Received: 1 September 2022 | Revised: 15 February 2023 | Accepted: 23 February 2023

DOI: 10.1002/edn3.402

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

SPECIAL ISSUE ARTICLE

Investigating the association between diet and infection with *Trichomonas gallinae* in the European turtle dove (*Streptopelia turtur*)

Rebecca E. Young[1] | Jenny C. Dunn[2] | Ian P. Vaughan[1] | John W. Mallord[3] | Chris J. Orsman[3] | Moussa Ka[4] | Mamadou B. Diallo[4] | Malang Sarr[4] | Hervé Lormée[5] | Cyril Eraud[5] | Orsolya Kiss[6] | Rebecca C. Thomas[7] | Keith C. Hamer[7] | Simon J. Goodman[7] | William O. C. Symondson[1]

[1]Cardiff School of Biosciences, Cardiff Univeristy, Cardiff, UK

[2]School of Life Sciences, Joseph Banks Laboratories, University of Lincoln, Lincoln, UK

[3]RSPB Centre for Conservation Science, The Lodge, Sandy, UK

[4]Nature Communauté Développement, Dakar, Senegal

[5]Office National de la Chasse et de la Faune Sauvage, Unité Avifaune Migratrice, Réserve de Chizé, Villiers-en-Bois, France

[6]Faculty of Agriculture, Institute of Animal Sciences and Wildlife Management, University of Szeged, Hódmezővásárhely, Hungary

[7]School of Biology, University of Leeds, Leeds, UK

Correspondence
Rebecca E. Young, Cardiff School of Biosciences, Cardiff Univeristy, Cardiff CF10 3AX, UK.
Email: rebecca.young@biology.ox.ac.uk

Present address
Rebecca E. Young, Durrell Wildlife Conservation Trust, Trinity, UK
Hervé Lormée and Cyril Eraud, Office Français de la Biodiversité, Unité Avifaune Migratrice, Direction de la Recherche et del'appui Scientifique, Villiers-en-Bois, France
Rebecca C. Thomas, Department of Animal and Plant Sciences, NERC Biomolecular Analysis Facility, University of Sheffield, Sheffield, UK

Funding information
British Society for Parasitology International Training and Fieldwork Award; Genetics Society Heredity Fieldwork Grant; Natural Environment Research Council GW4+ Studentship, Grant/Award Number: NE/L002434/1; Royal Society for the Protection of Birds project CASE partner

Abstract

Trichomonas gallinae, a protozoan parasite infecting a wide range of birds, has been a cause for conservation concern since its recognition as an emerging infectious disease, having had notable impacts on several avian species, including causing widespread mortality in greenfinches (*Chloris chloris*), and chaffinches (*Fringilla coelebs*), and hindering the recovery of the endangered pink pigeon (*Nesoenas mayeri*). Horizontal transmission between birds congregating at feeding sites, such as those provided for conservation and species management purposes, is an important driver in the spread of *T. galliane*. Supplementary feeding is a key conservation intervention for the European turtle dove (*Streptopelia turtur*), which is declining across its range, driven at least partially by a loss of natural food resources. Due to the link between *T. gallinae* transmission and supplementary feeding, we consider the prevalence of this parasite among European turtle dove in relation to diet, in the first study to analyze these two factors in the decline of this species together. Using birds sampled from breeding and wintering grounds, the dietary composition of individuals was compared to the presence of *T. gallinae*, and specific *T. gallinae* strains. Dietary variation was summarized into two axes using detrended correspondence analysis; neither was associated with the presence of *T. gallinae* or any specific strains. 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 due to an increase in contact between potentially infected and uninfected birds gathering at high densities at feeding sites. Significant dietary overlap was observed between infected and uninfected individuals in all sites, with almost complete dietary overlap being observed in one breeding site. The level of dietary overlap between individuals infected with specific strains fluctuated between sampling seasons, indicating no consistent relationship between diet and infection status.

KEYWORDS
diet, high-throughput sequencing, parasitology, supplementary feeding, *Trichomonas galliane*, turtle dove

1 | INTRODUCTION

1.1 | Importance of disease as a conservation issue

In recent decades, emerging infectious diseases (EIDs) have increasingly become recognized as an important conservation issue, contributing to the decline of species across a wide range of taxa worldwide (Daszak et al., 2000; McCallum & Dobson, 1995). From the amphibian population crashes caused by chytridiomycosis to the extinctions of endemic Hawaiian birds linked to avian malaria, EIDs have the potential to spread rapidly among populations, resulting in mass mortality, local extinctions, and contributing to global species decline (Berger et al., 1998; Cunningham & Daszak, 1998; McCallum, 2012; Warner, 1968).

One of the most damaging epidemics for wild bird populations of recent years is the widespread epidemic of trichomonosis in greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*). The spread of this disease, caused by the protozoan parasite *Trichomonas gallinae*, began in western England and Wales in 2005, and spread across large areas of mainland Europe, with outbreaks of trichomonosis in finches occurring in Ireland and across mainland Europe, in Northern Germany, Austria, France, and Slovenia (Amin et al., 2014; Marx et al., 2017; McBurney et al., 2015). Trichomonosis has 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 affects a wide range of avian orders, including Passeriformes, Columbiformes, and Falconiformes (Amin et al., 2014; Bunbury et al., 2008; Sansano-Maestre et al., 2009), with at least 23 strains of the parasite of varying virulence identified (Thomas et al., 2022). It infects one of the UK's most rapidly declining species, the European turtle dove (*Streptopelia turtur*), and has been linked to both adult and nestling mortality in this species (Stockdale et al., 2015). While this parasite is commonly spread via direct transmission, passing both horizontally, between adults during courtship, and vertically, from parent to offspring during feeding of crop milk (Bunbury et al., 2007), the congregation of birds at artificial feeding sites is believed to have been a key contributor to the rapid spread of *T. gallinae*, transmitted through contaminated food and water resources at these sites (Lennon et al., 2013; McBurney et al., 2015; Robinson et al., 2010; Thomas, 2017).

1.2 | Disease transmission routes and feeding behavior

Transmission route is an important factor when assessing the impacts of disease on a population, and understanding both inter and intraspecific transmission of disease is vital in species management (Herrera & 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 behavior (De Castro & Bolker, 2005; Herrera & Nunn, 2019).

Certain behaviors increase or decrease the risk of disease transmission, and one factor that has been attributed to an increase in the spread of disease is the congregation of animals. Wildlife often aggregates in small areas in 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 & Litvaitis, 2011), increasing reproductive success (Robb, McDonald, Chamberlain, & Bearhop, 2008) 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. Providing food resources can result in less severe declines being observed in target species of over-winter supplementary feeding programs (Siriwardena et al., 2007), and increased breeding success as a result of earlier laying dates and increased fledging success (Robb, McDonald, Chamberlain, Reynolds, et al., 2008). However, there are important negative implications of larger inter and intraspecific aggregations at feeding sites, such as 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 and pathogenic spillover (Lawson et al., 2011). Close contact in the form of aggressive behaviors has been observed with animal aggregation at feeding sites in the banded mongoose (*Mungos mungo*), and is believed to be associated with increased *Mycobacterium tuberculosis* infection (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*) (Tryland et al., 2019). *Trichomonas galliane* outbreaks have been reported in several different species of columbiforms. 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). More recently, a large outbreak of *T. galliane* infection was recorded in a wintering population of woodpigeons (*Columba palumbus*), where approximately 2600 woodpigeons died in a wintering site in Spain in 2001 (Höfle et al., 2004). It is suggested that the congregation of woodpigeons at game bird feeders may have contributed to the disease outbreak, as birds have been seen gathering at such feeders when yields of natural food resources have been poor.

Increased indirect pathogen transmission is common when animals congregate at feeding sites as a result of infective stages being shed in the environment from infected hosts, via routes such as feces and saliva (Murray et al., 2016). The length of time such stages can persist in the environment varies between organisms and with environmental factors (Turner et al., 2021; Walther & Ewald, 2004), 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 behavior 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).

With widespread habitat degradation observed across the world resulting in reduced availability of natural food sources, birds are increasingly feeding opportunistically at man-made sites and garden bird feeders (Browne & Aebischer, 2003; Dunn et al., 2018; Lawson et al., 2018; Robb, McDonald, Chamberlain, & Bearhop, 2008). For example, the turtle dove, an obligate granivore feeding on naturally occurring weed seeds, has lost a great deal of its natural food resources as a result of agricultural intensification, and a dietary switch has been observed, with this species now feeding on far higher levels of cultivated seeds than previously observed (Browne & Aebischer, 2003; Dunn et al., 2018). There is evidence of turtle doves feeding at supplementary feeding sites, as Dunn et al. (2018) detected hemp (*Cannabis sativa*), niger (*Guizotia abyssinica*), and sorghum (*Sorghum bicolor*), common components of supplementary seed mix, in the diet of turtle doves in the UK. In addition, Browne and Aebischer (2003) recorded turtle doves feeding primarily at anthropogenic food sources, such as spilt grain, animal feed and grain stores, and there are anecdotal reports of turtle doves being seen under garden bird feeders (Dunn et al., 2018). The use of feeding sites results in much higher rates of inter- and intra-specific interactions between birds than would occur under natural foraging behaviors (Lawson et al., 2018). This increased contact facilitates the spread of parasites, such as *T. gallinae*, and other wildlife pathogens, with a common factor observed in all geographic areas where trichomonosis has emerged being mortality where birds gather at feeding and watering sites (McBurney et al., 2015; Robinson et al., 2010).

As well as being commonplace in residential gardens, with millions of households provisioning food in this way every year (Robb, McDonald, Chamberlain, & Bearhop, 2008), supplementary feeding is commonly used by game managers to encourage high densities of species for hunting (Gortázar et al., 2006), and 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 (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).

Supplementary feeding is commonly used for turtle doves, both as 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). Baiting is also used to attract game birds for hunting (Rocha & Quillfeldt, 2015). Turtle doves are widely hunted across much of mainland Europe and utilize bait which may be laid to attract them, or other game species (Rocha et al., 2022). 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. Here, we therefore bring together two key elements of research 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 (Young, 2022), we investigated the relationship between the proportion of the diet consisting of cultivated crop seeds and the presence of *T. gallinae*, assessing whether there was any association between diet consumed and strain of *T. gallinae* detected, in particular, a positive association between infection with *T. galliane* and consumption of non-natural food resources, indicating the potential use of supplementary feed sites.

2 | MATERIALS AND METHODS

2.1 | Datasets

Dietary data and parasite infection datasets used in this study were collected from turtle doves from three countries, across both wintering and breeding grounds (Appendix 1: Figure A1) as

part of a wider study (Young, 2022). A wintering population of turtle doves was sampled using mist nets at the Beer Sheba Project (Beer Sheba Project, 2002), an agricultural resource center encompassing a 100 ha *Acacia* woodland near Sandiara (14°22′ N, 16°48′ W), Senegal between November and March over four winters (2014/2015–2017/2018). 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), early in the breeding season. In France, samples were collected in spring 2017 (May 15–30) using baited potter traps from 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 (Appendix 1: Figure A2). In Hungary, birds were sampled in spring 2018 (May 20–June 7) using mist nets from Balotaszállás-Öttömos (46°16′ N, 19°35′ W), consisting of a mix of plantations and native forest (Appendix 1). While 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.

Full details of sampling, DNA extraction, and DNA sequencing can be found in Appendix 2. In short, for dietary analysis, fecal samples were collected from sterile bird bags in which birds were held after capture, and dried to preserve DNA (Rayé et al., 2011). DNA was extracted using either a QIAmp DNA Stool Mini Kit, or QIAmp Fast Stool Mini Kit (Qiagen), following the manufacturer's protocols with minor modifications to maximize DNA yield, as detailed in Dunn et al. (2016). Two extraction negatives, containing nuclease-free water instead of sample DNA, were included in every extraction batch to detect potential contamination. To test for *T. gallinae* infection, samples were collected from the oral cavity, esophagus, and crop of turtle doves using a sterile swab, which was then used to inoculate individually labeled InPouch™ culture kits (BioMed) and incubated at 37°C for 7 days to allow parasite proliferation. Following incubation, culture medium was preserved with an equal volume of 100% ethanol. All culture samples were processed using the following protocol, and the absence or presence of *T. gallinae* was confirmed by PCR. Culture medium was processed as per Riley et al. (1992) in order to remove culture medium from sample and isolate parasites if they are present. Following the isolation step, all samples underwent the DNA extraction protocol, using one of two methods, either modified ammonium acetate method (Nicholls et al., 2000) or using a DNeasy Blood and Tissue kit (Qiagen). All samples were tested using PCR for the presence of *T. gallinae* DNA.

For dietary analysis, the second internal transcribed spacer of nuclear ribosomal DNA (hereafter ITS2) region was amplified using the UniPlant primer pair (F: 5′-TGTGAATTGCARRATYCMG-3′; R: 5′-CCCGHYTGAYYTGRGGTCDC-3′) (Moorhouse-Gann et al., 2018), and for sequencing of *T. gallinae*, the ITS1/5.8S/ITS2 ribosomal region of the genome (hereafter referred to as the ITS region) was amplified from oral swab samples using the TFR1/TFR2 primer pair (F: 5′-TGCTTCAGTTCAGCGGGTCTTCC-3′; R: 5′-CGGTAGGT GAACCTGCCGTTGG-3′) (Felleisen, 1997). PCR products from oral swab samples were electrophoresed through a 1% gel, stained using SYBR®Safe (Thermo Fisher Scientific) in 0.5× TBE buffer and visualized using UV light to determine the infection status of birds. The presence of *T. gallinae* infection was indicated by the occurrence of a band of the appropriate amplicon size. Where no amplicon was observed, the sample was run through PCR a second time to confirm the absence of *T. gallinae*.

For sequencing, all primers were labeled with MID-tags (Multiple Identifiers) to provide a unique combination of tags for each DNA sample. Each plate of MID-tagged PCR product was combined into a single pool of uniquely identifiable samples, with approximately equal concentration per sample. Pools were cleaned using SPRIselect beads (Beckman Coulter) following the manufacturer's instructions, and the library preparation for Illumina sequencing was carried out on this pool using NEXTflex Rapid DNA-seq Kit (Bioo Scientific), following the manufacturer's protocols. The library was sequenced using a V2 chip, with 2 × 250 bp paired-end reads on an Illumina MiSeq sequencer (Illumina). Bioinformatic analyses were carried out using a custom pipeline designed for analysis of diet metabarcoding data from Illumina MiSeq (Drake et al., 2021). Full description of bioinformatics analysis is given in Appendix 3.

An additional dataset, collected for another study in the UK, was analyzed in order to incorporate the strain Type A, a high-virulence strain which was detected in the previous study in the UK, but did not occur in the samples collected in this study (Dunn et al., 2018; Thomas et al., 2022). Data were collected in the UK from 12 farmland sites across Essex, Suffolk, Cambridgeshire, and Norfolk in 2011–2014. Sampling and DNA extraction were conducted following the same protocols used in sample collection from Senegal, France, and Hungary, and full details of sampling are given in Dunn et al. (2018). Due to the application of different bioinformatic processing pipelines and data cleaning in the two datasets, UK data were analyzed separately from data collected on the breeding grounds in this study.

2.2 | Dietary variation and infection status

All statistical analyses were carried out in R, version 4.0.1 (R Core Team, 2020). Due to differences in the amplification efficiency of DNA from different plant species, the presence or absence of each taxonomic unit within each sampling unit (frequency of occurrence), rather than read count, was used in all statistical analyses (Lamb et al., 2018).

An index of bird body condition was calculated using a 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 an approximate linear increase in weight through the daily sampling period was observed in this study. Residuals from the model represented the condition of individual birds.

To assess the relationship between diet and infection status, detrended correspondence analysis (DCA) was first carried out to summarize dietary data, based on the presence or absence of dietary taxa within each bird sampled, to capture the source of dietary variation in a small number of variables, to be included in a generalize linear model (GLM). Analysis was carried out using the "decorana" function in the R package *vegan* (Oksanen, 2020) and visualized using functions from the *ggplot2* package (Wickham, 2016).

A high proportion of dietary items occur in a very low number of samples, which 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. Four different thresholds representing how frequently items occurred in the diet were tested (taxa occurring in the diet of at least 3%, 5%, 7%, and 10% of birds sampled) to determine the most appropriate threshold for defining "frequently occurring taxa" (Appendix 4). DCA was conducted on each subset and the scores of samples inspected to assess for outliers within the sample. The optimum threshold for DCA was determined based on the absence of outliers in the data, as defined by Bendixen (2003).

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 (Appendix 5: Figure A4). A GLM was fitted with the presence or absence of *T. gallinae* as the dependent variable, using the binomial family and logit link function. Independent variables included in the model were season, body condition index, and the coordinate scores for DCA1 and DCA2. This model was repeated to assess individual strains of *T. gallinae*, with the presence or absence of GEO, Tcl-1, Type C, and Type IIIc being the dependent variable in four respective models. A reduced dataset 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.

2.3 | Dietary overlap

Dietary overlap between infected and uninfected birds, and birds infected with different strains of *T. gallinae*, was visualized 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, 2020) was used to estimate differences between the presence of genera in individual samples, using Jaccard distance. "Ordispider" was used to visualize 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 et al., 2015)) with 9999 repeats. Pairwise overlap was also assessed between specific strains.

Pianka's niche overlap was repeated as above 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 underestimating the overlap (Linton et al., 1981).

2.4 | Source of food and *Trichomonas gallinae* infection

The effect of different food sources was assessed by considering dietary items categorized as "wild" or "cultivated" based on the likely source of food items (Appendix 6). Cultivated seeds included crop plants, animal fodder, and seeds common in supplementary feed, and wild were those naturally occurring in the environment. The effect of the proportion of wild seeds in the diet upon the infection probability by *T. gallinae* was assessed using a binomial GLM. The 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, modeling 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 of the dominant food type occurring in the diet. As quantitative diet data were not available, the proportion of taxonomic units detected in the diet classified as cultivated or wild was used as a proxy to indicate whether an individual's diet was 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 classed as wild and cultivated was the same.

3 | RESULTS

3.1 | Dietary variation and infection status

A total of 190 birds sampled from the breeding and wintering grounds, from which diet and *T. gallinae* infection status data were successfully obtained were assessed to investigate the relationship between diet and parasite infection (Table 1). The overall prevalence of *T. galliane* infection across the dataset was 70%, but varied from 49% to 100% between seasons (Table 1). Five strains of *T. gallinae* were very rare within this study, occurring in just one (Tcl-TD,

Country	Year	Sampling season	Total *N* sampled	% Infected with *Trichomonas galliane*
Senegal	2014/2015	Winter 1 (W1)	24	100.0
Senegal	2015/2016	Winter 2 (W2)	12	66.7
Senegal	2016/2017	Winter 3 (W3)	28	64.3
Senegal	2017/2018	Winter 4 (W4)	58	82.8
France	2018	Spring 1 (S1)	57	49.1
Hungary	2019	Spring 2 (S2)	11	63.6
Total			190	70
UK	2011–2014	UK dataset	35	91.4

TABLE 1 Number of birds sampled across breeding and wintering grounds within the present study.

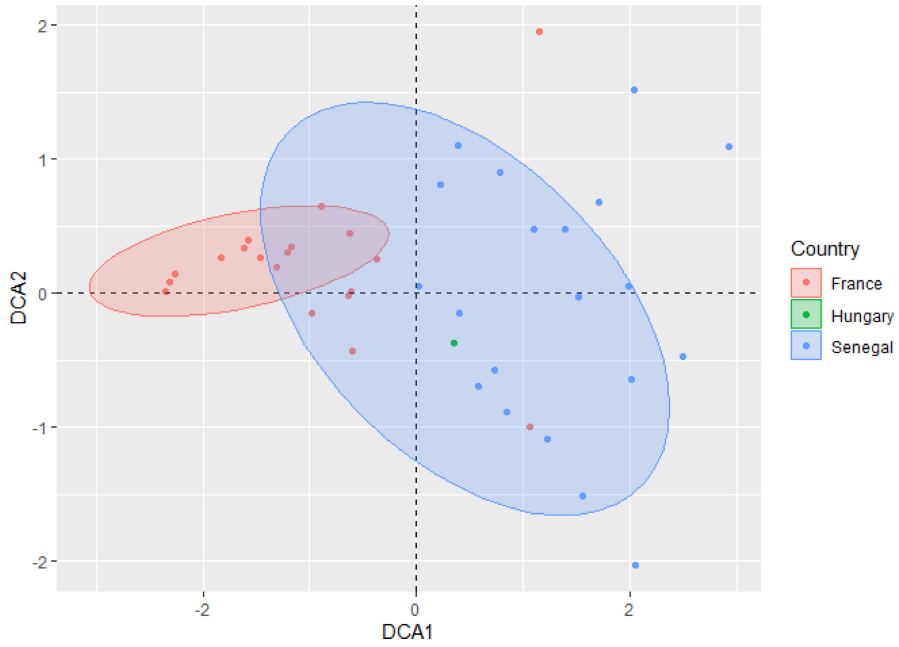

FIGURE 1 Biplot illustrating the variance accounted for by DCA1 and DCA2, with the associated eigenvalues of 0.827 and 0.568, respectively. Shaded ellipses represent 95% confidence interval for countries with more than one sample.

GEO-TD3, Type IIIc-TD, ESWD), two (GEO-TD2), three (TtI-TD), or four birds (GEO-TD), reflecting 0.6%, 1.1%, 1.7%, and 2.3% of the total number of birds sampled, respectively, and, due to their low occurrence, were not included in strain-specific analysis. Data from an additional 35 birds sampled in the UK as part of another body of work were analyzed (Dunn et al., 2018; Thomas et al., 2022).

When accounting for outliers in the dataset following threshold testing to remove uncommon taxa from analysis (Appendix 4), the threshold for the total proportion of samples within which a taxon 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 explains the geographical separation of samples, with the majority of samples from Senegal having positive values on dimension 1, and birds sampled from France largely having negative values (Figure 1). Following the removal of outliers, only one sample from Hungary was retained in analysis. When visualizing the distribution of taxa consumed (Figure 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 bird 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.

DCA1 and DCA2 explained the majority of the variation observed in this dataset; therefore, only they were included as explanatory variables in GLM analysis (Appendix 5).

The final dataset contained only one sample from Hungary, which 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 the presence of *T. gallinae* (GLM: $LRT_4 = 33.494$, $p < 0.001$). When considering individual strains, no variables were significantly associated with the presence of Tcl-1 or Type C, but season was significantly associated with the presence of GEO (GLM: $LRT_4 = 48.809$, $p < 0.001$)

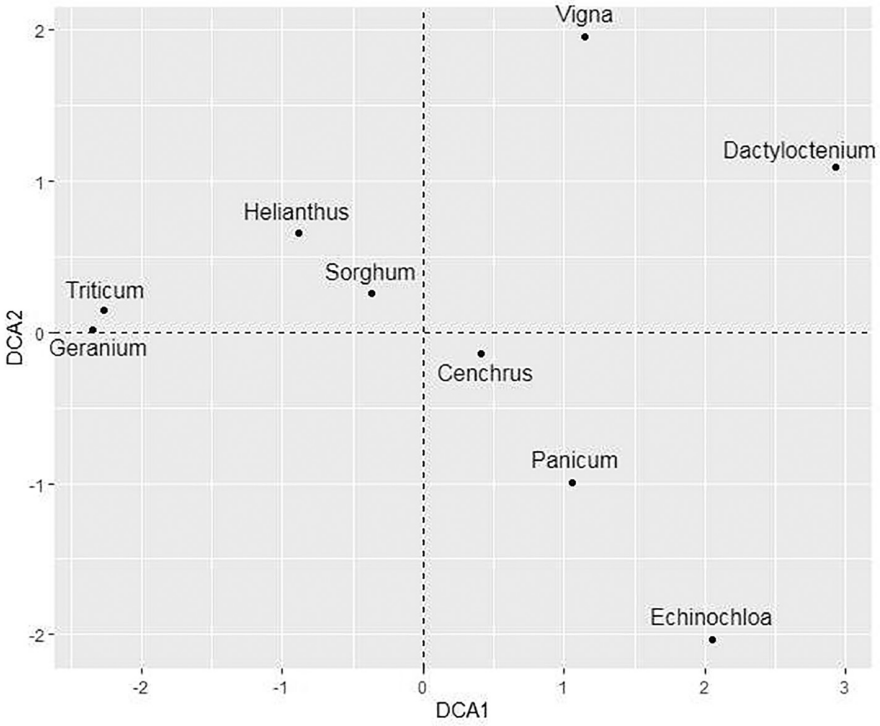

FIGURE 2 Biplot showing the distribution of dietary taxa across DCA1 and DCA2.

and Type IIIc (GLM: $LRT_4 = 13.5121$, $p = 0.009$). The variables explaining dietary variation (DCA1 and DCA2) were not associated with the presence of any strain of *T. gallinae*.

3.2 | Dietary overlap

Pianka's niche overlap suggests significant dietary overlap between infected and uninfected individuals on the wintering and breeding grounds. The lowest overlap was observed on the wintering grounds, with almost complete overlap identified on the breeding grounds (Table 2). When visualizing dietary segregation, centroids are close together, supporting this result of little difference in the diet between infected and uninfected individuals (Figure 3).

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) (Table 2). The dietary overlap was highest in France (S1), where almost complete dietary overlap was observed between infected and uninfected individuals and the lowest dietary overlap between infected and uninfected individuals was observed in data collected from the UK (Table 2).

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.

TABLE 2 Pianka's niche overlap analysis on the similarity of diet between individuals infected and uninfected with *Trichomonas galliane* within difference sampling season.

Sampling season	Pianka	*p*	SES
Wintering grounds overall	0.577	≤0.001	4.632
Breeding grounds overall	0.943	≤0.001	8.592
W3	0.874	≤0.001	7.898
W4	0.824	≤0.001	7.602
S1	0.957	≤0.001	8.365
UK	0.770	≤0.001	5.479

Note: W3 and W4 refer to the wintering seasons in Senegal of 2016/17 and 2017/18, respectively, S1 refers to spring sampling season in France (2017).

The average pairwise overlap was greater in the breeding grounds than in the wintering grounds (Table 3), but both exhibited reasonably strong overlap of dietary items consumed by individuals infected with different strains of *T. gallinae*. When visualizing 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 4). 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 4b).

Again, when considering sampling seasons separately, W2 and S2 were excluded due to small sample sizes. Within individual sampling seasons, low dietary overlap was detected in W1 and W3 (Table 3), supported by the distribution of centroids in Figure 5a,b. Much higher dietary overlap between birds infected with different strains was observed in W4 and S1 (Table 3, Figure 5c,d).

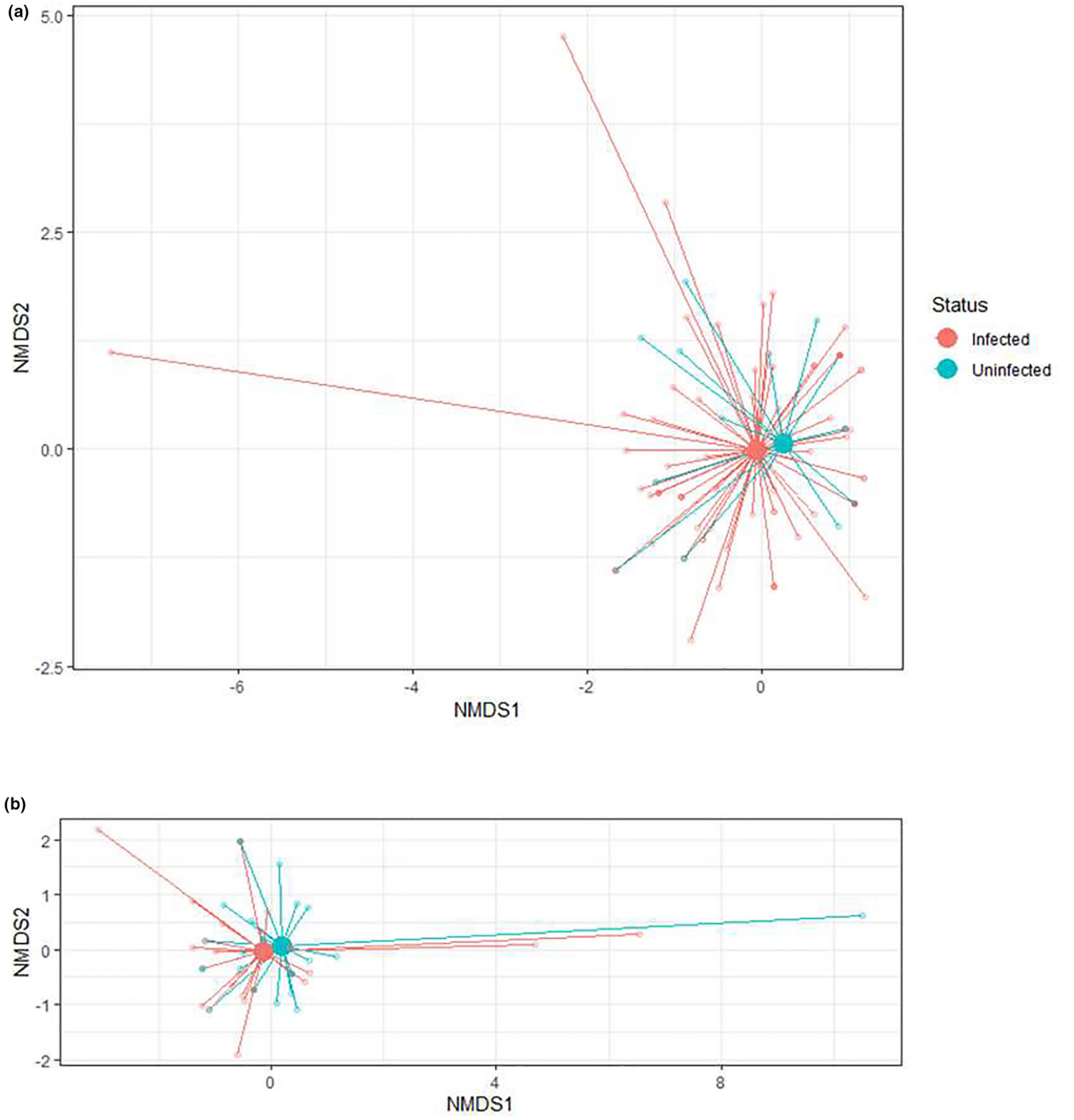

FIGURE 3 Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves (a) in the wintering grounds, differentiated by status of infection with *Trichomonas galliane*. Stress = 0.07, $N = 122$; (b) in the mainland European breeding grounds, differentiated by status of infection with *T. gallinae*. Stress = 0.07, $N = 71$. 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.

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, and 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 visualizing these data, the overlap of centroids observed in UK data is not as strong as that of samples from W4 and S1 (Figure 6).

TABLE 3 Pianka's niche overlap analysis on the similarity of diet between individuals infected with different strains of *Trichomonas galliane* (GEO, Type C, Type IIIc and Tcl-1) within difference sampling season.

Season	Pianka	*p*	SES
Wintering grounds overall	0.765	≤0.001	21.265
Breeding grounds overall	0.807	≤0.001	22.218
W1	0.418	0.001	6.459
W3	0.460	≤0.001	9.464
W4	0.785	≤0.001	22.325
S1	0.957	≤0.001	8.635

Note: W3 and W4 refer to the wintering seasons in Senegal of 2016/17 and 2017/18, respectively, and S1 refers to spring sampling season in France (2017).

3.3 | 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: $Dev_5 = 33.608$, $p \leq 0.001$), as well as strain-specific associations for GEO (GLM: $Dev_5 = 46.65$, $p \leq 0.001$) and Type IIIc (GLM: $Dev_5 = 22.71$, $p \leq 0.001$). The proportion of wild seeds consumed was not associated with 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 7).

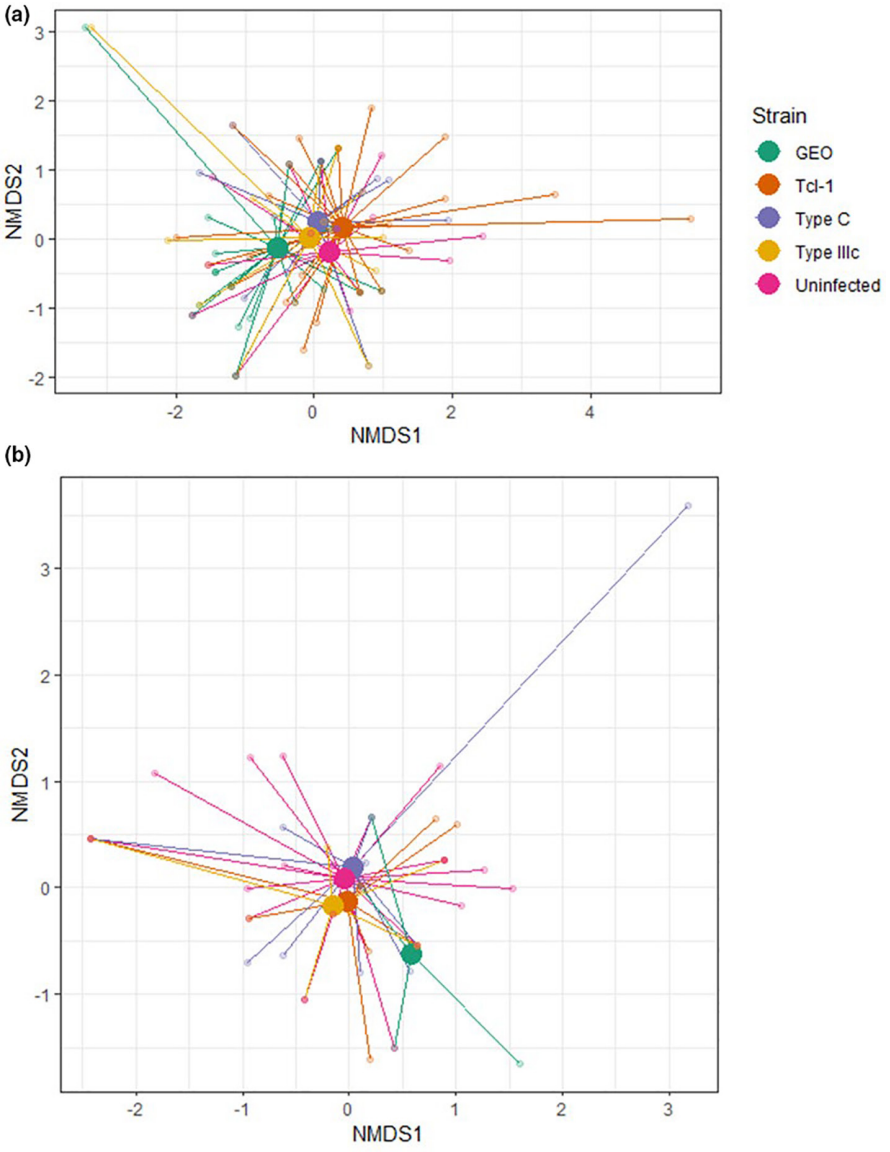

FIGURE 4 Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves across birds sampled (a) in the wintering grounds, differentiated by the strains of *Trichomonas galliane* present. Stress = 0.066, $N = 110$; (b) in the mainland Europe breeding grounds, differentiated by the strains of *T. gallinae* present. Stress = 0.086, $N = 63$.

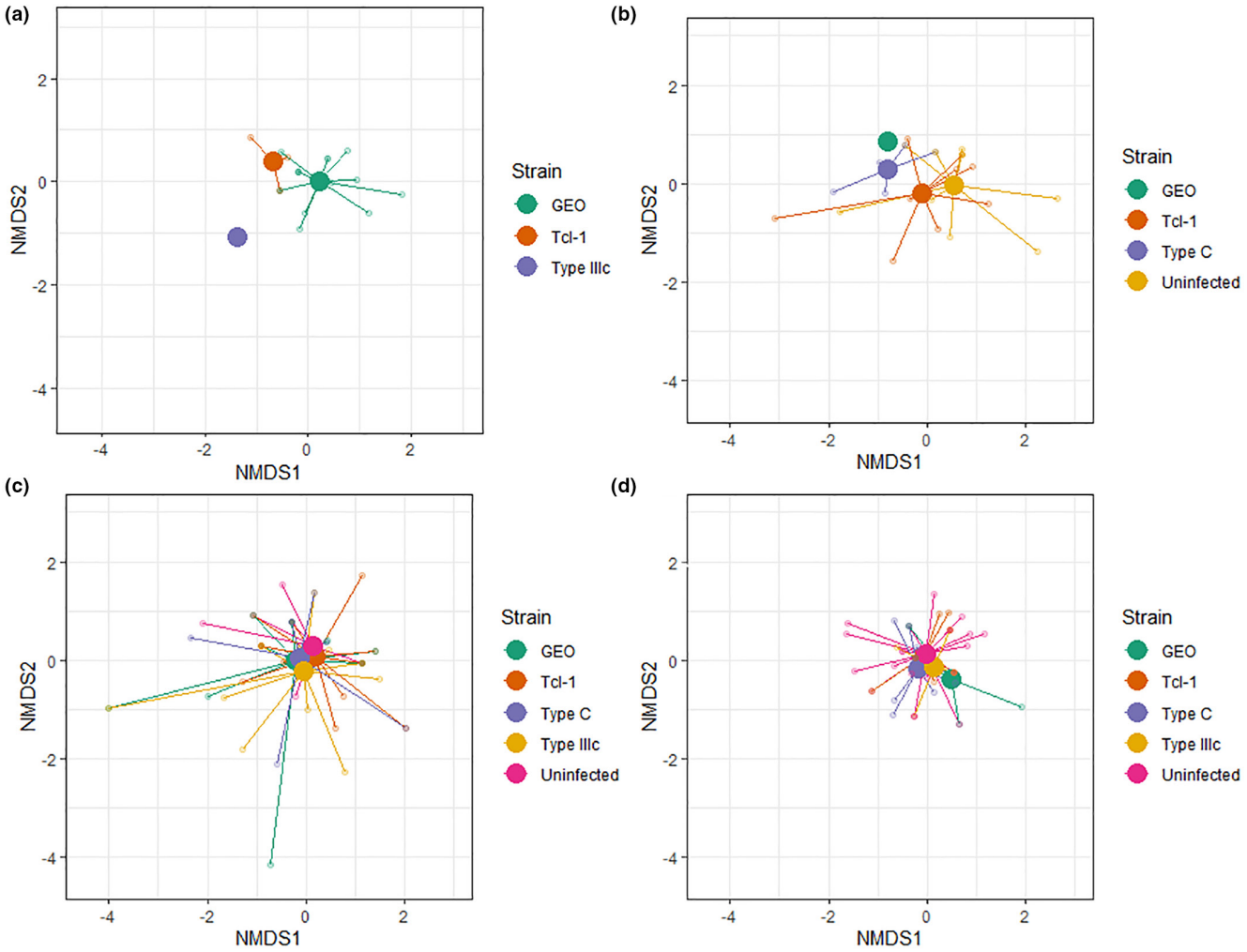

FIGURE 5 Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves across birds sampled in the breeding grounds, differentiated by the strains of *Trichomonas galliane* present. (a) Winter 2014/15 (W1), (b) winter 2016/17 (W3), (c) winter 2017/18 (W4), (d) spring, France (S1).

4 | DISCUSSION

While there is a large body of research reporting the increased transmission of disease at supplementary food sites, this study specifically investigated 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 associated with 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.

4.1 | The relationship between rate of infection and consumption of cultivated seed

While *T. gallinae* is commonly transmitted during courtship, and the feeding of crop-milk to offspring, based on the findings of numerous previous studies implicating individuals congregating at supplementary feeding sites in the increased spread of disease (Dhondt et al., 2007; Fogell et al., 2019; McBurney et al., 2015; Pennycott et al., 1998), we investigated the associations between diet and the presence of *T. gallinae*. We hypothesized that infection with *T. gallinae* would be positively associated with the proportion of cultivated seeds in the diet. This is because a higher proportion of cultivated seed was expected to indicate increased use of supplementary food resources, and thus increased chance of contact with other individuals. We did not find evidence to support this, 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. However, the effect of supplementary feeding on infection should not be ruled out and remains a key consideration in actions involving provisioning of food resources. A number of potential explanations for the lack of association within the present study are discussed.

Over half of uninfected birds sampled had a diet dominated by cultivated seed expected to be obtained through supplementary feeding. This finding may be related to the types of foraging and

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences
WILEY

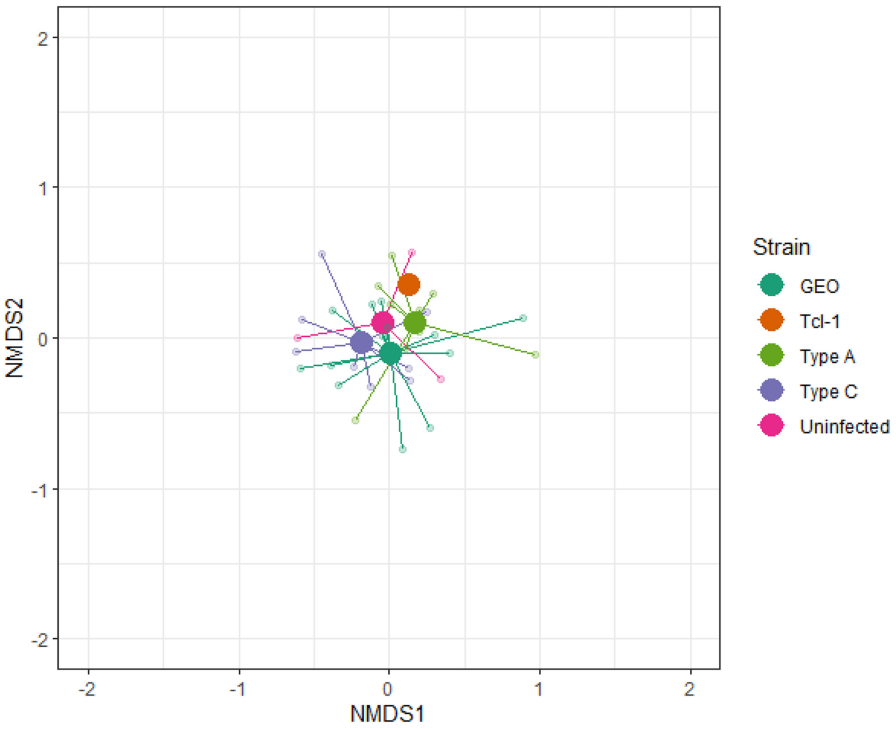

FIGURE 6 Spider plot derived from non-metric multidimensional scaling of genera consumed by turtle doves across birds sampled in the UK, differentiated by the strains of *Trichomonas galliane* present. Stress = 0.246, *N* = 34.

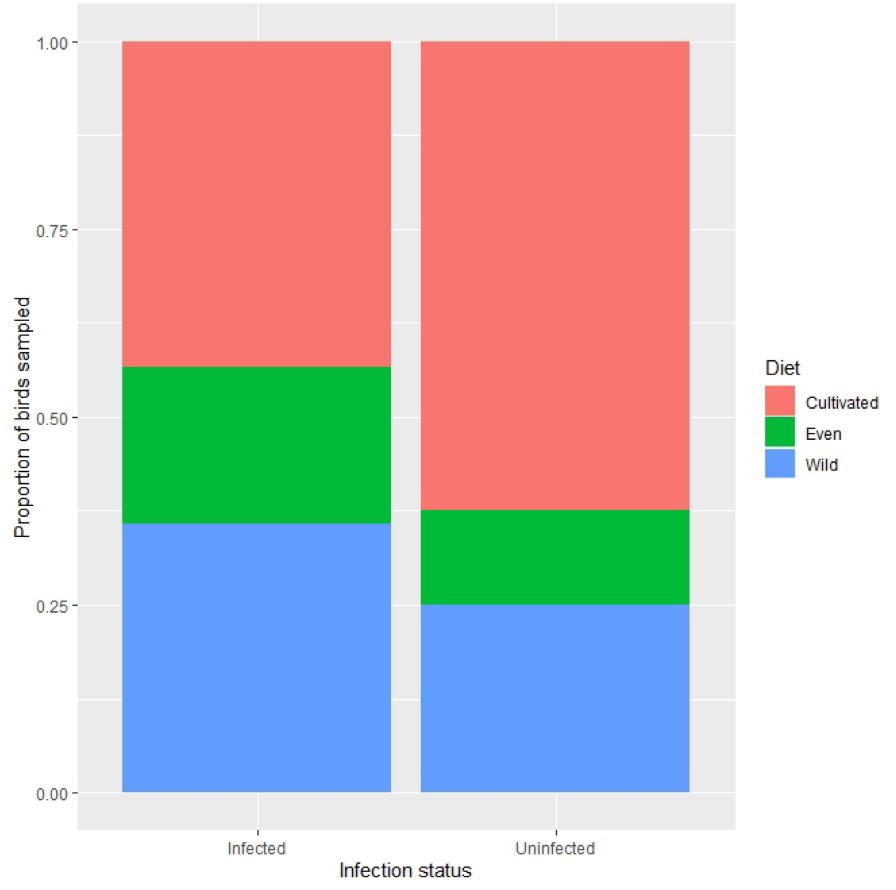

FIGURE 7 Proportion of total birds (*N* = 190) infected or uninfected with *Trichomonas galliane*, where the taxonomic units detected in the diet were dominated by wild or cultivated seeds, or with an even split of wild and cultivated food sources. Calculated based on the proportion of dietary items consumed by individuals accounted for by wild seeds. Infected birds, *n* = 134, uninfected birds, *n* = 56.

method of supplementary food delivery. The rationale for our expectation 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 (McBurney et al., 2015; Robinson et al., 2010; Thomas, 2017). This congregation behavior is commonly observed at artificial food sites, such as garden bird feeders (McBurney et al., 2015), and supplementary feeding sites, intended

to support high densities of game species for hunting, such as partridges and pheasants (Phasianidae), ducks (Anatidae), and doves and pigeons (Columbidae) (Gortázar et al., 2006; Millán et al., 2003).

It is possible that a relationship between supplementary food and infection was not observed in this study due to the nature of supplementary food delivery, as it did not result in the congregation of animals. Bait was used to attract individuals into potter traps in France. Once they entered the trap and triggered the door, solitary individuals were shut in traps until they were removed. This contrasts with previously reported incidences of transmission at supplementary feeding stations, where large numbers of individuals congregate, facilitating disease transmission, as birds were not seen to congregate around potter traps. There may still have been an elevated risk of disease transmission at feeding sites, as *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) and 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 at this site.

In Senegal, birds feeding on cultivated seed were unlikely to be congregating at feeding sites, 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 were commonly detected in turtle dove diet in the wintering grounds (Young, 2022). It is therefore most likely that birds were obtaining this food resource from harvested fields, rather than supplementary feed sites. While birds do feed in groups and mix with other columbids (mourning dove [*Streptopelia decipiens*] and Namaqua dove [*Oena capensis*]) in harvested fields, these are spread over a wide area of arable land, rather than dense congregations observed at single bird feeding stations. 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 while 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 on breeding grounds, reducing the risk of disease transmission in this instance. In wintering sites, such as sub-Saharan Africa, water sources are suggested to pose a higher risk of disease transmission, as water is generally scarcer, leading to high densities of birds congregating at watering holes, increasing both the likelihood of parasites being deposited in pools by infected hosts, and infective agents being picked up by uninfected hosts (Amin et al., 2014). Typically, when in Africa, turtle doves go to drink at first light or sunset, when large congregations of birds are observed (Browne & Aebischer, 2005).

In European sampling sites, it is unlikely that individuals were obtaining cultivated grain from agricultural fields as they were observed to be in Senegal, due to the timing of sampling. The two most prevalent cultivated crops in our sampling site in Senegal, millet and sorghum, are harvested from September to November (GIEWS, 2020), resulting in an abundance of spilt grain early in the wintering season, when many of the birds in Senegal were sampled. In contrast, turtle doves caught in Europe were sampled early in the breeding season and pre-harvest, before cultivated crops in the surrounding agricultural landscape would have matured. Therefore, such food resources would not be as readily available in the environment in European sampling sites as in Africa; thus, they most likely are from supplementary food sources in Europe. Anecdotal evidence from yearly sampling at the French field sites indicates that post-harvest, turtle doves do forage in harvested fields, as they are very difficult to catch, not coming into even baited traps when there is an abundance of seed in agricultural fields (personal observation).

Another potential explanation for the lack of association between consumption of cultivated resources and *T. galliane* infection may 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, finding that a higher density of bird feeders would result in individuals staying at feed sites for longer, increasing pathogen transmission. They also observed 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.

There is evidence that prior exposure to milder strains of *T. galliane* can infer protection against future infection. 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. 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*. 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. One aspect that the majority of 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* (Forzan et al., 2010; Lawson et al., 2006; Marx et al., 2017; Sansano-Maestre et al., 2009; Thomas, 2017). One exception is work carried out by Bunbury et al. (2008) on the Mauritian pink pigeon, which repeatedly tested birds over a 2-year period for the presence of *T. gallinae*, finding evidence of an increase in infection rate with increased host age and a negative impact on survival in birds consistently testing positive for this parasite. However, many questions regarding the dynamics of this parasite infection remain unanswered. 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, and if this varies between species of host and parasite strains. Greater monitoring of disease and understanding these host–parasite dynamics would help shed further light on disease transmission, and potential risk factors, such as congregation at supplementary feeding sites, and help inform management plans for declining species.

As well as these potential behavioral explanations, it is possible that birds in better condition as a result of utilizing abundant cultivated food resources were more capable of fighting off infection, as associations have been shown between immune system functioning and body condition (Becker & Hall, 2014; Chandra, 1999; Møller et al., 1998). Previous dietary analysis on these data (Young, 2022) detected a positive correlation between body condition and the proportion of diet accounted for by cultivated seed. Multiple other studies investigating the effects of supplemental feeding have reported improvement of health metrics of birds, including increased antioxidant levels, more rapid feather growth, reduced stress indicators, improved body condition index, improved innate immune defense, and improved fat score (Galbraith et al., 2016; Wilcoxen et al., 2015). Furthermore, some commonly fed seeds, such as *Helianthus* sp., have a high calorific value (Hullar et al., 1999), which may help improve body condition by alleviating nutritional stress (Becker & Hall, 2014). These findings support the suggestion that use of supplementary food may improve body condition, and therefore boost immune function, reducing host susceptibility to *T. gallinae*. However, it is important to note that, while 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. While this association between body condition and infection status was not supported in the current study, it is still a key consideration that should be addressed when providing supplementary food (Galbraith et al., 2016; Wilcoxen et al., 2015).

4.2 | *Trichomonas gallinae* strains in relation to diet

There was variation in both the *T. gallinae* strain composition and the dietary composition between the sites sampled in this study (Young, 2022); it was hypothesized 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 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, we found little support for any association, with significant dietary overlap between infected and uninfected individuals. 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 2 years in Senegal, and high dietary overlap occurring in 1 year in Senegal, and in France. This lack of support for dietary items being linked to specific strains suggests that variation in the strains previously detected in supplementary food resources may reflect another unknown environmental factor or simply reflect variation in the parasite community composition, rather than an association with dietary items.

While we were unable to find evidence supporting a link between diet and infection with *T. gallinae*, 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. While 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.

AUTHOR CONTRIBUTIONS

The conception or design of the study: WOCS, JCD. The acquisition, analysis or interpretation of the data: REY (field work, lab work, analysis and interpretation); WOCS, JCD, IPV, JWM (analysis and interpretation); CJO; MK; MBD; MS (acquisition of data – field sampling in Senegal); HL; CE (acquisition of data – field sampling in France); OK (acquisition of data – field sampling in Hungary); RCT, JCD KCH, SJG (acquisition of data – provided UK dataset). Writing of the manuscript: REY.

ACKNOWLEDGMENTS

Natural Environment Research Council GW4+ Studentship NE/L002434/1; Royal Society for the Protection of Birds project CASE partner, Genetics Society Heredity Fieldwork Grant; British Society for Parasitology International Training and Fieldwork Award.

CONFLICT OF INTEREST STATEMENT

No conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be made available via online repository following acceptance of the manuscript.

ORCID

Rebecca E. Young https://orcid.org/0000-0002-6292-5813

REFERENCES

Amin, A., Bilic, I., Liebhart, D., & Hess, M. (2014). Trichomonads in birds – A review. *Parasitology*, *141*, 733–747.

Becker, D., & Hall, R. (2014). Too much of a good thing: Resource provisioning alters infectious disease dynamics in wildlife. *Biology Letters*, *10*, 20140309.

Beer Sheba Project. (2002). http://beershebaproject.org/ [Accessed 13 March 2023]

Bendixen, M. (2003). A practical guide to the use of correspondence analysis in marketing research. *Marketing Research On-Line*, *1*, 16–36.

Berger, L., Speare, R., Daszak, P., Green, D., Cunningham, A., Goggin, C., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., & Hines, H. B. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, *95*, 9031–9036.

Browne, S., & Aebischer, N. (2003). Habitat use, foraging ecology and diet of turtle doves *Streptopelia turtur* in Britain. *Ibis*, *145*, 572–582.

Browne, S., & Aebischer, N. (2005). Studies of West Palearctic birds: Turtle dove. *British Birds*, *98*, 58–72.

Bucher, E. H. (1992). The causes of extinction of the passenger pigeon. *Current Ornithology*, *9*, 1–36.

Bunbury, N., Jones, C., Greenwood, A., & Bell, D. (2008). Epidemiology and conservation implications of *Trichomonas gallinae* infection in the endangered Mauritian pink pigeon. *Biological Conservation*, *141*, 153–161.

Bunbury, N., Jones, C. G., Greenwood, A. G., & Bell, D. J. (2007). *Trichomonas gallinae* in Mauritian columbids: Implications for an endangered endemic. *Journal of Wildlife Diseases*, *43*, 399–407.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*, 1–9.

Chandra, R. (1999). Nutrition and immunology: From the clinic to cellular biology and back again. *Proceedings of the Nutrition Society*, *58*, 681–683.

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*, i884–i890.

Cunningham, A. A., & Daszak, P. (1998). Extinction of a species of land snail due to infection with a microsporidian parasite. *Conservation Biology*, *12*, 1139–1141.

Daszak, P., Cunningham, A., & Hyatt, A. (2000). Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science*, *287*, 443–449.

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*, *14*, 306–323.

De Castro, F., & Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters*, *8*, 117–126.

Dhondt, A. A., Dhondt, K. V., Hawley, D. M., & Jennelle, C. S. (2007). Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Parasitology*, *36*, 205–208.

Drake, L., Cuff, J., Young, R., Marchbank, A., Chadwick, E., & Symondson, W. (2021). An assessment of minimum sequence copy thresholds for identifying and reducing the prevalence of artefacts in dietary metabarcoding data. *Methods in Ecology and Evolution*, *13*, 694–710.

Dunn, J. C., Morris, A. J., & Grice, P. V. (2015). Testing bespoke management of foraging habitat for European turtle doves *Streptopelia turtur*. *Journal for Nature Conservation*, *25*, 23–34.

Dunn, J. C., Stockdale, J. E., McCubbin, A., Thomas, R. C., Goodman, S. J., Grice, P. V., Morris, A. J., Hamer, K. C., & Symondson, W. O. C. (2016). Non-cultured faecal and gastrointestinal seed samples fail to detect *Trichomonad infection* in clinically and sub-clinically infected columbid birds. *Conservation Genetics Resources*, *8*, 97–99.

Dunn, J. C., Stockdale, J. E., Moorhouse-Gann, R. J., McCubbin, A., Hipperson, H., Morris, A. J., Grice, P. V., & Symondson, W. O. C. (2018). The decline of the turtle dove: Dietary associations with body condition and competition with other columbids analysed using high-throughput sequencing. *Molecular Ecology*, *27*, 3386–3407.

Edgar, R. (2016). UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv*, *15*, 081257.

Ewen, J. G., Walker, L., Canessa, S., & Groombridge, J. J. (2015). Improving supplementary feeding in species conservation. *Conservation Biology*, *29*, 341–349.

Felleisen, R. (1997). Comparative sequence analysis of 5·8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. *Parasitology*, *115*, 111–119.

Flint, B., Hawley, D., & Alexander, K. A. (2016). Do not feed the wildlife: Associations between garbage use, aggression, and disease in banded mongooses (*Mungos mungo*). *Wiley Online Library*, *6*, 5932–5939.

Fogell, D. J., Groombridge, J. J., Tollington, S., Canessa, S., Henshaw, S., Zuel, N., Jones, C. G., Greenwood, A., & Ewen, J. G. (2019). Hygiene and biosecurity protocols reduce infection prevalence but do not improve fledging success in an endangered parrot. *Scientific Reports*, *9*, 1–10.

Forzan, M., Vanderstichel, R., Melekhovets, Y., & McBurney, S. (2010). Trichomonas in finches from the Canadian Maritime provinces - an emerging disease. *Canadian Veterinary Journal*, *51*, 391.

Galbraith, J. A., Stanley, M. C., Jones, D. N., & Beggs, J. R. (2016). Experimental feeding regime influences urban bird disease dynamics. *Wiley Online Library*, *48*, 700–713.

GIEWS. (2020). FAO GIEWS Country Brief on Senegal [Online]. Available at: https://www.fao.org/giews/countrybrief/country.jsp?code=SEN&lang=en [Accessed 25 January 2022].

Gortázar, C., Acevedo, P., Ruiz-Fons, F., & Vicente, J. (2006). Disease risks and overabundance of game species. *European Journal of Wildlife Research*, *52*, 81–87.

Gotelli, N. J., Hart, E. M., & Ellison, A. M. (2015). *EcoSimR: Null model analysis for ecological data*. R package version 0.1.0. http://github.com/gotellilab/EcoSimR

Herrera, J., & Nunn, C. L. (2019). Behavioural ecology and infectious disease: Implications for conservation of biodiversity. *Philosophical Transactions of the Royal Society B*, *374*, 20180054.

Höfle, U., Gortázar, C., Ortiz, J. A., Knispel, B., & Kaleta, E. F. (2004). Outbreak of trichomoniasis in a woodpigeon (*Columba palumbus*) wintering roost. *European Journal of Wildlife Research*, *50*, 73–77.

Hullar, I., Meleg, I., Fekete, S., & Romvari, R. (1999). Studies on the energy content of pigeon feeds I. Determination of digestibility and metabolizable energy content. *Poultry Science*, *78*, 1757–1762.

Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H. J., & Tappu, R. (2016). MEGAN community edition-interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS Computational Biology*, *12*, e1004957.

Lamb, P., Hunter, E., Pinnegar, J., Creer, S., Davies, R., & Taylor, M. (2018). How quantitative is metabarcoding: A meta-analytical approach. *Molecular Ecology*, *28*, 420–430.

Lawson, B., Cunningham, A. A., Chantrey, J., Hughes, L. A., John, S. K., Bunbury, N., Bell, D. J., & Tyler, K. M. (2011). A clonal strain of *Trichomonas gallinae* is the aetiologic agent of an emerging avian epidemic disease. *Infection, Genetics and Evolution*, *11*, 1638–1645.

Lawson, B., Cunningham, A., Chantrey, J., Hughes, L., Kirkwood, J., Pennycott, T., & Simpson, V. (2006). Epidemic finch mortality. *The Veterinary Record*, *159*, 367.

Lawson, B., Robinson, R., Toms, M., Risely, K., Macdonald, S., & Cunningham, A. (2018). Health hazards to wild birds and risk factors associated with anthropogenic food provisioning. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *373*, 20170091.

Lennon, R. J., Dunn, J. C., Stockdale, J. E., Goodman, S. J., Morris, A. J., & Hamer, K. C. (2013). Trichomonad parasite infection in four species of Columbidae in the UK. *Parasitology*, *140*, 1368–1376.

Lighten, J., van Oosterhout, C., Paterson, I. G., Mcmullan, M., & Bentzen, P. (2014). Ultra-deep Illumina sequencing accurately identifies MHC class IIb alleles and provides evidence for copy number variation in the guppy (*Poecilia reticulata*). *Molecular Ecology Resources*, *14*, 753–767.

Linton, L. R., Davies, R. W., & Wrona, F. J. (1981). Resource utilization indices: An assessment. *The Journal of Animal Ecology*, *50*, 283.

Marx, M., Korner-Nievergelt, F., & Quillfeldt, P. (2016). Analysis of ring recoveries of European turtle doves *Streptopelia turtur* – Flyways, migration timing and origin areas of hunted birds. *Acta Ornithologica*, *51*, 55–70.

Marx, M., Reiner, G., Willems, H., Rocha, G., Hillerich, K., Masello, J. F., Mayr, S. L., Moussa, S., Dunn, J. C., Thomas, R. C., & Goodman, S. J. (2017). High prevalence of *Trichomonas gallinae* in wild columbids across western and southern Europe. *Parasites and Vectors*, *10*, 1–11.

McBurney, S., Kelly-Clark, W., Forzán, M., Lawson, B., Tyler, K., & Greenwood, S. (2015). Molecular characterization of *Trichomonas gallinae* isolates recovered from the Canadian Maritime provinces' wild avifauna reveals the presence of the genotype responsible for the European finch trichomonosis epidemic and additional strains. *Parasitology*, *142*, 1053–1062.

McBurney, S., Kelly-Clark, W. K., Forzán, M. J., Vanderstichel, R., Teather, K., & Greenwood, S. J. (2017). Persistence of *Trichomonas gallinae* in birdseed. *Avian Diseases*, *61*, 311–315.

McCallum, H. (2012). Disease and the dynamics of extinction. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*, 2828–2839.

McCallum, H., & Dobson, A. (1995). Detecting disease and parasite threats to endangered species and ecosystems. *Trends in Evolution and Ecology*, *10*, 190–194.

Millán, J., Gortazar, C., & Villafuerte, R. (2003). Does supplementary feeding affect organ and gut size of wild red-legged partridges *Alectoris rufa*? *Wildlife Biology*, *9*, 229–233.

Møller, A., Christe, P., Erritzøe, J., & Mavarez, J. (1998). Condition, disease and immune defence. *Oikos*, *83*, 301–306.

Moorhouse-Gann, R., Dunn, J., De Vere, N., Goder, M., Cole, N., Hipperson, H., & Symondson, W. (2018). New universal ITS2 primers for high-resolution herbivory analyses using DNA metabarcoding in both tropical and temperate zones. *Scientific Reports*, *8*, 1–15.

Moyers, S. C., Adelman, J. S., Farine, D. R., Thomason, C. A., & Hawley, D. M. (2018). Feeder density enhances house finch disease transmission in experimental epidemics. *Royal Society B: Biological Sciences*, *373*, 2170090.

Murray, M. H., Becker, D. J., Hall, R. J., & Hernandez, S. M. (2016). Wildlife health and supplemental feeding: A review and management recommendations. *Biological Conservation*, *204*, 163–174.

Nicholls, J. A., Double, M. C., Rowell, D. M., & Magrath, R. D. (2000). The evolution of cooperative and pair breeding in thornbills *Acanthiza* (Pardalotidae). *Journal of Avian Biology*, *31*, 165–176.

Oksanen, J. (2020). *FGB et al. vegan: Community ecology package*.

Orams, M. B. (2002). Feeding wildlife as a tourism attraction: A review of issues and impacts. *Tourism Management*, *23*, 281–293.

Pennycott, T. W., Ross, H. M., Mclaren, I. M., Park, A., Hopkins, G. F., & Foster, G. (1998). Causes of death of wild birds of the family Fringillidae in Britain. *Vetenary Records*, *143*, 155–158.

Pianka, E. (1986). *Ecology and natural history of desert lizards*. Princeton University Press.

Porazinska, D. L., Sung, W., Giblin-Davis, R. M., & Thomas, W. K. (2010). Reproducibility of read numbers in high-throughput sequencing analysis of nematode community composition and structure. *Molecular Ecology Resources*, *10*, 666–676.

POWO. (2019). *Plants of the World Online | Kew Science* [Online]. https://powo.science.kew.org/

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Rayé, G., Miquel, C., Coissac, C., Redjadj, E., Loison, A., & Taberlet, P. (2011). New insights on diet variability revealed by DNA barcoding and high-throughput pyrosequencing: Chamois diet in autumn as a case study. *Ecological Research*, *26*, 265–276.

Riley, D. E., Roberts, M. C., Takayama, T. O. M., & Krieger, J. N. (1992). Development of a polymerase chain development of a polymerase chain reaction-based diagnosis of *Trichomonas vaginalis*. *Journal of Clinical Microbiology*, *30*, 465–472.

Robb, G., McDonald, R., Chamberlain, D., & Bearhop, S. (2008). Food for thought: Supplementary feeding as a driver of ecological change in avian populations. *Frontiers in Ecology and the Environment*, *6*, 476–484.

Robb, G., McDonald, R., Chamberlain, D., Reynolds, S., Harrison, T., & Bearhop, S. (2008). Winter feeding of birds increases productivity in the subsequent breeding season. *Biology Letters*, *4*, 220–223.

Robinson, R. A., Lawson, B., Toms, M. P., Peck, K. M., Kirkwood, J. K., Chantrey, J., Clatworthy, I. R., Evans, A. D., Hughes, L. A., Hutchinson, O. C., John, S. K., Pennycott, T. W., Perkins, M. W., Rowley, P. S., Simpson, V. R., Tyler, K. M., & Cunningham, A. A. (2010). Emerging infectious disease leads to rapid population declines of common British birds. *PLoS One*, *5*, e12215.

Rocha, G., & Quillfeldt, P. (2015). Effect of supplementary food on age ratios of European turtle doves (*Streptopelia turtur* L.). *Animal Biodiversity and Conservation*, *38*, 11–21.

Rocha, G., Tizado, E. J., & Sánchez-García, C. (2022). Hunting levels of turtle dove (*Streptopelia turtur*) at sites where food is provided: Implications for sustainable harvesting. *Animals*, *12*, 2106.

Sansano-Maestre, J., Garijo-Toledo, M. M., & Gómez-Muñoz, M. (2009). Prevalence and genotyping of *Trichomonas gallinae* in pigeons and birds of prey. *Avian Pathology*, *38*, 201–207.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*, 7537–7541.

Siriwardena, G. M., Stevens, D. K., Anderson, G. Q. A., Vickery, J. A., Calbrade, N. A., & Dodd, S. (2007). The effect of supplementary winter seed food on breeding populations of farmland birds: Evidence from two large-scale experiments. *Journal of Applied Ecology*, *44*, 920–932.

Sorensen, A., van Beest, F. M., & Brook, R. K. (2014). Impacts of wildlife baiting and supplemental feeding on infectious disease transmission risk: A synthesis of knowledge. *Preventive Veterinary Medicine*, *113*, 356–363.

Stabler, R. M. (1948). Protection in pigeons against virulent *Trichomonas gallinae* acquired by infection with milder strains. *The Journal of Parasitology*, *34*, 150–153.

Stabler, R. M. (1954). Trichomonas gallinae: a review. *Experimental Parasitology*, *3*, 368–402.

Stockdale, J. E., Dunn, J. C., Goodman, S. J., Morris, A. J., Sheehan, D. K., Grice, P. V., & Hamer, K. C. (2015). The protozoan parasite *Trichomonas gallinae* causes adult and nestling mortality in a declining population of European turtle doves, *Streptopelia turtur*. *Parasitology*, *142*, 490–498.

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For biodiversity research and monitoring*. Oxford University Press.

Thomas, R. C. (2017). *Molecular epidemiology of trichomonas gallinae in turtle doves (Streptopelia turtur)*. University of Leeds.

Thomas, R. C., Dunn, J. C., Dawson, D. A., Hipperson, H., Horsburgh, G. J., Morris, A. J., Orsman, C., Mallord, J., Grice, P. V., Hamer, K. C., & Eraud, C. (2022). Assessing rates of parasite coinfection and spatiotemporal strain variation via metabarcoding: Insights for the conservation of European turtle doves *Streptopelia turtur*. *Molecular Ecology*, *31*, 2730–2751.

Tryland, M., Nymo, I. H., Romano, J. S., Mørk, T., Klein, J., & Rockström, U. (2019). Infectious disease outbreak associated with supplementary feeding of semi-domesticated reindeer. *Frontiers in Veterinary Science*, *6*, 126.

Turner, W. C., Kamath, P. L., Van Heerden, H., Huang, Y. H., Barandongo, Z. R., Bruce, S. A., & Kausrud, K. (2021). The roles of environmental variation and parasite survival in virulence–transmission relationships. *Royal Society Open Science*, *8*, 210088.

Walther, B. A., & Ewald, P. W. (2004). Pathogen survival in the external environment and the evolution of virulence. *Biological Reviews of the Cambridge Philosophical Society*, *79*, 849–869.

Warner, R. E. (1968). The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. *The Condor*, *70*, 101–120.

Weidman, T., & Litvaitis, J. A. (2011). Can supplemental food increase winter survival of a threatened cottontail rabbit? *Biological Conservation*, *144*, 2054–2058.

Wickham, H. (2016). *Ggplot2-elegant graphics for data analysis*. [Online]. https://scholar.google.com/scholar_lookup?hl=en&publication_year=2016&author=H.+Wickham&title=Ggplot2%3A+Elegant+-Graphics+for+Data+Analysis

Wilcoxen, T. E., Horn, D. J., Hogan, B. M., Hubble, C. N., Huber, S. J., Flamm, J., Knott, M., Lundstrom, L., Salik, F., Wassenhove, S. J., & Wrobel, E. R. (2015). Effects of bird-feeding activities on the health of wild birds. *Conservation Physiology*, *3*, 1–13.

Young, R. E. (2022). *Saving an iconic species from extinction in the UK: Interactions between diet, parasites and environmental change* (PhD thesis). Cardiff University.

How to cite this article: Young, R. E., Dunn, J. C., Vaughan, I. P., Mallord, J. W., Orsman, C. J., Ka, M., Diallo, M. B., Sarr, M., Lormée, H., Eraud, C., Kiss, O., Thomas, R. C., Hamer, K. C., Goodman, S. J., & Symondson, W. O. C. (2023). Investigating the association between diet and infection with *Trichomonas gallinae* in the European turtle dove (*Streptopelia turtur*). *Environmental DNA*, *00*, 1–24. https://doi.org/10.1002/edn3.402

APPENDIX 1

SAMPLING LOCATION INFORMATION

Senegal (14°22′ N, 16°48′ W): an enclosed area including 100ha of naturally regenerated *Acacia seyal* forest, small allotments growing fruit and vegetables, and a small reservoir. In 2017, it was estimated that around 40,000 turtle doves, along with other species of resident African doves, were roosting here (personal observation). 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.

France, Chizé Forest (46°07′ N, 0°25′ W): covers around 3435 ha, a mixture of mature and deciduous woodland, with areas of scrub.

France, Aulnay Forest (46°02′ N, 0°14′ W): covers around 2870 ha, 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 (Appendix 1: Figure A1).

France, Ile d'Oléron (45°93′ N, 01°28′ W): a 174 km^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; Appendix 1: Figure A1).

Bait was used to catch birds in France. 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*).

Hungary, Balotaszállás-Öttömos (46°16′ N, 19°35′ W): 4000 ha, 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.

APPENDIX 2

DNA EXTRACTION AND SEQUENCING

FECAL SAMPLES FOR DIET ANALYSIS

DNA was extracted using either a QIAmp DNA Stool Mini Kit, or QIAmp Fast Stool Mini Kit (Qiagen). The extraction protocol is largely the same between the two kits, with the only change being

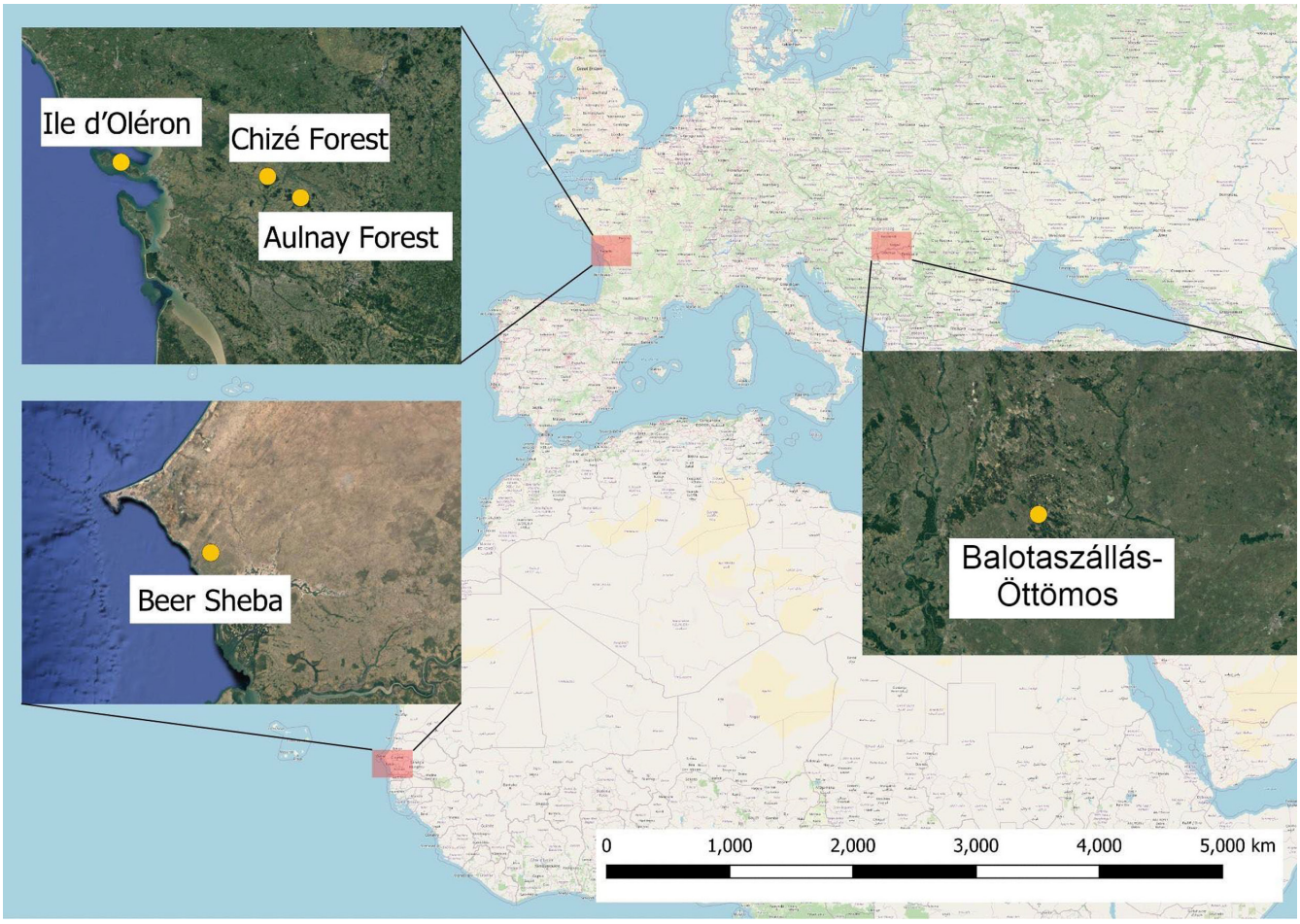

FIGURE A1 Turtle dove sampling locations in Europe and Senegal.

a replacement of the InhibitEX tablets with an InhibitEX buffer in the new kit. Extractions were conducted following the manufacturers' protocols, including all recommended steps, with the following modifications, based on Dunn et al. (2016). (i) a sterile pestle was used to homogenize fecal samples in buffer ASL; (ii) samples were incubated at 70°C for 30 min; (iii) drying step was increased to 3 min centrifugation; (iv) final elution in 100 µL buffer AE.

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 (187–387 bp) to maximize amplification of degraded plant DNA from fecal samples. Both forward and reverse primers were labeled with MID-tags (Multiple Identifiers) to provide a unique combination of tags for each DNA sample. PCRs were carried out in 25 µL reaction volumes, consisting of 12.5 µL Multiplex PCR Master Mix (Qiagen), 2.5 µL nuclease free water, 2.5 µL each of 0.01 M UniPlantF and UniPlantR MID-tagged primers (Eurofins), and 5 µL 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™ Thermal Cycler (Thermo Fisher Scientific), using the following thermocycling protocol: 95°C (15 min); 40 cycles of 95°C (30 s), 58°C (90 s), 72°C (90 s); 72°C (10 min). Concentration of amplified DNA was determined by testing 5 µL 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

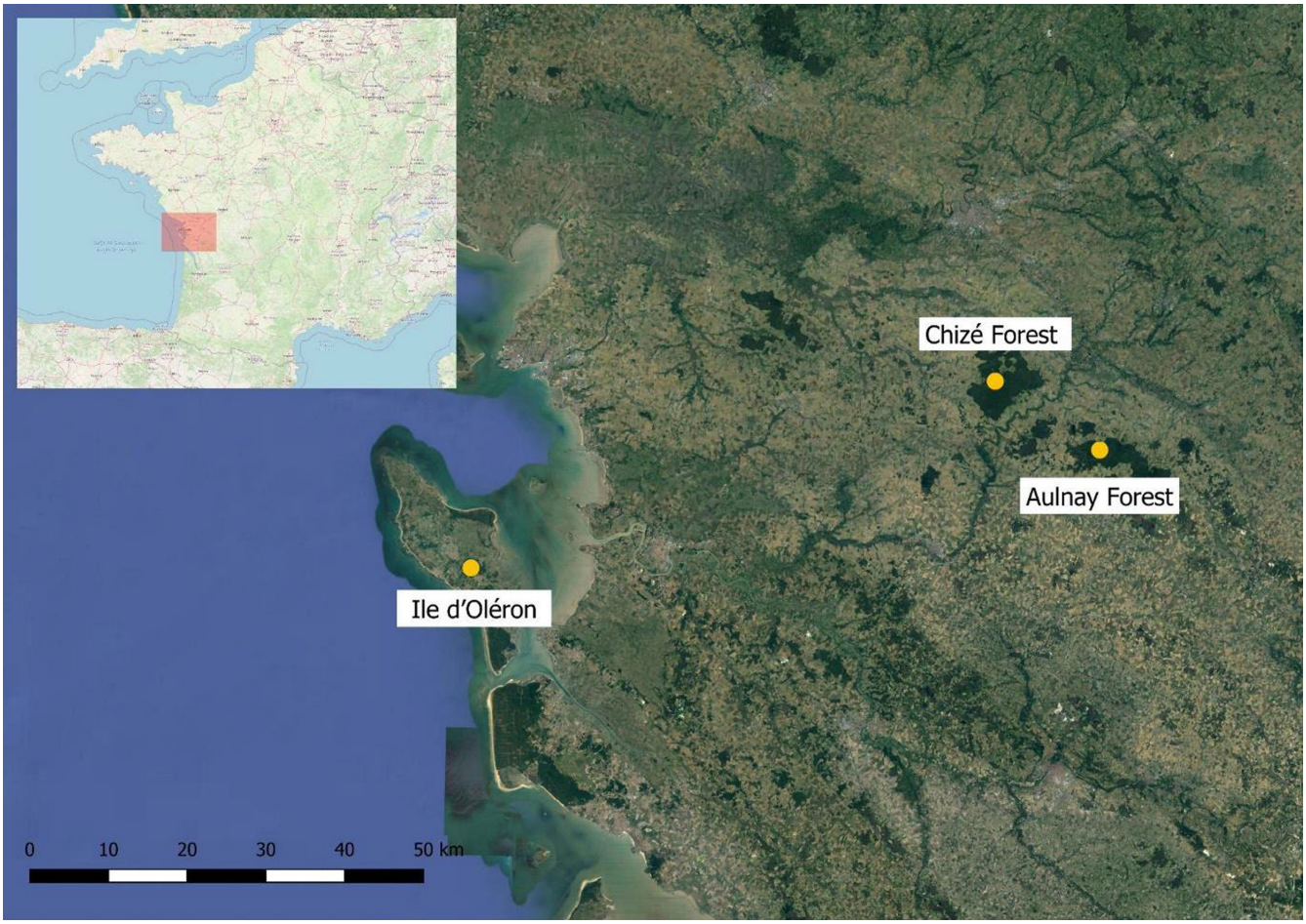

FIGURE A2 Sampling sites in France.

add to the pools was the average of all sample volumes added to the pool.

In preparation for high-throughput sequencing, following pooling of samples, the quality of the pooled samples and the size of amplicons were checked via TapeStation2200 (Agilent). Pools were cleaned using SPRIselect beads (Beckman Coulter) following the manufacturer's protocols, at a ratio of 1:1.1, to retain fragments of the desired size range (207–400 bp). Once cleaned, the concentration of DNA was measured for each pool using a Qubit dsDNA High-sensitivity Assay (Thermo Fisher 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), following the manufacturer's protocols.

ORAL SWABS FOR ASSESSING *TRICHOMONAS GALLINAE* INFECTION

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

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) depending on the date of extraction. For the ammonium acetate method, the pellet was obtained from 2.5 mL of culture medium, for extraction using DNeasy Blood and Tissue kit, 200–400 μL of culture medium was used. Samples from the UK dataset, and DNA extracted in 2018 used the modified ammonium acetate method, whereby isolated parasite samples were centrifuged at 3200 rpm for 5 min, the supernatant discarded and the pellet retained. 250 μL of Digsol buffer (20 mM EDTA, 50 mM Tris, 120 mM NaCl, 1% SDS, pH 8.0) and 20 μL Proteinase K (Qiagen) were added and samples were incubated overnight at 37°C to allow digestion. Following digestion, 300 μL of 4M ammonium acetate was added, mixed by vortexing, and incubated at room temperature for 30 min, vortexing every 10 min. Samples were centrifuged at 13,000 rpm for 10 min and the supernatant transferred into a new tube, discarding the pellet. A volume of 1 mL 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 min. The supernatant was discarded and 500 μL 70% ethanol was added to the DNA pellet before being spun at 13,000 rpm for 5 min to wash the pellet. The supernatant was discarded and the pellet was left to air-dry until no ethanol remained, typically taking 1–2 h. The DNA pellet was dissolved in 20–50 μL of low TE buffer (0.1 mM EDTA, 10 mM 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 50 μL TE buffer. Two extraction negatives were included for every extraction, whereby all reagents were added but no sample was included in order to detect contamination in extraction buffers.

Polymerase chain reaction was used to determine the infection status of each sample. The TFR1/TFR2 primer pair (Felleisen, 1997) were used to amplify the 400 bp 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 1.8 μL Multiplex PCR Master Mix (Qiagen), 0.225 μL of 10 μM forward and reverse primers (Merck KGaA), and 2.25 μL nuclease free water, giving a reaction volume of 4.5 μL, to which 0.5 μL DNA was added. A negative control of molecular-grade water and a positive control known to contain *T. gallinae* DNA were included in each PCR run.

The following touchdown PCR thermal cycling program was performed using SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). The program consisted of initial denaturing for 15 min at 95°C, 11 cycles of 1 min at 94°C, 30 s at 66°C (decreasing by 1°C every cycle until 56°C), 1 min at 72°C, followed by 24 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C, with a final elongation step of 10 min at 72°C. PCR products were electrophoresed though a 1% gel, stained using SYBR®Safe (Thermo Fisher Scientific) in 0.5× TBE buffer and visualized using UV light. The presence of *T. gallinae* infection was indicated by the occurrence of a band of the appropriate amplicon size when visualized. In instances where no amplicon was observed, the sample was run through PCR a second time to confirm the absence of *T. gallinae*.

Samples which tested positive for *T. gallinae* were prepared for high-throughput sequencing by making up 25 μL reaction volume consisting of 10 μL Multiplex PCR Master Mix (Qiagen), 9 μL nuclease free water, 2.5 μL each of 3 μM TFR1[f] and TFR2[r] primers (Eurofins), and 1 μL template DNA. Both forward and reverse primers were labeled 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 1 μL nuclease-free water), arranged as for fecal samples. 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 for diet analysis, 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 (0.18–40.5 ng/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 for DNA extracted from fecal samples, with one modification: in the initial cleaning of the pools using SPRIselect beads (Beckman Coulter), a ratio of 1:0.9 was used to remove fragments smaller than the desired 420 bp.

APPENDIX 3

BIOINFORMATIC ANALYSIS AND DATA CLEANING

Bioinformatic analyses were carried out on both diet and parasite infection datasets 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. 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 (170 bp for DNA extracted from fecal samples, 380 bp for DNA extracted from oral swabs) 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). For DNA extracted from fecal samples, one primer

mismatch was permitted; for DNA extracted from oral swabs, 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). This was necessary for analyzing parasite samples as even one base mismatch leads to the assignment of a new strain of *Trichomonas gallinae*. 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). 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% for DNA from fecal samples and 97% for DNA from oral samples 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 analyzed 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.

Data processing and cleaning following this initial bioinformatic processing differed for DNA extracted from fecal samples for diet analysis and DNA extracted from oral swabs for *T. gallinae* analysis.

For DNA from fecal samples, 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, and 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.

For DNA from oral swab samples, any sequences that did provide a 100% match to published *T. galliane* strains were assigned a new strain identifier, based on the closest published match.

Cleaning the dataset

There were two stages to the cleaning of data to remove artifacts from the final dataset, 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, and 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: artifacts created during PCR and sequencing, and is carried out on a per-sample basis. Artifacts generally occur in far lower proportions than genuine sequences, and 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 amongst 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, artifacts are more likely to pass filtering thresholds in samples with higher read counts, as artifacts 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 4276 to 172,171) facilitating a standardized clean-up across all samples.

In order to designate a filtering threshold for DNA extracted from fecal samples, 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. 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. In order to balance false positives and false negatives, a slightly more conservative threshold of 4% was selected, which removed most false positives from positive controls, without running the risk of removing too many genuine reads. 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 replaced 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

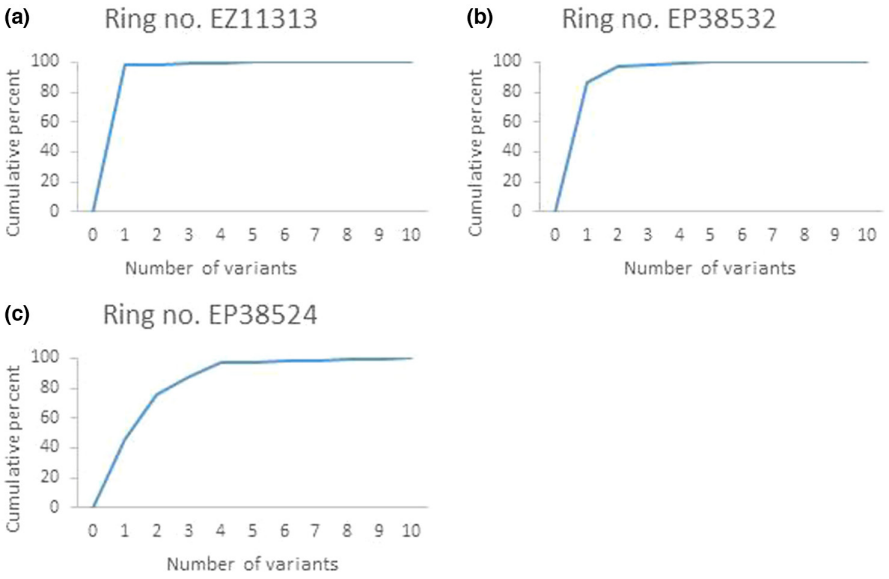

FIGURE A3 Examples of the cumulative proportion curves showing sequence depth accounted for by variants in a sample. (a) 98.3% of sequences are from a single variant, creating clear inflection point so additional variants accounting for remaining 1.7% variation are removed as artifacts. (b) Two possible inflection points, so more subjective: if using point at 1 variant, 89.5% of variation accounted for by one variable, and therefore, threshold is 10.5%, if using point at 2 variants, threshold is 3%; (c) cannot discern clear inflection point.

data filtering, fungal and bacterial taxa were removed, as were any taxa which were not identified to at least family level.

For *T. galliane* DNA isolated from oral swabs, no positive controls were included; therefore, the filtering threshold was set at 0.3%, as per as per Taberlet et al. (2018). After removing maximum contamination and low proportion reads from samples, there were still reads present in very low numbers, indicating the retention of some artifacts or polymorphic gene copies. An additional filtering step was therefore required for *T. gallinae* DNA. This is because the genomic marker widely used in studies of *Trichomonas* species (ITS region) encodes the small 5.8S rRNA subunit (SSU), as well as the flanking internal transcription spacers. 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, as all reads generated during sequencing are returned (Porazinska et al., 2010). Porazinska et al. (2010) investigated the repeatability of using HTS to analyze 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 1 bp. Without extensive use of technical repeats, these polymorphisms cannot be distinguished from artifacts, but are likely to account in part to the high number of zOTUs differing from known sequences returned from sequencing in this study.

To further clean the data, 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 artifacts occur in much lower numbers than genuine reads, the presence of a clear inflection point (as seen in Appendix 3: Figure A3a) indicated the divide between a true sequence and an artifact, and any reads occurring beyond this inflection point would be considered artifacts and not included

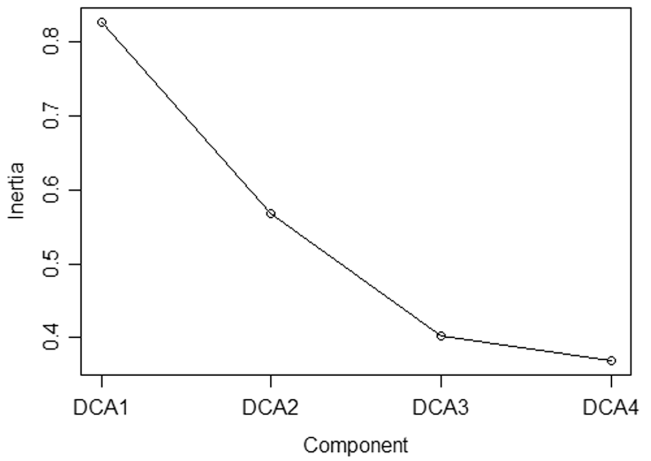

FIGURE A4 Scree value illustrating the eigenvalues of the four DCA dimensions. DCA1 and DCA2 were included in GLM models.

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 (Appendix 3: Figure A3b), 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 (Appendix 3: Figure A3c). 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, and thus, the maximum percentage accounted for by a clear inflection point was used as the threshold for all samples to determine co-infection.

Different thresholds were tested for cleaning steps two and three. Analyses were conducted on datasets 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 >0.3% per sample; (step 3) zOTUs accounting for a minimum of 7% of reads per sample.

APPENDIX 4

DETERMINING FREQUENTLY OCCURRING TAXA

TABLE A1 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

APPENDIX 5

DCA DIMENSION SELECTION

APPENDIX 6

WILD AND CULTIVATED DIETARY ITEMS

Dietary items consumed by turtle doves were categorized into "cultivated" (29 taxonomic units), consisting of crop plants, those cultivated for animal fodder and seeds commonly provided in supplementary feed, and "wild" (90 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, and 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

Taxon	Taxon rank	Family	Status	Country consumed in
Acer campestre	Species	Sapindaceae	Wild	France
Achyranthes aspera	Species	Amaranthaceae	Wild	Senegal
Adansonia digitata	Species	Malvaceae	Wild	Senegal
Agrostis pallens	Species	Poaceae	Wild	France
Alkanna	Species	Boraginaceae	Wild	Hungary
Alkanna tinctoria	Species	Boraginaceae	Wild	Hungary
Alopecurus myosuroides	Species	Poaceae	Wild	Hungary
Amaranthus	Genus	Amaranthaceae	Wild	Senegal
Amaranthus albus	Species	Amaranthaceae	Wild	Hungary
Ambrosia	Genus	Asteraceae	Wild	Hungary
Ambrosia artemisiifolia	Species	Asteraceae	Wild	Hungary
Anchusa arvensis	Species	Boraginaceae	Wild	France
Arachis	Genus	Fabaceae	Cultivated	Senegal
Blainvillea rhomboidea	Species	Asteraceae	Wild	Senegal
Boerhavia erecta	Species	Nyctaginaceae	Wild	Senegal
Brachiaria ramosa	Species	Poaceae	Wild	Senegal
Brassica	Genus	Brassiceae	Brassica	France
Brassica napus	Species	Brassiceae	Cultivated	France
Brassica rapa	Species	Brassiceae	Cultivated	France
Buglossoides	Genus	Boraginaceae	Wild	Hungary
Bulbostylis	Genus	Cyperaceae	Wild	Senegal
Bulbostylis hispidula	Species	Cyperaceae	Wild	Senegal
Cannabis sativa	Species	Cannabaceae	Cultivated	Senegal, France, Hungary
Carduus	Genus	Asteraceae	Wild	Hungary
Carduus tenuiflorus	Species	Asteraceae	Wild	France
Cenchrinae	Subtribe	Poaceae	Cultivated	Senegal, Hungary
Cenchrus americanus	Species	Poaceae	Cultivated	Senegal

Taxon	Taxon rank	Family	Status	Country consumed in
Cenchrus polystachios	Species	Poaceae	Wild	Senegal
Chenopodiastrum hybridum	Species	Amaranthaceae	Wild	Hungary
Chenopodium	Genus	Amaranthaceae	Wild	France, Hungary
Combretum bracteosum	Species	Combretaceae	Wild	Senegal
Cucumis	Genus	Cucurbitaceae	Cultivated	Senegal
Cucumis maderaspatanus	Species	Cucurbitaceae	Cultivated	Senegal
Cucumis melo	Species	Cucurbitaceae	Cultivated	Senegal
Cucurbita pepo	Species	Cucurbitaceae	Cultivated	Senegal
Dactyloctenium aegyptium	Species	Poaceae	Wild	Senegal
Datura stramonium	Species	Solanaceae	Wild	France, Hungary
Daucus	Genus	Apiaceae	Cultivated	Senegal
Digitaria	Genus	Poaceae	Wild	Senegal
Echinochloa	Genus	Poaceae	Wild	Senegal
Eragrostis	Genus	Poaceae	Wild	Senegal
Erodium cicutarium	Species	Gerraniaceae	Wild	France
Eucalyptus	Genus	Myrtaceae	Wild	Senegal
Euphorbia	Genus	Euphorbiaceae	Wild	Hungary
Euphorbia cyparissias	Species	Euphorbiaceae	Wild	Hungary
Euphorbia helioscopia	Species	Euphorbiaceae	Wild	France
Euphorbia pseudoesula	Species	Euphorbiaceae	Wild	Hungary
Euphorbia seguieriana	Species	Euphorbiaceae	Wild	Hungary
Euphorbia stepposa	Species	Euphorbiaceae	Wild	Hungary
Fagus	Genus	Fagaceae	Wild	France
Fraxinus	Genus	Oleaceae	Wild	France
Geranium dissectum	Species	Gerraniaceae	Wild	France
Geranium molle	Species	Gerraniaceae	Wild	France
Geranium rotundifolium	Species	Gerraniaceae	Wild	France
Guizotia abyssinica	Species	Asteraceae	Cultivated	Senegal
Gymnosporia senegalensis	Species	Celastraceae	Wild	Senegal
Hedera hibernica	Species	Araliaceae	Wild	France
Helianthus	Genus	Asteraceae	Cultivated	Senegal, France
Helianthus annuus	Species	Asteraceae	Cultivated	France, Hungary
Hibiscus sabdariffa	Species	Malvaceae	Cultivated	Senegal
Indigofera	Genus	Fabaceae	Wild	Senegal
Ipomoea coptica	Species	Convolvulaceae	Wild	Senegal
Juglans	Genus	Juglandaceae	Cultivated	Senegal, France
Loliinae	Subtribe	Poaceae	Wild	France
Lolium	Genus	Poaceae	Wild	France
Ludwigia erecta	Species	Onagraceae	Wild	Senegal
Mangifera indica	Species	Mango	Cultivated	Senegal
Medicago arabica	Species	Fabaceae	Wild	France
Melochia corchorifolia	Species	Malvaceae	Wild	Senegal
Mercurialis annua	Species	Euphorbiaceae	Wild	France
Merremia	Genus	Convolvulaceae	Wild	Senegal
Merremia aegyptia	Species	Convolvulaceae	Wild	Senegal
Montia fontana	Species	Montiaceae	Wild	France
Nymphaea	Genus	Nymphaeaceae	Wild	Senegal

Taxon	Taxon rank	Family	Status	Country consumed in
Nymphaea guineensis	Species	Nymphaeaceae	Wild	Senegal
Nymphaea lotus	Species	Nymphaeaceae	Wild	Senegal
Nymphaea lotus var. thermalis	Species	Nymphaeaceae	Wild	Senegal
Nymphaea micrantha	Species	Nymphaeaceae	Wild	Senegal
Panicum (Europe)	Genus	Poaceae	Cultivated	Hungary
Panicum miliaceum (Europe)	Species	Poaceae	Cultivated	France
Panicum (Africa)	Genus	Poaceae	Wild	Senegal
Paspalum	Genus	Poaceae	Wild	Senegal
Poa	Genus	Poaceae	Wild	France
Poa infirma	Species	Poaceae	Wild	France
Poaceae	Family	Poaceae	Wild	France
Potentilla	Genus	Rosaceae	Wild	France
Prunus	Genus	Rosaceae	Wild	France
Puccinellia	Genus	Poaceae	Wild	Hungary
Quercus	Genus	Fagaceae	Wild	France
Quercus robur	Species	Fagaceae	Wild	France
Ranunculus parviflorus	Species	Ranunculaceae	Wild	France
Rubus	Genus	Rosaceae	Wild	France
Sambucus ebulus	Species	Adoxaceae	Wild	France
Senna	Genus	Fabaceae	Wild	Senegal
Senna obtusifolia	Species	Fabaceae	Wild	Senegal
Sida spinosa	Species	Malvaceae	Wild	Senegal
Silene	Species	Caryophyllaceae	Wild	Hungary
Solanum	Genus	Solanaceae	Cultivated	Senegal
Solanum tuberosum	Species	Solanaceae	Cultivated	Senegal
Sonchus oleraceus	Species	Asteraceae	Wild	Senegal
Sorghum	Genus	Poaceae	Cultivated	Senegal, France
Sorghum halepense	Species	Poaceae	Cultivated	Hungary
Stellaria media	Species	Caryophyllaceae	Wild	France
Tamarix	Genus	Tamaricaceae	Wild	Senegal
Trianthema portulacastrum	Species	Aizoaceae	Wild	Senegal
Trifolium	Genus	Fabaceae	Wild	France
Triticeae	Tribe	Poaceae	Cultivated	France
Triticum	Genus	Poaceae	Cultivated	France
Triticum aestivum	Species	Poaceae	Cultivated	France
Triticum monococcum subsp. *Aegilopoides*	Species	Poaceae	Cultivated	France
Triumfetta	Genus	Malvaceae	Wild	Senegal
Tuberaria macrosepala	Species	Cistaceae	Wild	France
Ulmus	Genus	Ulmaceae	Wild	France
Ulmus minor	Species	Ulmaceae	Wild	France
Urochloa mosambicensis	Species	Poaceae	Wild	Senegal
Urtica dioica	Species	Urticaceae	Wild	Hungary
Verbascum	Species	Scrophulariaceae	Wild	Hungary
Vicia faba	Species	Fabaceae	Cultivated	France
Vigna radiata	Species	Fabaceae	Cultivated	France
Zornia glochidiata	Species	Fabaceae	Wild	Senegal