infection than previously observed (Thomas 2017). There are indications both from the current study, and that of Thomas (2017), that certain strains co-occur less frequently than would be expected if infection were random, and there have been no detected instances of strains co-occurring more frequently than expected. This suggests that there may be a mechanism acting to suppress co-infection with multiple strains. Examples of such mechanisms which have been observed in other systems and could be acting here include competition between parasite strains within the host and effects of the host's immune response (Dobson 1985; Magalhães *et al.* 2019).

It has been shown in an *in vitro* study of co-infection with two strains of *Trypanosoma cruzii* in human cells, that there was a ten-fold decrease in the rate of co-infection after 72 hours (Magalhães *et al.* 2019). Understanding the cause of the negative co-infections detected among strains of *T. gallinae* would be valuable in the context of understanding how prior or concurrent infection with different strains affects host response to infection. It has been previously shown that infection with mild strains of *T. gallinae* can result in protection against mortality caused by more virulent strains (Stabler 1948). These findings present a promising basis that infection with milder strains confers future protection against mortality; however, this was based on a very small sample size, and before the time of strain genotyping.

6.4 Future work

6.4.1 Nutrient content of dietary resources

Whilst supplementary seed is commonly provided to wildlife, there is limited evidence that such seeds contain adequate nutritional resources to support the nutritional needs for development, survival and reproduction of nestling and adult turtle doves (Murray *et al.* 2016). Detrimental health effects have

been associated with high rates of supplementary food provisioning, including sperm abnormalities being associated with a diet high in sunflower seeds and higher gastrointestinal parasite loads being associated with protein deficient diets, as a result of reduced immune function (Ezenwa 2004; Støstad *et al.* 2019).

Seeds commonly provided to birds include peanuts, sunflower seeds, sorghum, maize and millet. There is limited evidence available as to how wild seeds and cultivated seeds compare in nutritional value, but protein intake has been highlighted as an issue for the successful growth and fledging of chicks reliant on cultivated seeds, with native seeds such as *Helianthus* sp. and *Croton* sp. having higher protein and fat content of sorghum (Pruitt *et al.* 2008). There is a paucity of information regarding the nutritional value of both cultivated and wild seeds, therefore it is difficult to identify specific nutritional consequences of the dietary switch from wild to cultivated seeds. In order to bridge this gap of knowledge, analysis of the macronutrient content of food resources would be highly beneficial. A recently developed protocol by Cuff *et al.* (2021) (MEDI) determines the protein, lipid and carbohydrate contents of invertebrate samples. Whilst it has not yet been tested on plant matter, if this protocol works for seeds, it would provide a cost effective and efficient way of obtaining macronutrient information for key food resources. Completing this analysis on a range of commonly consumed cultivated and wild seeds, such as those identified in this study, would facilitate a comparison between the nutritional value of wild and supplementary food resources. This could be used to determine shortfalls in nutrition occurring when natural resources are unavailable, and to provide a basis to advise on the most appropriate food to use when food provisioning is necessary for species survival. Such information would be beneficial across species management projects which are reliant on supplementary feeding in order to provide the best food possible to support vulnerable populations.

6.4.2 Population dynamics of *Trichomonas gallinae*

One aspect that almost all studies of *T. gallinae* on wild birds have in common, the present study included, is that they are only able to provide a snapshot of the infection status of birds carrying *T. gallinae* (Lawson *et al.* 2006; Sansano-

Maestre *et al.* 2009; Forzan *et al.* 2010; Marx *et al.* 2017; Thomas 2017). This leaves many questions regarding the dynamics of this parasite infection unanswered. For example, it is unclear how rapidly birds are able to clear infection of this parasite, how likely they are to become re-infected and within what time-frame this may occur, if this varies between species of host and parasite strains and the implications for repeated infection on host survival. One exception is work carried out by Bunbury *et al.* (2008a) on the Mauritian pink pigeon. This work tested birds every two months over a two year period for the presence of *T. gallinae.* Endemic to Mauritius, this population is ideal for disease monitoring for several reasons. The small population, spatial confinement and regular use of aviaries for supplementary feeding makes it easier to repeatedly capture and sample all birds within this limited population (Bunbury *et al.* 2008a). Furthermore, as a recovering population, these birds are intensively monitored. Records are kept of population trends and life-history details of individuals; at the time of this study, virtually the entire population had accurate sex and age data, and over 99% of adult pink pigeons were ringed with both metal rings and unique colour combination rings (Bunbury *et al.* 2008b; Carl Jones, *personal communication*). This population gives the unique opportunity to gain a full and detailed picture of infection dynamics in relation to host life-history. This previous study in pink pigeons detected the prevalence of *T. gallinae* within the population, an increase in infection rate with increased host age and a negative impact on survival in birds consistently testing positive for this parasite (Bunbury *et al.* 2008a). However, this study only reported the presence or absence of infection, and no genetic sequencing of *T. gallinae* was carried out. By processing isolated parasite samples using HTS, it would be possible to expand on the initial work conducted by Bunbury *et al.* to answer more in depth questions about this parasite's population dynamics. Using parasite strain information, it would be possible to discern if individuals repeatedly testing positive for *T. gallinae* is likely a result of a prolonged, or repeated infection; with a change in the strain detected suggesting a new infection, rather than prolonged. Furthermore, one could assess which strains are abundant, whether certain strains are more likely to be associated with pathogenicity, whether individuals become re-infected with the same strains and if prior exposure can have implications for

the pathogenicity of subsequent infections. This latter point relates to the previously discussed work by Stabler (1984), which suggested protective effects of prior exposure to milder *T. gallinae* strains. A more in depth understanding of how infection of different strains manifests in hosts previously exposed to the parasite could hold great potential for the development of vaccination programme to protect the most vulnerable species against declines caused by this parasite. Individual bird's genetics could also be considered, as specific genes and the rate of inbreeding may be associated with the host's susceptibility to infection. Work in this system could also build on the co-infection work in the present study, investigating the rate of co-infection in another avian species, and assessing if infection with multiple strains, over single strain infection, has implications for survival.

Finally, due to the necessity of supplementary feeding to support the pink pigeon population, the correlation between use of supplementary feeding and parasite infection could also be further investigated using this study system. By recording the frequency with which individual birds use feeders and how many conspecifics they come into contact with, it would be possible to investigate more thoroughly the implications of the use of bird feeders on disease transmission within this population, and potentially trial interventions and feeder designs to mitigate the spread of disease. This system also provides the opportunity to investigate intraspecific contact at bird feeders, which is also known to be a driver in disease transmission. Initial work has been carried out in this area, studying the presence of *T. gallinae* in alternative hosts, such as the Madagascar turtle dove (*Streptopelia picturata*), the spotted dove (*Streptopelia chinensis*) and the zebra dove (*Geopelia striata*) (Bunbury *et al.* 2007), however, this work again lacks information about genetic diversity of the *T. gallinae* strains infecting these species.

6.5 Concluding remarks

Through the multi-disciplinary approach of this thesis, I have presented the first in-depth study into the diet, rate of *Trichmonas gallinae* infection, and how these two drivers of species decline are associated with each other in the European turtle dove, on both the breeding and wintering grounds. It has provided the basis for further advice to improve species management through

supplementary feeding, which can be applied to this species, and others, as well as identifying areas for future research.

Through analysis of diet and habitat use, I have shown that both wild and cultivated seeds now play a key role in the diet of turtle doves, and highlighted the importance of preserving wild seed availability in the wintering grounds, where there are fewer schemes in place for species conservation. *Trichomonas gallinae* remains prevalent in the populations studied in this thesis and new strains have been identified, further increasing knowledge on the genetic diversity of this parasite. The lack of significant association between parasite infection and diet is important and I suggest is a result of feeding behaviour, which can be used to inform supplementary feeding methods for species reliant on this form of management.

Appendix 1 – Supplementary information relating to Chapter 2

1.1 DNA extraction and sequencing of bait seeds

Seeds from the seed mix in France were identified by Sanger sequencing. Seeds were homogenised using a TissuLyser II (Qiagen, Hilden, Germany) with a stainless-steel bead (~3-7mm in diameter). DNA extraction was carried out using Qiagen DNeasy blood and tissue kit, following manufacturers protocols. Extracted DNA was amplified by polymerase chain reaction (PCR) for sequencing. The primer pair S2F (5'-ATGCGATACTTGGTGTGAAT-3') and S3R (5'-GACGCTTCTCCAGACTACAAT-3') (Chen *et al.* 2010) were used to amplify a 387-547bp amplicon from the ITS2 region. PCRs were 15µl, consisting of 7.5µl Multiplex PCR Master Mix (Qiagen), 3.9µl nuclease free water, 0.3µl of each of 10µM forward and reverse primers (Merck KGaG, Darmstadt, Germany) and 3µl template DNA. Thermocyling profile was as follows: initial denaturation at 95°C (10 minutes), 40 cycles of 95°C (30 seconds), 56°C (30 seconds), 72°C (1 minute); final extension at 72°C (10 minutes). Following PCR product Sanger sequencing (Eurofins, Ebersberg, Germany), chromatograms were manually checked for errors, and sequences were aligned and trimmed using BioEdit v7.0.5.3 (Hall 1999). Resulting sequences were compared to the GenBank BLAST database (NCBI) and identifications of seeds were assigned based on the highest match (minimum 90%). Assignments were made to species level where possible, but when there was more than one top hit, assignment was made to the highest taxonomic rank in common between top hits.

1.2 Age differentiation

In the breeding grounds, birds were caught early in the breeding season, thus were all at least one year old and classified as adults. In Senegal, age was differentiated by studying the stage of moult of birds, and age classification assigned based on EURING aging codes (Baker *et al.* 1993). Birds caught in November-December and defined as 3 (definitely hatched during current calendar year) and those caught January – March and defined as 5 (definitely hatched during previous calendar year) were classified as juvenile, as they

were all in their first year and first wintering season. Individuals defined as 4 (hatched before current calendar year – exact year unknown) were classified as adult if they were caught in November-December. However, if they were caught in January-March, it was not possible to determine whether these were first year or mature birds, thus were classified as unknown. Birds defined as 6 (Hatched before last calendar year – exact year unknown) were classified as adults.

1.3 Sexing PCR

A number of approaches was tested to determine the sex of birds sampled using DNA-based sex identification. Sexing PCR uses two conserved chromo-helicase-DNA-binding (CHD) genes, which are located on avian sex chromosomes (ZW in females, ZZ in males) (Griffiths *et al.* 1998). The two genes amplified are CHD-W, located on the W chromosome and thus only present in females, and CHD-Z, found on the Z chromosome and occurring in both sexes. Whilst size varies between avian taxa, the size of amplicons of CHD-W and CHD-Z generally differ, resulting in a single CHD-Z band in males but a second CHD-W band in females (Griffiths *et al.* 1998).

Initial sex identification PCRs were carried out using DNA extracted from faecal samples because these were already available for all birds used in dietary analysis and DNA extracted from faecal DNA generally contains a high proportion of host DNA (Ando *et al.* 2020). The P8/ P2 primer pair (Griffiths *et al.* 1998, Table S2), which have been shown to successfully differentiate sex in a wide range of avian taxa, including Columbiformes (Griffiths *et al.* 1998; Jensen *et al.* 2003). The PCR mix consisted of 2.5µl Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 1.3µl nuclease free water, 0.1µl forward and reverse primers (Merck KGaG, Darmstadt, Germany) and 1µl template DNA. All PCRs included a PCR negative containing 1µl molecular grade water instead of template DNA. PCR was carried out using SimpliAmp™ Thermal Cycler (ThermoFisher Scientific, Waltham, USA) with the following thermal profile: denaturing at 95°C for 15 minutes, 40 cycles of 94°C for 45 seconds, 48°C for 45 seconds, 72°C for 45 seconds, and a final elongation step of 72°C for 10 minutes. Each PCR was run twice in with the intention of confirming sex result obtained. PCR products were electrophoresed through a 2% gel, stained

using SYBR®Safe (Thermo Fisher Scientific, Paisley, UK), for 40 minutes, using a current 110 amps and voltage of 110 volts in 0.5X TBE buffer. Bands were then visualised using UV light.

Sex identification PCRs were run on DNA from 158 turtle dove faecal samples, despite promising results in early testing, of these 38 samples failed to produce any bands in either replicate, and a further 27 samples failed in one of the two replicates.

As it was possible this high failure rate was a result of degraded or poor quality DNA in faecal samples, we tested the primer set Z37B, which was specifically developed for sex-typing birds using DNA from degraded samples (Dawson *et al.* 2015, Table S2). PCRs were conducted using a 5µl reaction volume consisting of 2µl Multiplex PCR Master Mix (Qiagen), 1.4µl nuclease free water, 0.08µl 10mM forward and reverse primers (Merck) and 1µl template DNA, following the thermal cycling protocol of 95°C (15 minutes), 35 cycles of 94°C (30 seconds), 56°C (90 seconds), 72°C (1 minute). PCR products were visualised as above, and no bands were present using this primer pair.

Due to the high failure rate of faecal samples, sex identification PCRs were run on DNA extracted from blood samples. DNA was extracted from turtle dove blood samples, preserved using Whatman FTA cards, using Qiagen blood and tissue kit (Qiagen), following manufacturer's instructions and eluting in 100µl of buffer AE. PCR with blood samples was conducted as for DNA from faecal samples, using the P2/P8 primer pair.

Sex identification PCRs were run on DNA from 81 blood samples, of these only two failed to produce reliable banding patterns in both replicates, and a further five in one replicate. Despite a higher success rate, results for sex determining PCRs gave inconsistent results for the sex in six of the 81 blood samples, and when compared with results from PCRs using DNA from faecal samples, a total of 13 birds did not yield consistent results for determined sex.

To try to further improve results, PCR was again performed on a subset of 20 blood samples, including 12 where sex had been previously determined based on behavioural traits, with the addition of Magnesium Chloride ($MgCl_2^+$) to improve DNA yield, tested at two concentrations: 1.0mM and 3.5mM (based

on results from Jensen *et al.* 2003). PCR mix consisted of 2.5µl Multiplex PCR Master Mix (Qiagen), 0.1µl forward and reverse primers (Merck) and 1µl template DNA. For 1.0mM $MgCl_2^+$, 0.2µl $MgCl_2^+$ and 1.1µl nuclease free water were added, for 3.5mM $MgCl_2^+$, 0.7µl $MgCl_2^+$ and 0.6µl nuclease free water were added. Thermal cycling was as previously described for P2/P8 primers. To further improve separation of CHD-W and CHD-Z bands, electrophoresis protocol described previously was adapted to use 3% agarose gel, a voltage of 90 volts, and run for 75 minutes.

Results varied between PCR reactions with different concentrations of $MgCl_2^+$, and obtained results failed to consistently support those of known sex birds, or of previous sex identification PCRs performed as part of this study (Table S1.1). Furthermore, an unexpected third band occurred, further decreasing our confidence in these results (Figure). Due to the repeated inconsistency of results and unusual band occurrence, we did not feel we could accurately or reliably determine sex of birds, therefore this variable is not considered in analysis.

Table S1.1: Results of sex identifying PCRs illustrating inconsistency of results obtained with

† known sex birds, †† previous PCRs from this study and ††† differing PCR conditions. Samples marked with * were faint bands on gel

Sex determined based on behaviour †	Previous PCR results ††		1.0mM $MgCl_2^+$ †††		3.5mM $MgCl_2^+$	
			Replicate 1	Replicate 2	Replicate 1	Replicate 2
F			F	F	fail	fail
M			M	M*	F*	M*
F			fail	fail	fail	fail
F			fail	F	M	M*
F			F	F	F	M*
M			fail	fail	fail	M*
M			M	M*	M	fail
M			fail	fail	fail	fail
F			M	fail	M	M*
M			F	F	F	F
M			F	F	F	F
F			fail	fail	fail	fail
	F	F	F	F	F	F
	M	M	M	fail	M	M

M	M	F	F*	M	M*
M	M	F	fail	fail	fail
F	F	M	fail	M*	M
M	M	F	F	F	M*
M	M	F	F	F	F
F	F	F	F	F	F

1.4 PCR and library preparation

Table S1.2: Primer pairs used for amplification of CHD region for sexing (P2/P8; Z37B) and ITS2 region to amplify DNA extracted from seeds (S2F/ S3R) and degraded DNA extracted from faecal samples from the European turtle dove (UniPlantF/UniPlantR)

Primer	Primer sequence (5'-3')	Product length (bp)	Citation
P8	CTCCCAAGGATGAGRAAYTG		Griffiths *et al.* 1998
P2	TCTGCATCGCTAAATCCTTT		
Z37B [f]	AACTGGTTGTAGGTATAGTGCAATTATG		Dawson *et al.* 2015
Z37B [r]	GATTACAAAGCCAATATGGATGC		

S2F	ATGCGATACTTGGTGTGAAT	387-547	Chen *et al.* 2010
S3R	GACGCTTCTCCAGACTACAAT		
UniPlantF	TGTGAATTGCARRATYCMG	187-387	Moorhouse-Gann *et al.* 2018
UniPlantR	CCCGHYTGAYYTGRGGTCDC		

1.5 Bioinformatics and data cleaning

Table S1.3: Percentage of samples exhibiting primer truncation following HTS

	Forward primer	Reverse primer
Forward sequencing	5.19%	7.61%
Reverse sequencing	7.41%	12.40%

1.5.1 Filtering threshold selection

The aim of data cleaning is to remove as many artefacts as possible, whilst retaining the maximum amount of genuine data. Selection of a filtering threshold is a delicate balance between too relaxed thresholds resulting in under filtering and a large number of false positives, versus being too stringent, causing over filtering of data and an abundance of false negatives. The positive controls were used to set a filtering threshold in our data set (Taberlet book). Each positive control consisted of a single seed sample, of one of three known species endemic to Mauritius, therefore not occurring in our data set. Each positive control should therefore contain reads from this species alone, and any reads of alternative taxonomic units occurring in this sample would be erroneous.

As read count differs between samples, it was not appropriate to set a filtering threshold based on raw read count, therefore read counts were converted to frequency of occurrence to reflect the relative read abundance of different taxonomic units within a sample. To remove all artefacts from positive controls required a filtering threshold of 11%. However, Drake *et al.* (*in prep*) demonstrated through the use of mock communities that setting thresholds too high could result in false negatives. Containing a mix of known DNA from taxa not expected to occur in the diet being examined, mock communities can be included in metabarcoding as another form of positive control (De Barba *et al.* 2014). As several known taxa should be included in these samples, they can be used not only to assess if filtering is too low and thus including false positives, but also if filtering thresholds have been set too high, resulting in the erroneous removal of taxa which should be present in this sample (Drake *et*

al. in prep). When testing filtering thresholds, Drake *et al. (in prep)* found that setting a threshold higher to filter out all false positives also removed target reads, as demonstrated by the loss of taxa from mock communities. Therefore, they advise lowering thresholds in order to give a balance between false positives and false negatives and avoid over filtering the data. We therefore assessed the efficiency of different, lower filtering thresholds.

When comparing different filtering thresholds, a number of aspects were considered. We looked at the number of reads removed at each threshold, the number of non-target reads in the positive controls, the number of identified taxa removed and the geographical range of taxa removed (Table S1.4).

Table S1.4: Changes to read counts when different thresholds are applied to remove internal contaminants on a per sample basis from turtle dove dietary data. With the exception of the column containing raw data, all thresholds are applied in conjunction with removal of maximum contamination and taxa such as bacteria and fungi are removed in order to make a decision based on the final data set which would be used in analysis.

	No filtering	1% threshold	2% threshold	3% threshold	4% threshold	5% threshold	8% threshold	11% threshold
Total read count	12,119,221	11,317,817	10,953,504	10,684,416	10,472,968	10,299,088	9,814,877	9,366,588
Mean reads removed per sample	NA	7.29	10.59	13.04	14.97	16.55	20.95	25.02
Max reads removed per sample	NA	1,665	2,927	4,473	4,473	4,637	9,233	16,209
No. reads removed	0	801,404	1,165,717	1,434,805	1,646,253	1,820,133	2,304,344	2,752,633
% of total reads remaining	100.00%	93.39%	90.38%	88.16%	86.42%	84.98%	80.99%	77.29%
No. non-target taxa	475	10	5	5	3	3	1	0

in positive controls								
No. remaining taxa	388	200	155	142	131	121	94	85

Data were filtered at 3%, 4% and 5% and the results of filtering were compared in order to assess which proportion was most appropriate. As there was little variation in the number of reads removed at these thresholds (85%-88% of total read count), we instead considered the taxa removed at each threshold.

When filtering threshold was set to 3%, there were 5 non target taxa in the positive controls and 145 taxa remained, which fell three non-target taxa in positive controls, and 134 identified taxa at a 4% threshold. Of the taxa removed, one was only identified to clade level (*Asterid*), therefore would have been removed following data cleaning, and a further two were taxa which were not recorded as present within the geographical sampling range (www.powo.kew) (give taxa), suggesting that these are likely false positives retained at the 3% threshold.

When increasing the filtering threshold from 4% to 5%, the number of non-target taxa in the positive controls was unchanged, but 10 more taxa were removed. Of those removed, none provided a strong argument for increasing the threshold to 5%, such as not being geographically viable as seen when comparing 3% and 4%. Nine out of ten of the taxa removed are within our sampling geographical range (powo), thus are viable true reads. One of the species removed (*Euphorbia stepposa*) is only recorded in three countries, Romania, Ukraine and North Caucasus (powo). This species was recorded in two samples, both from Hungary. Birds in Hungary were captured close to the border with Romania (approximately 50km from border), therefore it is possible that this weed species may have spread across the border into Hungary through movement of animals and people between the two countries, but not yet been recorded as present in Hungary in the database we were using. We determined that the presence of this species in two birds sampled in Hungary, the only field site in close proximity to one of this species' native countries, but in no others from other field sites, supported this as a true read. This suggests that 5% filtering thresholds begins to remove true reads, thus we set the threshold at 4%.

We provide further support for using this threshold by comparing the geographical range of all taxa identified with where birds determined to have eaten that seed were caught. If seeds were consistently occurring outside the

expected geographical range, this would indicate that the threshold could have been set too low. Of a total of 219 sequenced faecal samples (including repeats) and 123 taxonomic units identified, there were a total of 612 dietary taxon presence counts. Of these, only 2.6% of taxa occurred in birds sampled from countries outside of their expected range, after filtering. We would expect that if reads occurring outside the expected range was a result of filtering thresholds being too low, that the read count for such taxa would account for a low proportion of the total read count per sample, therefore a slight increase of the filtering threshold would remove these reads. However this was not necessarily the case, for example, according to Plants of the world online, *Helianthus* sp. is not present in Senegal, however in one sample it accounted for 66.9% of reads, too high a proportion to be an artefact. Furthermore, *Euphorbia pseudoesula* was detected in the diet of six birds sampled in Hungary, with a minimum frequency of occurrence of 47.0%, despite only being reported as occurring in France out of our study sites (powo). It is therefore more likely that the reason for these taxa occurring outside the expected geographical range is that this database is not fully up to date for all species. This suggestion is supported by the fact that 11 of the 16 taxa detected outside their reported geographical range were detected in birds from Senegal, where information on flora present is likely to be less extensively reported.

Table S1.5: Read counts from Illumina Miseq following different stages of data processing

Stage of data processing	Number of reads	Percentage of raw reads retained
Raw reads from Illumine Miseq	17,143,308	100%
Passed quality threshold and were merged	15,409,859	89.9%
Successfully assigned to a sample	12,284,963	71.7%
Passed denoising threshold	12,119,221	70.7%
Retained following removal of contamination and artefacts	9,806,737	57.2%

1.6 Taxa identified

Table S1.6: Taxonomic units identified in this study, including percent of diet in which unit occurred in each country sample

				Percentage of birds in which taxa occurred		
Taxon	**Taxon rank**	**Family**	**Status**	**Senegal (n=128)**	**France (n=63)**	**Hungary (n=12)**
Acer campestre	Species	Sapindaceae	Wild	-	1.6	-
Achyranthes aspera	Species	Amaranthaceae	Wild	2.3	-	-
Adansonia digitata	Species	Malvaceae	Wild	0.8	-	-
Agrostis pallens	Species	Poaceae	Wild	-	1.6	-
Alkanna	Species	Boraginaceae	Wild	-	-	8.3
Alkanna tinctoria	Species	Boraginaceae	Wild	-	-	8.3
Alopecurus myosuroides	Species	Poaceae	Wild	-	-	8.3
Amaranthus	Genus	Amaranthaceae	Wild	0.8	-	-
Amaranthus albus	Species	Amaranthaceae	Wild	-	-	8.3
Ambrosia	Genus	Asteraceae	Wild	-	-	16.7
Ambrosia artemisiifolia	Species	Asteraceae	Wild	-	-	8.3
Anchusa arvensis	Species	Boraginaceae	Wild	-	1.6	-

Arachis	Genus	Fabaceae	Cultivated	3.1	-	-
Blainvillea rhomboidea	Species	Asteraceae	Wild	7.8	-	-
Boerhavia erecta	Species	Nyctaginaceae	Wild	0.8	-	-
Brachiaria ramosa	Species	Poaceae	Wild	2.3	-	-
Brassica	Genus	Brassiceae	Brassica	-	1.6	-
Brassica napus	Species	Brassiceae	Cultivated	-	1.6	-
Brassica rapa	Species	Brassiceae	Cultivated	-	1.6	-
Buglossoides	Genus	Boraginaceae	Wild	-	-	8.3
Bulbostylis	Genus	Cyperaceae	Wild	0.8	-	-
Bulbostylis hispidula	Species	Cyperaceae	Wild	0.8	-	-
Cannabis sativa subsp. Indica	Species	Cannabaceae	Cultivated	0.8	1.6	8.3
Carduus	Genus	Asteraceae	Wild	-	-	8.3
Carduus tenuiflorus	Species	Asteraceae	Wild	-	4.8	-
Cenchrinae	Subtribe	Poaceae	Cultivated	17.2	-	16.7
Cenchrus americanus	Species	Poaceae	Cultivated	32.0	-	-
Cenchrus polystachios	Species	Poaceae	Wild	0.8	-	-
Chenopodiastrum hybridum	Species	Amaranthaceae	Wild	-	-	8.3

Chenopodium	Genus	Amaranthaceae	Wild	-	3.2	8.3
Combretum bracteosum	Species	Combretaceae	Wild	0.8	-	-
Cucumis	Genus	Cucurbitaceae	Cultivated	1.6	-	-
Cucumis maderaspatanus	Species	Cucurbitaceae	Cultivated	0.8	-	-
Cucumis melo	Species	Cucurbitaceae	Cultivated	0.8	-	-
Cucurbita pepo	Species	Cucurbitaceae	Cultivated	0.8	-	-
Dactyloctenium aegyptium	Species	Poaceae	Wild	14.8	-	-
Datura stramonium	Species	Solanaceae	Wild	-	1.6	8.3
Daucus	Genus	Apiaceae	Cultivated	0.8	-	-
Digitaria	Genus	Poaceae	Wild	0.8	-	-
Echinochloa	Genus	Poaceae	Wild	18.0	-	-
Eragrostis	Genus	Poaceae	Wild	0.8	-	-
Erodium cicutarium	Species	Gerraniaceae	Wild	-	1.6	-
Eucalyptus	Genus	Myrtaceae	Wild	0.8	-	-
Euphorbia	Genus	Euphorbiaceae	Wild	-	-	50.0
Euphorbia cyparissias	Species	Euphorbiaceae	Wild	-	-	33.3

Euphorbia helioscopia	Species	Euphorbiaceae	Wild	-	6.3	-
Euphorbia pseudoesula	Species	Euphorbiaceae	Wild	-	-	50.0
Euphorbia seguieriana	Species	Euphorbiaceae	Wild	-	-	16.7
Euphorbia stepposa	Species	Euphorbiaceae	Wild	-	-	16.7
Fagus	Genus	Fagaceae	Wild	-	1.6	-
Fraxinus	Genus	Oleaceae	Wild	-	1.6	-
Geranium dissectum	Species	Gerraniaceae	Wild	-	1.6	-
Geranium molle	Species	Gerraniaceae	Wild	-	20.6	-
Geranium rotundifolium	Species	Gerraniaceae	Wild	-	4.8	-
Guizotia abyssinica	Species	Asteraceae	Cultivated	0.8	-	-
Gymnosporia senegalensis	Species	Celastraceae	Wild	0.8	-	-
Hedera hibernica	Species	Araliaceae	Wild	-	1.6	-
Helianthus	Genus	Asteraceae	Cultivated	2.3	41.3	8.3
Helianthus annuus	Species	Asteraceae	Cultivated	-	1.6	8.3
Hibiscus sabdariffa	Species	Malvaceae	Cultivated	0.8	-	-
Indigofera	Genus	Fabaceae	Wild	0.8	-	-
Indigofera colutea	Species	Fabaceae	Wild	0.8	-	-

Ipomoea coptica	Species	Convolvulaceae	Wild	1.6	-	-
Juglans	Genus	Juglandaceae	Cultivated	0.8	1.6	-
Loliinae	Subtribe	Poaceae	Wild	-	3.2	-
Lolium	Genus	Poaceae	Wild	-	4.8	-
Ludwigia erecta	Species	Onagraceae	Wild	0.8	-	-
Mangifera indica	Species	Mango	Cultivated	1.6	-	-
Medicago arabica	Species	Fabaceae	Wild	-	1.6	-
Melochia corchorifolia	Species	Malvaceae	Wild	7.0	-	-
Mercurialis annua	Species	Euphorbiaceae	Wild	-	7.9	-
Merremia	Genus	Convolvulaceae	Wild	2.3	-	-
Merremia aegyptia	Species	Convolvulaceae	Wild	0.8	-	-
Montia fontana	Species	Montiaceae	Wild	-	1.6	-
Nymphaea	Genus	Nymphaeaceae	Wild	0.8	-	-
Nymphaea guineensis	Species	Nymphaeaceae	Wild	0.8	-	-
Nymphaea lotus	Species	Nymphaeaceae	Wild	3.1	-	-
Nymphaea lotus var. thermalis	Species	Nymphaeaceae	Wild	3.9	-	-
Nymphaea micrantha	Species	Nymphaeaceae	Wild	0.8	-	-
Panicum (Europe)	Genus	Poaceae	Cultivated	-	-	8.3

Panicum miliaceum (Europe)	Species	Poaceae	Cultivated	-	11.1	-
Panicum (Africa)	Genus	Poaceae	Wild	22.6	-	-
Panicum miliaceum (Africa)	Species	Poaceae	Wild	-	-	-
Paspalum	Genus	Poaceae	Wild	1.6	-	-
Poa	Genus	Poaceae	Wild	-	4.8	-
Poa infirma	Species	Poaceae	Wild	-	1.6	-
Poaceae	Family	Poaceae	Wild	-	3.2	-
Potentilla	Genus	Rosaceae	Wild	-	1.6	-
Prunus	Genus	Rosaceae	Wild	-	3.2	-
Puccinellia	Genus	Poaceae	Wild	-	-	8.3
Quercus	Genus	Fagaceae	Wild	-	1.6	-
Quercus robur	Species	Fagaceae	Wild	-	1.6	-
Ranunculus parviflorus	Species	Ranunculaceae	Wild	-	1.6	-
Rubus	Genus	Rosaceae	Wild	-	1.6	-
Sambucus ebulus	Species	Adoxaceae	Wild	-	1.6	-
Senna	Genus	Fabaceae	Wild	1.6	-	-
Senna obtusifolia	Species	Fabaceae	Wild	8.6	-	-

Sida spinosa	Species	Malvaceae	Wild	0.8	-	-
Silene heuffelii	Species	Caryophyllaceae	Wild	-	-	8.3
Solanum	Genus	Solanaceae	Cultivated	3.1	-	-
Solanum tuberosum	Species	Solanaceae	Cultivated	1.6	-	-
Sonchus oleraceus	Species	Asteraceae	Wild	0.8	-	-
Sorghum	Genus	Poaceae	Cultivated	32.0	33.3	-
Sorghum halepense	Species	Poaceae	Cultivated	-	-	8.3
Stellaria media	Species	Caryophyllaceae	Wild	-	3.2	-
Tamarix	Genus	Tamaricaceae	Wild	0.8	-	-
Trianthema portulacastrum	Species	Aizoaceae	Wild	1.6	-	-
Trifolium	Genus	Fabaceae	Wild	-	1.6	-
Triticeae	Tribe	Poaceae	Cultivated	-	36.5	-
Triticum	Genus	Poaceae	Cultivated	-	74.6	-
Triticum aestivum	Species	Poaceae	Cultivated	-	1.6	-
Triticum monococcum subsp. Aegilopoides	Species	Poaceae	Cultivated	-	22.2	-
Triumfetta	Genus	Malvaceae	Wild	0.8	-	-
Tuberaria macrosepala	Species	Cistaceae	Wild	-	1.6	-

Ulmus	Genus	Ulmaceae	Wild	-	1.6	-
Ulmus minor	Species	Ulmaceae	Wild	-	1.6	-
Urochloa mosambicensis	Species	Poaceae	Wild	10.2	-	-
Urtica dioica	Species	Urticaceae	Wild	-	-	8.3
Verbascum	Species	Scrophulariaceae	Wild	-	-	8.3
Vicia faba	Species	Fabaceae	Cultivated	-	1.6	-
Vigna radiata	Species	Fabaceae	Cultivated	-	1.6	-
Vigna unguiculata	Species	Fabaceae	Cultivated	10.9	-	-
Zornia glochidiata	Species	Fabaceae	Wild	0.8	-	-

1.7 Statistical analysis

Table S1.7: Pairwise comparisons of mean species richness of dietary items consumed by turtle doves sampled between seasons from Tukey's post-hoc test. Significant pairwise interactions highlighted in bold.

Season comparison	Estimate	St. Error	t-value	*p*
S1-S2	0.058	0.167	0.348	1.000
W1-S1	-0.147	0.135	1.081	0.884
W2-S1	-0.051	0.164	0.308	1.000
W3-S1	-0.288	0.137	2.099	0.277
W4-S1	**-0.567**	**0.115**	**4.926**	**<0.001 *****
W1-S2	-0.205	0.192	1.065	0.890
W2-S2	-0.109	0.213	0.510	0.996
W3-S2	-0.346	0.193	1.790	0.460
W4-S2	**-0.625**	**0.178**	**3.505**	**0.006 ****
W2-W1	0.096	0.190	0.507	0.996
W3-W1	-0.141	0.167	0.847	0.956
W4-W1	-0.420	0.149	2.818	0.051
W3-W2	-0.237	0.190	1.245	0.807
W4-W2	**-0.516**	**0.175**	**2.943**	**0.036 ***
W3-W4	-0.279	0.150	1.856	0.418

Table S1.8: Significant effects of season on dietary items consumed after correcting for multiple testing when considering all dietary items, including bait, using the most simple model

	Full data set		
Dietary item	Likelihood Ratio Test	*P*	Country
Sorghum	20.5	0.005	Senegal, France
Triticum	141.5	0.001	France
Cenchrus	50.4	0.001	Senegal
Helianthus	62.3	0.001	Senegal, France, Hungary
Echinochloa	38.5	0.001	Senegal
Dactyloctenium	33.2	0.001	Senegal
Vigna	19.9	0.005	Senegal, France
Geranium	35.3	0.001	France
Senna	18.7	0.009	Senegal
Blainvillea	26.3	0.001	Senegal
Melochia	19.1	0.007	Senegal
Nymphaea	14.6	0.046	Senegal

Table S1.9: Significant effects of season on dietary items consumed after correcting for multiple testing in the full model, comparing results when bait seed is excluded from analysis against full data set.

	Full data set			**Bait excluded**			
Dietary item	Likelihood Ratio Test	*P*	Significance	Likelihood Ratio Test	*P*	Significance	Country
Sorghum	20.5	0.004	**	42.6	0.001	***	Senegal, France
Triticum	141.5	0.001	***	Excluded	Excluded	-	France
Cenchrus	50.4	0.001	***	38.4	0.001	***	Senegal
Helianthus	62.3	0.001	***	11.2	0.154	-	Senegal, France, Hungary
Echinochloa	38.5	0.001	***	32.1	0.001	***	Senegal
Dactyloctenium	33.2	0.001	***	28.0	0.002	**	Senegal
Vigna	19.9	0.005	**	16.8	0.026	*	Senegal, France
Geranium	35.3	0.001	***	48.2	0.001	***	France
Urochloa	0	1	-	37.0	0.001	***	Senegal
Senna	18.7	0.007	**	15.7	0.026	*	Senegal
Blainvillea	26.3	0.001	***	23.7	0.002	**	Senegal

Melochia	19.1	0.006	**	16.7	0.026	*	Senegal
Nymphaea	14.6	0.046	*	12.4	0.011	*	Senegal
Mercurialis	12.0	0.141	-	16.0	0.026	*	France

Table S1.10: Pairwise comparisons of mean body condition of turtle doves sampled between seasons from Tukey's post-hoc test when all consumed genera are included. Significant pairwise interactions highlighted in bold.

Season comparison	Estimate	St. Error	t-value	p
S1-S2	**-9.438**	**3.047**	**3.097**	**0.025 ***
W1-S1	**-20.802**	**2.209**	**9.416**	**<0.001 *****
W2-S1	**-14.656**	**2.938**	**4.989**	**<0.001 *****
W3-S1	**-19.424**	**2.124**	**9.147**	**<0.001 *****
W4-S1	**-10.307**	**1.692**	**6.093**	**<0.001 *****
W1-S2	**-11.364**	**3.366**	**3.376**	**0.011 ***
W2-S2	-5.218	3.883	1.344	0.747
W3-S2	**-9.986**	**3.310**	**3.017**	**0.031 ***
W4-S2	-0.869	3.051	0.285	1.000
W2-W1	6.146	3.267	1.881	0.400
W3-W1	1.378	2.560	0.538	0.994
W4-W1	**10.495**	**2.215**	**4.739**	**<0.001 *****
W3-W2	-4.769	3.210	1.486	0.658
W4-W2	4.349	2.942	1.478	0.663
W3-W4	**9.118**	**2.129**	**4.282**	**<0.001 *****

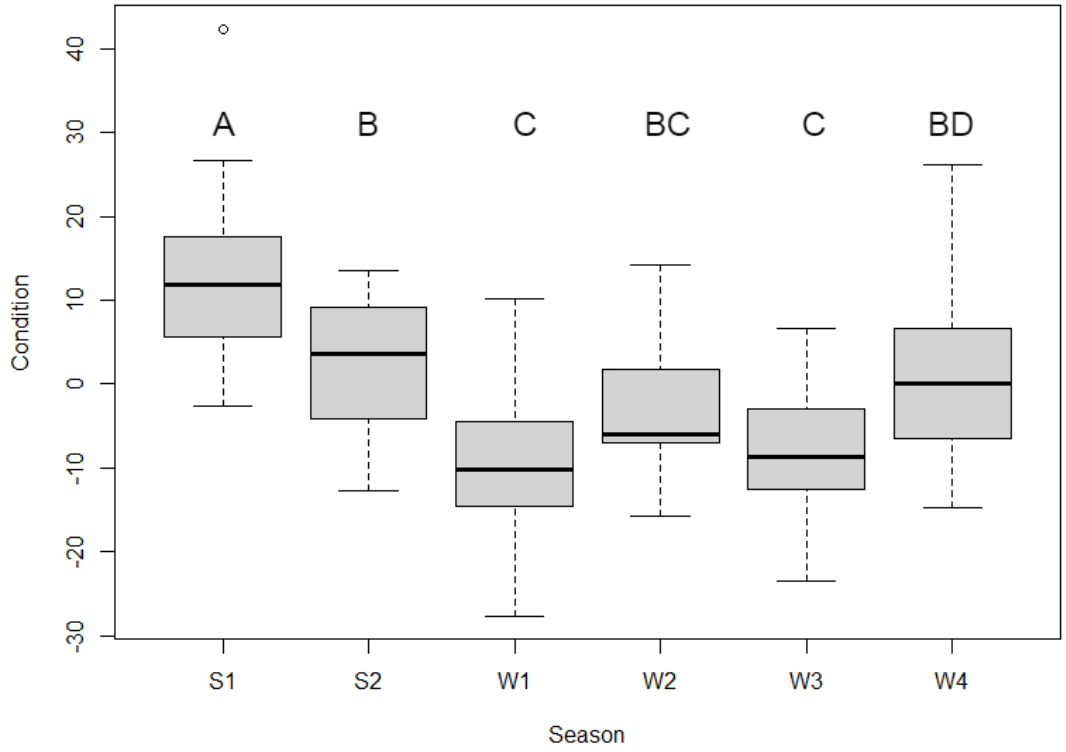


Figure S1.1: Variation in body condition of turtle doves across six sampling seasons when excluding birds sampled in France whose diet consisted solely of bait seed (ANOVA: $F=23.150_{5,167}$, $p<0.001$). Significant pairwise differences in mean body condition determined by Tukey's post-hoc test and are indicated by letters. For full breakdown of p-values associated with plot pairwise differences see Supplementary Information Table S11.

Table S1.11: Pairwise comparisons of mean body condition of turtle doves sampled between seasons from Tukey's post-hoc test when excluding birds sampled in France whose diet consisted solely of bait seed. Significant pairwise interactions highlighted in bold.

Season comparison	Estimate	St. Error	t-value	p
S1-S2	**-9.471**	**3.053**	**3.102**	**0.025 ***
W1-S1	**-21.668**	**2.385**	**9.086**	**<0.001 *****
W2-S1	**-15.521**	**3.053**	**5.084**	**<0.001 *****
W3-S1	**-20.290**	**2.308**	**8.791**	**<0.001 *****
W4-S1	**-11.172**	**1.931**	**5.786**	**<0.001 *****
W1-S2	**-12.197**	**3.217**	**3.792**	**0.003 ****
W2-S2	-6.051	3.739	1.618	0.575
W3-S2	**-10.819**	**3.160**	**3.423**	**0.009 ****
W4-S2	-1.701	2.897	0.587	0.991
W2-W1	6.146	3.217	1.911	0.387
W3-W1	1.378	2.520	0.547	0.994
W4-W1	**10.495**	**2.180**	**4.813**	**<0.001 *****
W3-W2	-4.769	3.160	1.509	0.647
W4-W2	4.349	2.897	1.501	0.652
W3-W4	**9.118**	**2.096**	**4.349**	**<0.001 *****

Appendix 2 - Supplementary information relating to Chapter 3

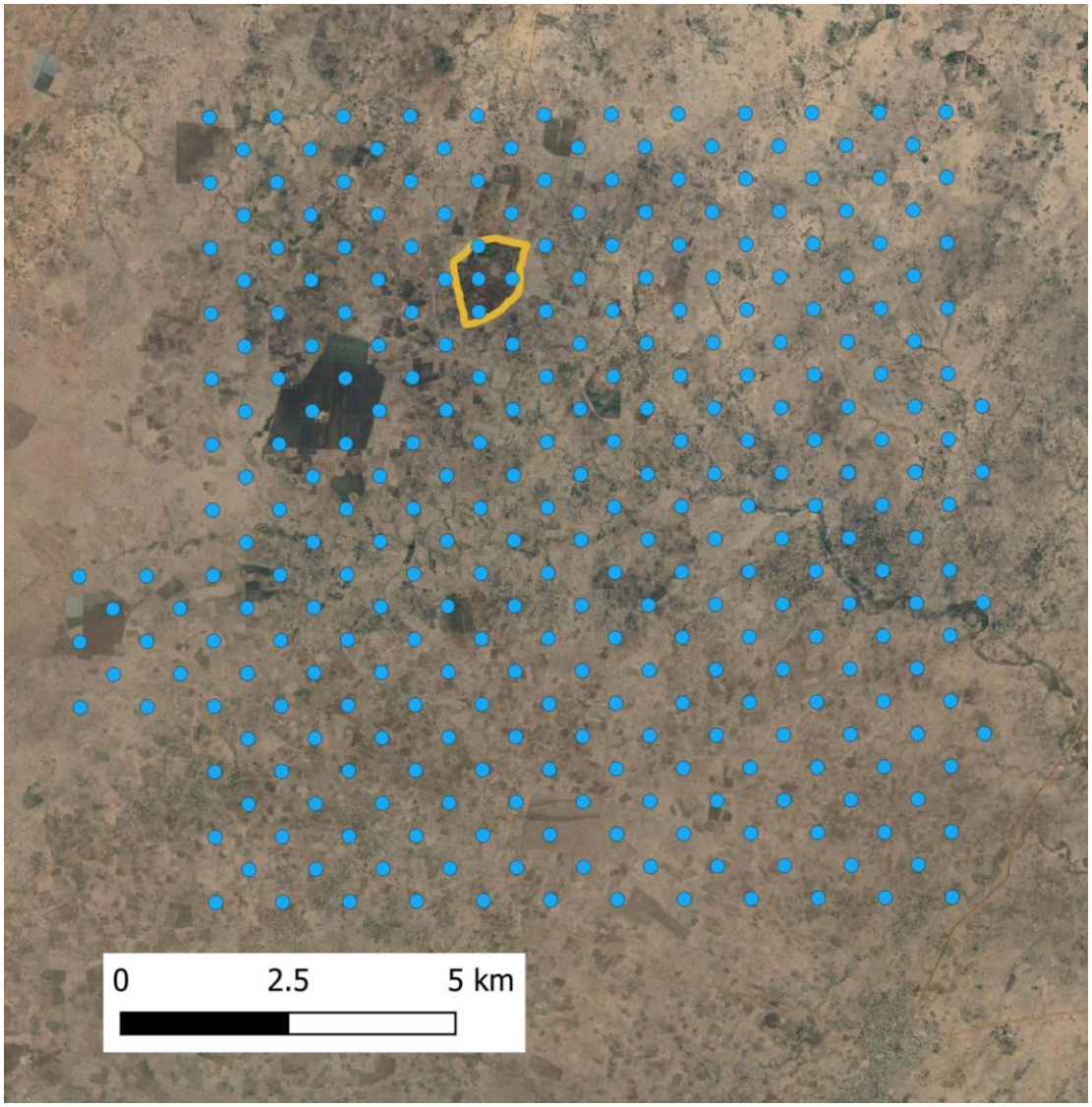


Figure S2.1: Grid used to select control points for environmental surveys. Area outlined in orange indicates Beer Sheba roost.

Grid to identify control points for habitat surveying. Initially, a 1km x 1km grid was overlaid on the study site, but the resulting grid was deemed too sparse, so a second 1km x 1km grid was overlaid, such that each point on the second grid was at the centre of the first 1km square, effectively resulting in a 709m x 709m grid. 10. In 2017/18 the grid was extended to encompass some new movements. However it was decided to add yet more points to the master

overlay, creating a 500mx500m grid, in order to create a larger number of possible controls in between clusters of bird locations.

Table S2.1: Information on the number of locations used in analysis. GPS and control locations surveyed were not included in environmental analysis from 2014/2015 because we did not have adequate sampling relocations from birds tagged in this year for analysis. Winter 2014/2015 was not included in seed identification for use in dietary choice analysis with EconullnetR because the sample size was small compared to other years. Habitat types from which seeds were collected included grassland, shrubland, crops, bare earth and wooded areas.

Winter	Number of GPS locations surveyed	Number of control points surveyed	Number of seed samples inspected
2015/16	211	165	73
2016/17	851	336	171
2017/18	1195	383	188

Table S2.2: Variables measured in environmental surveying, method used for data collection and statistical analyses for which data were used. Variables used in GLMMs. Due to the distribution of data across levels in factors, some were grouped together to improve stability of models. Grouped levels are indicated in bold

Habitat variable	**Categories**	**Method of reporting - all based on 100m radius around GPS point/control point**	**Grouping for analysis - terms highlighted in bold have been grouped for more effective analysis**	**Statistical analysis data used for**
Dominant land use	Village/human site Bare ground Dense savannah woodland >40% Open savannah woodland <40% Shrub land Grassland Arable - agricultural Plantation - agricultural Nat/ agricultural mosaic Water body	Three most dominant land uses, based upon proportion of area covered by specified land use, reported within each area, and ranked based upon which had the highest proportion of area attributed to this land use. Results in dominant land use 1, dominant land use 2, dominant land use 3. Where there were not three different type of land uses represented within the area, only one or two land uses are reported	Arable Bare earth Grassland Natural/agricultural mosaic Shrub land **Trees:** consisting of dense woodland (>40% trees), open savannah (<40% trees) and arable plantations **Human:** consisting of village and urban **Water:** consisting of water body and flooded/wetland	Compositional analysis GLMM

	Flooded/wetland Urban Other (specify)			
Dominant vegetation	Bare ground Forbs Grasses Shrubs Trees	As reported for dominant land use, but for dominant vegetation type		Compositional analysis GLMM
Class of tree cover	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65% 6 = >65%	Ranked based on the proportion of area covered by trees.	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65% 6 = >65%	GLMM
Class of forb cover	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65%	Ranked based on proportion of area covered by forbs	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65%	GLMM

	6 = >65%		6 = >65%	
Class of shrub cover	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65% 6 = >65%	Ranked based on proportion of area covered by shrubs	**2 = 0-4%** **2 = 0-4%** 3 = 4-15% 4 = 15-40% **5 = >40%** **5 = >40%**	GLMM
Class of grass cover	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65% 6 = >65%	Ranked based on proportion of area covered by grass	**3=0-15%** **3=0-15%** **3=0-15%** 4 = 15-40% 5 = 40-65% 6 = >65%	GLMM
Class of bare ground	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% 5 = 40-65% 6 = >65%	Ranked based on proportion of area of naturally bare ground	1 = 0% 2 = 1-4% 3 = 4-15% 4 = 15-40% **5 = >40%** **5 = >40%**	GLMM
	1 = 0% 2 = 1-4%		1 = 0% 2 = 1-4%	GLMM

Class of bare earth within grassland	3 = 4-15% 4 = 15-40% 5 = 40-65% 6 = >65%	Ranked based on proportion of area within a grassland comprising bare earth	3 = 4-15% 4 = 15-40% **5 = >40%** **5 = >40%**	
Crop type	Pumpkin Aubergine Okra Watermelon Nursery Onion Hibiscus Sorghum Millet Cocoa Coffee Plantain Maize Peanut	Up to six crops recorded within an area. For each crop reported, the following information was collected	Bean Hibiscus Fallow Millet Peanut Sorghum **Other crops:** consisting of uncommon crops, including cassava, okra, onion, pepper, pumpkin, tomato and watermoelon. Note, not all listed crops were reported at all, e.g. cocoa, thus not in this category **Natural area:** consisting of bare earth, grass, shrubs	Compositional analysis

		and trees, sometimes reported amongst cropped areas	
	Tomato Cassava Rice Sweet potato Irish potato Chilli pepper Cabbage Beans Fallow Tilled soil Other		
% cover	0-100%	Percentage of the area covered with specified crop	Compositional analysis
% of crop harvested	0-100%	Percentage of area within specified crop comprising bare ground	GLMM
% bare ground within crop area	0-100%	Percentage area within specified crop comprising bare earth	GLMM

Table S2.3: Full breakdown of GPS fixes used in home-range analysis. Individuals highlighted in bold had fixes over a period greater than one month, therefore were divided into the presented shorter increments for home-range analysis.

Bird ID	Home range ID	Year	Season	Fix start date	Fix end date	N days	N fixes
Adama	A2	2016/17	2	01/12/2016	07/12/2016	6	69
Bakel	B2	2016/17	2	01/03/2017	16/03/2017	15	141
Dewi	D2	2016/2017	2	02/03/2017	21/03/2017	18	235
Françoise		**2015/2016**	**1**	**21/11/2015**	**20/02/2016**	**67**	**215**
Françoise	F1.1	2015/2016	1	21/11/2015	10/12/2015	19	114
Françoise	F1.2	2015/2016	1	11/12/2015	29/12/2015	18	71
Françoise	F1.3	2015/2016	1	21/01/2016	20/02/2016	30	30
Jeremy	J3	2017/2018	3	20/11/2017	17/12/2017	27	242
Kousmar	K1	2015/2016	1	22/02/2016	26/02/2016	4	38
Linguère	L1	2015/2016	1	22/02/2016	15/03/2016	22	137
N'Dioum	N1	2015/2016	1	27/02/2016	15/03/2016	17	121

Nianing	N3	2017/2018	3	23/11/2017	02/12/2017	9	59
Ouro Sogui	O1	2015/2016	1	05/03/2016	16/03/2016	11	153
Palmarin	P1	2015/2016	1	05/03/2016	15/03/2016	10	133
Quincy	Q1	2015/2016	1	05/03/2016	16/03/2016	11	142
Ranerou	R2	2016/2017	2	18/11/2016	14/12/2016	26	165
Richard-Toll	R3	2017/2018	3	27/11/2017	17/12/2017	20	204
Sandiara	S2	2016/2017	2	19/11/2016	24/11/2016	5	53
Sokone		**2017/2018**	**3**	**27/11/2017**	**17/03/2018**	**107**	**1300**
Sokone	S3.1	2017/2018	3	27/11/2017	26/12/2017	29	359
Sokone	S3.2	2017/2018	3	27/12/2017	26/01/2018	30	365
Sokone	S3.3	2017/2018	3	27/01/2018	26/02/2018	30	361
Sokone	S3.4	2017/2018	3	27/02/2018	17/03/2018	18	215
Tamba	T3	2017/2018	3	17/02/2018	12/03/2018	22	272
Vieux	V3	2017/2018	3	20/02/2018	04/03/2018	12	54
XXX		**2016/2017**	**2**	**28/11/2016**	**01/03/2017**	**90**	**857**

XXX	X2.1	2016/2017	2	28/11/2016	27/12/2016	29	248
XXX	X2.2	2016/2017	2	28/12/2016	27/01/2017	30	306
XXX	X2.3	2016/2017	2	28/01/2017	27/02/2017	30	303
Zeinab	Z3	2017/2018	3	28/02/2018	16/03/2018	16	201
Ziguinchor		**2016/2017**	**2**	**01/12/2016**	**23/03/2017**	**109**	**1086**
Ziguinchor	Z2.1	2016/2017	2	01/12/2016	31/12/2016	30	200
Ziguinchor	Z2.2	2016/2017	2	01/01/2017	31/01/2017	30	334
Ziguinchor	Z2.3	2016/2017	2	01/02/2017	28/02/2017	27	301
Ziguinchor	Z2.4	2016/2017	2	01/03/2017	23/03/2017	22	251

Table S2.4: *h* values calculated using the ad hoc method, used when estimating home-range across the full data set

Bird ID	h_{ref}	$h_{ad\ hoc}$	Iteration
Adama	629.78	629.78	100%
Bakel	1370.50	1370.50	100%
Dewi	473.21	473.21	100%
Françoise	30317.90	30317.90	100%
Jeremy	1794.13	1794.13	100%
Kousmar	1156.72	240.19	50%
Linguère	4403.09	4403.09	100%
NDioum	3979.43	805.05	90%
Nianing	4724.50	4724.50	100%
Ouro_Sogui	1853.34	1853.34	100%
Palmarin	3058.92	3058.92	100%
Quincy	1347.21	1347.21	100%
Ranerou	6502.69	588.84	70%
Richard_Toll	9857.43	9857.43	100%
Sandiara	2048.95	457.41	70%
Sokone	4071.49	4071.49	100%
Tamba	7074.46	1035.27	90%
Vieux	543.60	543.60	100%
XXX	5929.74	5929.74	100%
Zeinab	1520.77	1520.77	100%
Ziguinchor	5446.22	5446.22	100%

Table S2.5: *h* values calculated using the ad hoc method, used when estimating home-range across the data set divided into 30 day increments

Bird ID	h_{ref}	$h_{ad\ hoc}$	Iteration
Adama_2	385.6261	385.6261	100%
Bakel_2	499.5263	499.5263	100%
Dewi_2	473.2077	473.2077	100%

Françoise_1.1	2884.539	2884.539	100%
Françoise_1.2	3783.264	3783.264	100%
Françoise_1.3	501.1278	451.015	90%
Jeremy_3	592.464	592.464	100%
Kousmar_1	480.3734	240.1867	50%
Linguère_1	836.1837	836.1837	100%
N'Dioum_1	894.5387	805.0549	90%
Nianing_3	1092.515	1092.515	100%
Ouro Sogui_1	623.6103	623.6103	100%
Palmarin_1	835.6924	835.6924	100%
Quincy_1	484.8051	484.8051	100%
Ranerou_2	841.1934	588.8354	70%
Richard-Toll_3	1098.311	1098.311	100%
Sandiara_2	653.4497	457.4148	70%
Sokone_3.1	833.9099	667.1279	80%
Sokone_3.2	897.2277	897.2277	100%
Sokone_3.3	613.9292	613.9292	100%
Sokone_3.4	611.6657	611.6657	100%
Tamba_3	7074.459	1035.267	90%
Vieux_3.2	298.957	298.957	100%
XXX_2.1	897.1792	717.7433	80%
XXX_2.2	932.7875	932.7875	100%
XXX_2.3	780.5918	780.5918	100%
Zeinab_3	480.43	480.43	100%
Ziguinchor_2.1	1577.661	1577.661	100%
Ziguinchor_2.2	537.1582	537.1582	100%
Ziguinchor_2.3	368.7228	368.7228	100%
Ziguinchor_2.4	346.5008	346.5008	100%

Table S2.6: Home-range calculated per individual bird, across the full period of data collection from GPS tags.

Bird ID	MCP_{95} (km^2)	MCP_{50} (km^2)	KDE_{95} (km^2)	KDE_{50} (km^2)	N sampling days	N fixes
Adama	3.91	0.02	6.30	0.94	6	69
Bakel	2.98	1.88	13.70	2.57	15	141
Dewi	2.91	0.24	4.73	0.57	18	235
Françoise	75.79	13.54	303.18	61.59	89	244
Jeremy	5.23	0.59	17.94	2.68	27	242
Kousmar	2.96	1.67	5.88	1.11	4	38
Linguère	11.29	4.27	44.03	7.73	22	137
NDioum	15.61	6.80	34.64	6.40	17	121
Nianing	10.45	0.18	47.24	9.33	9	59
Ouro_Sogui	5.49	2.85	18.53	3.43	11	153
Palmarin	9.23	0.91	30.59	5.48	10	133
Quincy	5.15	1.40	13.47	2.92	11	142
Ranerou	26.30	1.28	51.18	3.85	26	165
Richard_Toll	47.15	16.59	98.57	19.63	20	204
Sandiara	4.66	2.40	13.02	2.74	5	53
Sokone	28.68	3.42	40.71	0.98	107	1300
Tamba	38.18	1.74	61.49	8.45	22	272

Vieux	1.92	0.02	5.44	0.95	12	54
XXX	50.85	2.96	59.30	4.04	90	878
Zeinab	8.64	0.15	15.21	1.84	16	201
Ziguinchor	60.47	1.28	54.46	3.43	108	1086
Minimum	1.92	0.02	4.73	0.57	4	38
Mean±S.E.	19.90±4.80	3.06 ±0.95	44.74±13.97	7.17±2.88	30.71±7.53	282.24±76.44
Maximum	75.79	16.59	303.18	61.59	108	1300

Table 2.7: Calculated areas of home-range and core area for each individual using both minimum complex polygon (MCP) and kernel density estimator (KDE) methods, where individuals with >30 days of GPS fixes are divided into shorter increments

Bird ID	$h_{ad\ hoc}$	MCP_{95} (km^2)	MCP_{50} (km^2)	KDE_{95} (km^2)	KDE_{50} (km^2)
Adama_2	385.63	3.91	0.02	6.30	0.94
Bakel_2	499.53	2.98	1.88	13.70	2.57
Dewi_2	473.21	2.91	0.24	4.73	0.57
Françoise_1.1	512.39	4.82	0.85	16.92	3.16
Françoise_1.2	363.62	6.06	0.32	9.03	1.31
Françoise_1.3	925.94	0.47	0.43	9.26	1.97
Jeremy_3	592.46	5.23	0.59	17.94	5.68
Kousmar_1	240.19	2.96	1.67	5.88	1.11
Linguère_1	836.18	11.29	4.27	44.03	7.73
N'Dioum_1	805.05	15.61	6.80	34.64	6.40

Nianing_3	1092.52	10.45	0.18	47.24	9.33
Ouro Sogui_1	623.61	5.49	2.85	18.53	3.43
Palmarin_1	835.69	9.23	0.91	30.59	5.48
Quincy_1	484.81	5.15	1.40	13.47	2.92
Ranerou_2	588.84	26.30	1.28	51.18	3.85
Richard-Toll_3	1098.31	47.15	16.59	98.57	19.63
Sandiara_2	457.41	4.66	2.40	13.02	2.74
Sokone_3.1	667.13	20.57	4.76	37.27	2.79
Sokone_3.2	897.23	15.78	2.18	46.01	4.75
Sokone_3.3	613.93	7.39	0.52	16.26	2.59
Sokone_3.4	611.67	17.95	0.56	23.58	2.54
Tamba_3	1035.27	38.18	1.74	61.49	8.45
Vieux_3.2	298.96	1.92	0.02	5.44	0.95

XXX_2.1	717.74	49.38	2.22	55.96	7.05
XXX_2.2	932.79	30.77	2.48	63.67	6.06
XXX_2.3	780.59	31.25	1.82	45.48	4.10
Zeinab_3	480.43	8.64	0.15	15.21	1.84
Ziguinchor_2.1	1577.66	74.29	10.73	204.95	35.09
Ziguinchor_2.2	537.16	22.35	2.67	25.50	3.80
Ziguinchor_2.3	368.72	5.28	1.18	9.16	1.26
Ziguinchor_2.4	346.50	4.47	0.34	7.13	0.93

Appendix 3 – Supplementary information relating to Chapter 4

3.1 PCR and library preparation

Table S3.1: Primer pairs used for PCR amplification of ITS region (TFR1, TFR2) and FeDH region (TrichhydFOR, TrichhydREV) of T. gallinae from European turtle doves

Primer	**Primer sequence (5'-3')**	**Product length (bp)**	**Citation**
TFR1	TGCTTCAGTTCAGCGGGTCTTCC	400	Gaspar da Silva *et al.* 2007
TFR2	CGGTAGGTGAACCTGCCGTTGG		
TrichhydFOR	GTTTGGGATGGCCTCAGAAT	1000	Lawson *et al.* 2011
TrichhydREV	AGCCGAAGATGTTGTCGAAT		

3.2 Bioinformatics and data cleaning

Table 3.2: Percentage of samples exhibiting primer truncation following HTS

	Forward primer	Reverse primer
Forward sequencing	7.34%	6.91%
Reverse sequencing	8.82%	9.3%

Table S3.3: Number of reads retained at different stages of bioinformatics processing

Stage of data processing	Number of reads	Percentage of raw reads retained
Raw reads from illumine Miseq	939,018	100%
Reads that passed quality threshold and were merged	812,288	86.8%
Reads successfully assigned to a sample	612,178	65.4%
Reads that passed denoising threshold	611,509	65.3%
Reads remaining after removing contamination and artefacts	426,748	45.6%

3.3 Filtering thresholds

Different filtering thresholds were tested at the second and third staged of data cleaning in order to determine the most appropriate threshold.

For the second stage of filtering (removal of artefacts based on frequency of occurrence of strains within a host, following the removal of maximum contamination), three thresholds were tested, 0.001, 0.003 and 0.005. These were determined based on testing one threshold high and lower than that suggested by Taberlet *et al.* (2018) (0.003). The use of cumulative frequency graphs of the read counts accounted for by different *T. gallinae* variants present in a sample to determine a clear inflection point was the third filtering step.

Rate of co-infection suggested by ± 0.001 is unrealistically high (Table S3.3), suggesting that this level of data cleaning is not stringent enough, therefore cumulative read depth graphs were not generated for this data set and this threshold is not considered further. Rate of co-infection suggested by ± 0.003 and ± 0.005 are very similar (Table S3.3). Values highlighted in bold were consistent across cleaning thresholds, indicating a stable data set. Whilst there was some variation in the level of significance, the significant effects of season upon the four dominant strains identified in this study did not change

according to which cleaning thresholds were applied (Table S3.4). When data sets were analysed using ‘cooccur’, significant correlations identified between pairs of strains did not differ between ± 0.003 and ± 0.005. The lower threshold of 0.003 was used for analysis as this value is advised by Taberlet *et al.* (2018). It is also very close to 10 reads as a proportion of the mean read count for this data set (0.0027). Removing samples with reads less than 10 was identified as a commonly used method of cleaning (Drake *et al.* 2021), thus this threshold follows this methods, whilst adjusting the value to follow a proportional approach.

Table S3.4: Comparison of the minimum, mean and maximum number of strains present in a single individual under different thresholds for data cleaning. Values marked ± indicate the thresholds applied at step two cleaning, based upon relative abundance of zOTUs within a sample, removing any zOTUs occurring at a proportion below the given threshold. Values marked § indicate thresholds applied at third cleaning step, based upon cumulative read depth graphs. Thresholds were assigned according to the presence of a clear inflection point. Some graphs had less clear inflection points making it more difficult to define a threshold. For this reason thresholds were set at different levels, with the highest percentage being the most conservative option.

	0.001±	0.003±				0.005±		
No. strains per individual	None §	None §	7% §	10.5% §	11.70% §	None §	7.20% §	10.50% §
Minimum	5	1	1	1	1	1	1	1
Mean	56.121	6.236	**1.220**	**1.179**	1.146	2.964	**1.241**	**1.196**
Maximum	110	29	**4**	**3**	3	23	**4**	**3**

Table S3.5: Results of binary GLM, modelling prevalence of the four dominant T. gallinae strains against sampling season, tested at different cleaning threshold. Values marked ± indicate the thresholds applied at the second stage of cleaning, values marked § indicate thresholds applied at third stage of cleaning.

Sampling season associated with 4 degrees of freedom. Probability values associated with model: significant effects of sampling season on prevalence of parasite strain given as ***=p<0.001, **=p≤0.01, *=p≤0.05. Dev = deviance, N = 111

Cleaning thresholds	Type GEO	C	Tcl-1	Type IIIc
0.001±	**	*		*
0.003±	**	*		*
0.003±, 7%§	*	*		**
0.003±, 10.5%§	*	*		**
0.003±, 11.7%§	*	*		**
0.005±	**	*		*
0.005±, 7%§	*	*		**
0.005±, 10.5%§	*	*		**

When considering the thresholds for the third stage of cleaning, there was little difference in the number of samples within which each variant occurred at the different filtering thresholds; although some rare strains did not occur with a higher threshold (Table S3.5). However, at these were rare and not included in further analysis, this did not effect ecological inferences made from these data.

Table S3.6: Number of samples in which strains were identified when different filtering thresholds were applied at the third stage of the data cleaning process

Strain	N samples identified in (7%)	N samples identified in (10.5%)	N samples identified in (11.7%)
Tcl-1	45	43	43
Type C	30	30	30
GEO	26	26	26
Type IIIc	27	26	24
GEO-TD	5	5	5
ESWD	1	1	1
GEO-TD2*	2	2	1
Type IIIc-TD*	1	0	0
GEO-TD3*	1	0	0
Tcl-TD*	1	1	0

When considering co-infection, there was some variation in the co-infection results at different filtering thresholds (Table S3.6). However, the rate of co-infection did not differ significantly between 7%, 10.5% and 11.7% filtering thresholds (GLM, Poisson distribution, dev = 0.28, p = 0.592). Applying different filtering thresholds did not affect the results of co-occurrence analysis, with the same significant effects being observed regardless of filtering threshold used (see supplementary info for tables of results). Due to the limited differences between the 7% and 10.5% filtering thresholds, 7% was applied to prevent over filtering of the data.

Table S3.7: Variation in co-infection levels when different filtering thresholds applied to data cleaning

				Number of strains present in host					
	N co-infected	Total N	% co-infected	2	3	4	Min	Mean	Max
7%	19	112	16.96	13	4	2	1	1.24	4
10.50%	17	112	15.18	12	5	0	1	1.20	3
11.70%	15	112	13.39	12	3	0	1	1.16	3

3.4 Pairwise comparison of body condition between seasons

Table S3.8: Pairwise comparisons of mean condition of all turtle doves sampled between sampling seasons from Tukey's post-hoc test.

Season comparison	Estimate	St. Error	t-value	*p*
S2 - S1	-8.680	3.598	-2.413	0.208
W1 - S1	**-14.897**	2.509	-5.937	**<0.001 *****
W2 - S1	**-19.493**	2.003	-9.733	**<0.001 *****
W3 - S1	**-10.099**	1.708	-5.913	**<0.001 *****
W1 - S2	-6.216	4.040	-1.539	0.520
W2 - S2	**-10.813**	3.747	-2.886	**0.032 ***
W3 - S2	-1.418	3.598	-0.394	0.994
W2 - W1	-4.597	2.718	-1.691	0.423
W3 - W1	4.798	2.509	1.192	0.293
W3 - W2	**9.395**	2.003	4.691	**<0.001 *****

3.5 Full co-occur results

Table S3.9: Full results table from co-occur, significant results in bold. Table shows the observed number of birds infected with each strain of a pairwise comparison, and co-infected with both; the probability of co-occurrence of strain pairs based on occurrence of each strain within the population; and expected number of individuals co-infected with both strains. P Lt and Gt represent the probabilities of the two strains co-occurring at a frequency less than, or greater than observed, respectively. Effect size refers to the standardised difference between the expected and observed frequency of co-occurrence.

Strain 1	Strain 2	Strain 1 #infected	Strain 2 #infected	Observed #infected	Probability of co-occurrence	Expected number co-infected	*p* Lt	*p* Gt	Effect size
Tcl-1	GEO	45	26	8	0.093	10.4	0.188	0.612	-0.021
Tcl-1	Type C	45	30	1	0.108	12.1	**<0.001**	1.000	-0.099
Tcl-1	Type IIIc	45	27	6	0.097	10.8	**0.023**	0.993	-0.043
Tcl-1	GEO-TD	45	5	2	0.018	2	0.683	0.671	0.000
GEO	Type C	26	30	0	0.062	7	**<0.001**	1.000	-0.063
GEO	Type IIIc	26	27	6	0.056	6.3	0.558	0.648	-0.003
GEI	GEO-TD	26	5	2	0.010	1.2	0.919	0.329	0.007
Type C	Type IIIc	30	27	0	0.065	7.2	**<0.001**	1.000	-0.064
Type C	GEO-TD	30	5	0	0.012	1.3	0.203	1.000	-0.012
Type IIIc	GEO-TD	27	5	3	0.011	1.2	0.988	0.090	0.016

3.6 Comparing bioinformatic pipeline with jMHC

Table S3.10: Minimum, mean and maximum read counts resulting from two methods of assessing samples for co-occurrence of T. gallinae, before data cleaning

	jMHC	Bioinformatic pipeline
Minimum read count per sample	4	662
Mean read count per sample	66.702	4767.226
Maximum read count per sample	184	12,506

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