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Baseline spatiotemporal patterns in the invertebrate diet of Eurasian Tree Sparrow *Passer montanus* chicks revealed using DNA metabarcoding

Hannah L. Baker [a], Matt Prior[b], Ewan H. Stenhouse [c], Amy Baldwin[c], Trudy Workman [c] and Anne E. Goodenough[a]

[a]School of Education, Health and Science, University of Gloucestershire, Cheltenham, UK; [b]Wiltshire Ornithology Society, Wiltshire, UK; [c]School of Biosciences, Cardiff University, Cardiff, UK

ABSTRACT

Capsule: DNA metabarcoding revealed that the diet of Eurasian Tree Sparrow chicks at three sites in the UK was dominated by ground beetles Carabidae, suggesting parental foraging was predominantly ground-based.

Aims: Most bird species in temperate environments rely on invertebrates as a food resource, so widespread and substantial invertebrate declines are important in the context of avian conservation. The Eurasian Tree Sparrow, a specialist farmland bird in the UK, has a wide dietary niche for most of the year but relies on invertebrates to feed its chicks. We present the first molecular analysis of the diet of nestling Eurasian Tree Sparrows using DNA high-throughput sequencing and metabarcoding.

Methods: We collected faeces from chicks at three sites on the Marlborough Downs (Wiltshire, UK) during the 2024 breeding season and successfully extracted and identified invertebrate DNA from 59 samples by amplifying the cytochrome-c oxidase subunit-I (COI) region.

Results: In total, 22 invertebrate species from five orders (Coleoptera, Diptera, Hymenoptera, Hemiptera and Araneae) were identified. Samples were dominated by Coleoptera (73.1% prevalence), with the ground beetles *Pterostichus melanarius* and *Poecilus cupreus* being the most common species (detected in 22.6% and 12.9% of samples, respectively). The number of invertebrate species per faecal sample was low, suggesting that parents were feeding chicks repeatedly the same species, and/or very short gut retention times in chicks.

Conclusion: Our results suggest that parental foraging was primarily ground-based, indicating the importance of bare ground, or sparce and short vegetation, in foraging areas during the breeding season. There were significant spatiotemporal patterns in dietary composition of sampled chicks between sites and habitats, indicating the importance of microhabitats and cropping patterns. We discuss the importance of these findings in relation to Eurasian Tree Sparrow conservation efforts.

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An estimated 80–90% of bird species in temperate environments rely on invertebrates as food (Nyffeler *et al.* 2018, Rosenberg *et al.* 2019). Some consume insects year-round either as obligate insectivores, such as the Spotted Flycatcher *Muscicapa striata* and Common Swift *Apus apus* (Kirby *et al.* 2005, Romanowski *et al.* 2024), or as part of an omnivorous diet, for example the Dunnock *Prunella modularis* and Common Whitethroat *Curruca communis* (Hewson & Noble 2009). Invertebrates form an important part of diet during key phases of the avian life cycle, such as during breeding when parents feed chicks protein-rich insect prey, e.g. the Eurasian Tree Sparrow *Passer montanus* (hereafter 'Tree Sparrow'; Summers-Smith 1995), and Yellowhammer *Emberiza citrinella* (Hart *et al.* 2006). Concern about invertebrate decline and the ecosystem-level cascade effects, including the impact on birds, has been highlighted with increasing urgency (Wilson 1987, Conrad *et al.* 2006, Hallmann *et al.* 2017, Cardoso *et al.* 2020, Wagner 2020).

Birds that feed upon insects are typically declining more rapidly than non-insectivorous birds; an analysis of 368 North American avian species (Rosenberg *et al.* 2019, Tallamy & Shriver 2021) suggested that the average population change per insect-dependent species between 1970 and 2017 was −9.70 million

CONTACT Anne E. Goodenough aegoodenough@glos.ac.uk

individuals *versus* +0.41 million individuals per non-insect-dependent species. To analyse likely causality rather than report correlation, Grames *et al.* (2023) undertook a global meta-analysis of 125 studies: the authors demonstrated that insect biomass is often a limiting resource during the breeding season, with food availability strongly influencing reproductive success and having a moderate influence on chick body-condition, which is an important proxy for post-fledging survival.

The foraging strategies of insectivorous birds, and the invertebrate prey they consume, vary widely (Morse 1971, Grubb 1979). Some species are specialists (e.g. aerial insectivores such as Hirundinidae; saproxylic insectivores such as Picidae). Others, including many farmland birds, have a generalist foraging strategy and a wide dietary niche (Holland *et al.* 2002), which increases the importance of birds choosing high-quality invertebrate prey while minimizing foraging time and energy expenditure (Hubbard *et al.* 1982, Razeng & Watson 2015, Piel *et al.* 2021, Nell *et al.* 2023). Optimal foraging is especially important whilst adults are feeding chicks, when chick survival, growth, and condition are determined by provisioning rate (Naef-Daenzer & Keller 1999, Senécal *et al.* 2021), prey size (Schwagmeyer & Mock 2008, Cauchard *et al.* 2021), and calorific and nutritional quality (Holland *et al.* 2002, Davies *et al.* 2022). Parents thus have a constant trade-off between prey quality and quantity when feeding their chicks (Wright *et al.* 1998). Choice of prey can be influenced by differential availability and the ease with which different taxa can be located and captured (Merilaita 2006), as well as individual experience and search image (Ishii & Shimada 2010, Iwashita *et al.* 2022).

Across Europe and North America, many farmland birds are in decline (Stanton *et al.* 2018, Rigal *et al.* 2023). Between 1990 and 2019, the Common Farmland Bird Index decreased by 27% on average across 25 EU Member States (European Environment Agency 2021). In the UK, the farmland bird indicator, based on 19 species, showed a long-term decrease of 61% in abundance between 1970 and 2022 (DEFRA 2025). Specialist farmland species are disproportionately affected, with an average long-term population trend of −73% compared to a long-term trend of −22% for generalist farmland species over the period 1970–2022 (DEFRA 2025). One species that is categorized as a specialist farmland species and undergoing especially severe decline in the UK is the Tree Sparrow, which has decreased by 93% between 1970 and 2022 (DEFRA 2025). In common with many UK farmland species, the decline of Tree Sparrow populations has been largely ascribed to the impact of agricultural intensification on food availability and foraging opportunities. Reduction in uncultivated and bare ground, removal of hedges to create larger fields, and change from mixed to monoculture farms, have all reduced invertebrate habitat and avian foraging opportunities (Donald *et al.* 2001, Stoate *et al.* 2001, Newton 2004, Butler *et al.* 2010, Traba & Morales 2019, Mancini *et al.* 2023, Rigal *et al.* 2023). Loss of arable weeds that support insects due to herbicide and insecticide use is also important drivers of decline (Boatman *et al.* 2004). These factors act synergistically with the switch from spring-sown to autumn-sown crops, reducing winter availability of seeds, which affects overwinter survival (Siriwardena *et al.* 2000, Stoate *et al.* 2001).

Colonially nesting Tree Sparrows are insectivorous during breeding (Summers-Smith 1995), with studies in the UK and Slovakia showing the diet of adults and chicks is 95–96% insect derived during this period (Holland *et al.* 2006). Adults show a preference for foraging on the ground, although during the breeding season they also prey upon invertebrates available in bushes and treetops (Deckert 1962). Current knowledge of the diet of Tree Sparrow chicks is based on visual identification from chick faecal samples and the ring-neck method, which uses neck-collars to take crop samples (Moreby & Stoate 2000). These methods have shown the following invertebrate orders are important: Acarina, Araneae, Coleoptera, Demaptera, Diptera, Ephemerotera, Gastropda, Hemiptera, Hymenoptera, Isopoda, Lepidoptera, Mantodea, Neuroptera, Odonata, Opiliones, Orthoptera, Plecoptera, Siphonaptera and Trichoptera (Krištín *et al.* 1995, Cummins *et al.* 2000, Orszácová *et al.* 2002, Field *et al.* 2008, McHugh *et al.* 2016). However, more detailed knowledge of diet is essential for understanding Tree Sparrow breeding ecology and optimizing conservation efforts, especially in the UK where the species is a declining farmland specialist.

Recent developments in high-throughput sequencing (HTS) and DNA metabarcoding are revolutionizing the study of avian diet (review by Hoenig *et al.* 2022). These allow a broader prey spectrum to be profiled and food items to be identified at higher taxonomic resolution than is usually possible using morphological identification during feeding or in excreted remains, especially for soft-bodied prey, which can be especially challenging to identify using non-molecular methods (Moreby 1988, Zurdo *et al.* 2024). Previous studies using DNA metabarcoding of avian faecal samples have focused on invertebrate prey of species such as

the Reed Warbler *Acrocephalus scirpaceus*, Hawfinch *Coccothraustes coccothraustes*, Blue Tit *Cyanistes caeruleus,* Great Tit *Parus major* and Swainson's Thrush *Catharus ustulatus* (Davies *et al.* 2022, Stenhouse *et al.* 2023, Höhn *et al.* 2024, Deckel *et al.* 2025).

Our multi-site study aims to provide comprehensive data on the diet of Tree Sparrow chicks throughout the breeding season by harnessing – for the first time for this species – DNA metabarcoding to analyse faecal samples. We explore spatiotemporal variation in the dietary composition of Tree Sparrow chicks to assess the relative importance of differential arthropod availability over time as the breeding season progresses and relative to variation over space, in relation to the agricultural characteristics of our study sites. As Tree Sparrows are undergoing a severe population decline, there is an urgent need to improve knowledge of the diet and an understanding of trophic requirements. The findings from this study will thus be crucial to inform conservation strategies for this species, while potentially also benefiting other declining farmland passerines.

Methods

Study site

Primary data were collected from three sites that supported Tree Sparrow colonies on the Marlborough Downs in Wiltshire, United Kingdom (Figure 1). We chose these study sites to include different foraging areas representative of the habitats typically used by Tree Sparrows (farmland, open woodland, and gardens) (Summers-Smith 1995). Site 1 was a residential property situated within arable farmland of spring cereals and Oilseed Rape *Brassica napus*; the colony was split between this dwelling and a nearby barn. The area adjacent to the colony nestboxes was a maintained garden with some wilder areas; there were boundary hedges bordered by field margins, with arable farmland and a small woodland beyond. Site 2 was an arable farm planted with spring cereals and Oil-Seed Rape and a small, mixed, open woodland; the colony was located on the edge of woodland beside a neighbouring barn, which was surrounded by field margins, farm tracks and bordered by a hedge. Site 3 was an arable farm with spring cereals; the colony was situated on and adjacent to a large barn flanked by double hedges, Bramble *Rubus fruticosus* agg. scrub, field margins and farm tracks. Each site had a pond within 500 m of the colonies. At all sites, the birds used nest boxes with a hole diameter of 28 mm made with a sloping roof (Height = 180–245 mm; Width = 140–150 mm; Depth = 140–170 mm) affixed between 1.35 and 1.90 m above the ground. The number of breeding pairs within nestboxes varied: Site 1 = 13, Site 2 = 11, Site 3 = 23.

Sample collection

We collected faecal samples from Tree Sparrows chicks three times in the 2024 breeding season, sampling early-season broods in May, mid-season broods in June/July, and late-season broods in July/August. To avoid repeated disturbance, samples were collected during routine ringing sessions by licenced ringers when chicks were 7–10 days post hatching. As chicks often defaecate on disturbance, samples were collected opportunistically on sterile paper when chicks were first gently removed from the nest. This avoided an increase in handling time and reduced the potential for contamination relative to the alternative approach of removing faecal material from bird holding bags (Knutie & Gotanda 2018). Samples were transferred immediately using enzyme-free pipette tips (Pipetman expert, Gilson, Dunstable, UK) to 1.5 ml PCR-clean Eppendorf safe-lock tubes (Eppendorf, Germany) containing 95% molecular grade ethanol (Extra Pure, Fisher Chemical™, Leicestershire, UK) and stored in a freezer at −20°C (Verkuil *et al.* 2022, Stenhouse *et al.* 2023, Zurdo *et al.* 2023). Each sample was assigned an identification number based on the site, sampling date and nest box number.

In total, 96 chicks were sampled. Of these, 83 faecal samples were from a single chick per breeding attempt, collected to ensure an optimal balance of samples from each of the sites (Site 1 = 27; Site 2 = 18; Site 3 = 37) and time (early-season broods = 32; mid-season broods = 27; late-season broods = 23). For a subset of 14 nests, a second chick was sampled to allow a comparison of dietary similarity within as well as between nests; these are henceforth referred to as double-sampled nests.

Laboratory processing

DNA extraction was undertaken for the 96 faecal samples in a dedicated laboratory using a Qiagen DNeasy PowerSoil Pro kit (Hilden, Germany), following the manufacturer's instructions. DNA was extracted in seven batches of 6–23 samples. One negative extraction control was undertaken per extraction batch, which contained no faecal material but for which all extraction steps were completed.

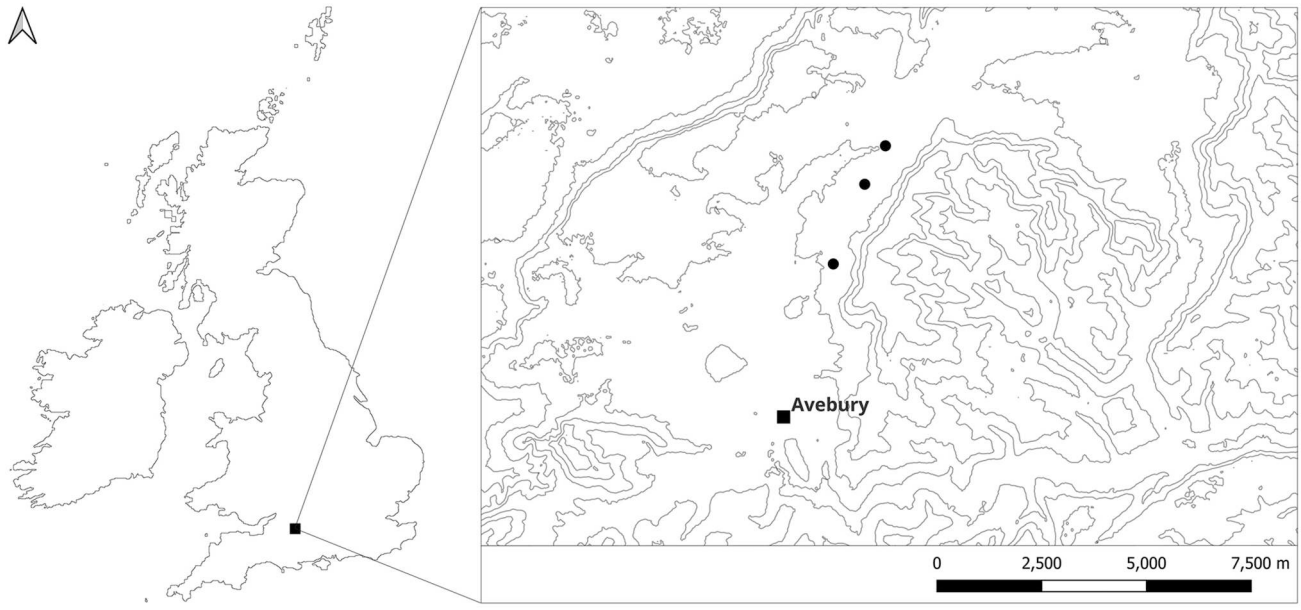

Figure 1. The approximate (to preserve landowner anonymity) locations of the study sites of the studied Tree Sparrow colonies. Image generated in QGIS using data from NASA Earthdata GIS (2025) for producing topographic contours.

To amplify a 306-bp fragment of the cytochrome-c oxidase subunit-I (COI) region in mitochondrial DNA that varies between invertebrate species (Davies *et al.* 2022), we used polymerase chain reaction (PCR) with primers: mlCOIintf, 5′ACGCTCGACAGGWACWGG WTGAACWGTWTAYCCYCC3′ (Leray *et al.* 2013) and Nancy, 5′ACTAGCAGTACCCGGTAAAATTAA AATATAAACTTC3′ (Simon *et al.* 1994). The PCR process followed Stenhouse *et al.* (2023) and involved amplification of the target region, with the addition to each sample of a unique combination of 10-bp molecular identifier tags (MID-tags) to identify samples bioinformatically. Each 25 µl PCR reaction contained 5 µl DNA template, 12.5 µl of multiplex PCR mix (Qiagen, Manchester, UK), 2.5 µl of each primer (0.2 µM each), and 2.5 µl of nuclease-free water (Qiagen). PCR thermocycler conditions comprised initial heating to 95°C for 15 min to denature the DNA, followed by 35 cycles of denature (30 s at 95°C) → anneal (90 s at 55°C) → extension (90 s extension at 72°C), before concluding with a single final extension step of 10 min at 72°C.

PCR was undertaken in two 96-well plates. Each plate contained 48 DNA samples that were drawn randomly from different sites and sampling dates to avoid any potential for procedural bias, 10 blanks (containing multiplex PCR mix and nuclease-free water), seven extraction negative controls (described above), eight PCR negatives (containing all PCR reagents but no extracted DNA) and 16 blanks which remained empty. Additionally, to check for tag-jumping between samples (i.e. artefactual exchange of DNA tags leading to decoupling of sequenced regions and sample ID), seven positive control samples consisting of the invertebrate species *Baetis harrisoni*, a mayfly commonly found across Africa and thus highly unlikely to be found in the study area, were added to each PCR.

To quantify the total DNA concentration of each sample, and to ensure the correct amplicon size was achieved, PCR products were run on a TapeStation 4200 with a D1000 ScreenTape (Agilent). To allow multiple samples to be sequenced together, which saved processing time and cost, as well as ensuring that higher concentration samples did not 'flood' the sequencer at the expense of lower concentration samples, the 96 samples were multiplexed (combined) into one of four pools. Multiplexing was based on the individual DNA concentrations of each sample to ensure that the concentration of each pool was approximately equal. For comparability, negative controls were multiplexed based on the average volume of the samples. Pools were cleaned using SPRIselect beads (Beckman Coulter, Brea, USA), with a left-side size selection using a 0.9:1 ratio. To check DNA concentration of the pools after cleaning, thereby ensuring that primer dimers had been removed, we used a Qubit Fluorometer with results being cross-checked using a TapeStation 4200 with a D1000 ScreenTape. The pool of MID-tagged samples was used for library preparation using the NEXTFLEX^{R} Rapid DNA-Seq Kit 2.0 following the

manufacturer's instructions (Revvity, Inc. Walton, MA, USA), suitable for pools with DNA concentrations of 1 ng to 1 μg. We measured the final DNA concentration for the prepared library using a Qubit Fluorometer and sequenced the library on an Illumina MiSeq desktop sequencer (Illumina, San Diego, CA, USA) with a Nano cartridge using 2 × 250 bp paired reads optimized for 8,000–10,000 reads per sample. Due to the unbalanced nature of the amplicon libraries, we added a 15% PhiX buffer to the sequencing run to improve crosstalk and phasing calculations.

Bioinformatics

We followed the bioinformatic pipeline for high-throughput sequencing data detailed in Drake *et al.* (2022). First, FastP (Chen *et al.* 2018) was used to discard reads of poor quality (< Q30), trim reads to a minimum length of 280 bp, and merge paired (forward and reverse) reads from MiSeq files. We then assigned read pairs to sample ID using the unique pair of MID-tags introduced during PCR and de-multiplexed samples using Mothur (Schloss *et al.* 2009), after which MID-tag and primer ends were removed. Finally, Unoise 3 within Usearch (Edgar 2016) was implemented to remove replicates, denoise the sequences, remove chimaeras, and cluster identical sequences into zero-radius Operational Taxonomic Units (zOTUs); a 100% clustering threshold was applied to avoid multiple species being grouped within an OTU.

Sequences were identified using taxonomic information from GenBank following alignment in Blastn (Camacho *et al.* 2009). A 97% identity threshold was used to capture the widest variety of invertebrates possible at the highest taxonomic resolution (Porter & Hajibabaei 2018). We used MEGAN Community Edition (Huson *et al.* 2016) to analyse the BLAST output and assign taxonomic identities to each zOTU, by using the lowest e-value (< 0.00001) and assigning the top hit to each sequence. If multiple taxa were suggested for a sequence, the feasibility of each candidate taxon being present within our study area was manually checked; this was never ambiguous, so there was no need to identify some taxa at levels coarser than species. We aggregated zOTUs that were assigned to the same taxon.

Data were cleaned following methods outlined in Drake *et al.* (2022). We applied a combined approach to remove, from all samples, the maximum read count in blanks and negative controls. Furthermore, we determined a threshold for read removal using the percentage at which artefacts assigned to the known positive controls (*Baetis harrisoni*) were removed from all samples. This process essentially allowed us to remove laboratory contaminants and sequencing errors from genuine samples using information from the blanks and controls, as well as removing sequences that occurred in low abundance due to tag-jumping or caused by over-represented taxa 'over-spilling' from one sample into another. For our dataset, a threshold of 1% was applied, removing low-frequency laboratory contaminants and sequencing errors. This threshold was determined by calculating the maximum read count of known contaminants and other clearly incorrect zOTUs across the dataset as a percentage of their respective total sample read count. Finally, *Baetis harrisoni* was removed from the dataset.

Data manipulation

The number of sequence reads was used to create a data matrix of each chick sampled and each invertebrate species identified. From this, a binary dataset was created using presence/absence to give information on the frequency of occurrence of each invertebrate species. Reducing data to a presence/absence framework and basing analysis on frequency of occurrence has been recommended as the most conservative approach when analyzing diet using metabarcoding (e.g. Symondson & Harwood 2014, Deagle *et al.* 2019), because factors other than the relative biomass of prey items can affect the sequence counts of different taxa, including differential rates of amplification within PCR reactions and sequencing (e.g. Amend *et al.* 2010, Deagle *et al.* 2010, Pompanon *et al.* 2012).

However, as highlighted by Deagle *et al.* (2019), when lab work is carefully standardized, as here, much of the difference in sequence counts within a sample will be biological (i.e. due to true differences in biomass of different prey items), so ignoring differences in sequence counts can inhibit ecological understanding. The existence of a significant relationship between sequence reads and biomass of different species in the original sample was quantified by Lamb *et al.* (2019) in a multi-taxon meta-analysis, and more recently, by Verkuil *et al.* (2022) using camera-recorded dietary information, which showed that sequence reads were a proxy (albeit an approximate one) for true diet composition.

Thus, in common with many recent diet studies using metabarcoding (e.g. Arazmi *et al.* 2025, Bender *et al.* 2025), we adopted a dual approach by also using sequence reads to weight the 'amount' of each

invertebrate species present within each sample to give relative abundance of each invertebrate species as a percentage – a metric known as 'relative read abundance'. This was done for each sample (each chick) individually, rather than across the whole dataset, to avoid any risk that sample-specific differences could bias data of relative read abundance. This was considered especially important in this study because chicks were sampled at different ages (day seven to day 10 post hatching) and thus the biomass of invertebrates consumed would be expected to differ between chicks based on their relative size. Calculating relative read abundance for each chick individually avoided such bias by ensuring the calculation focused only on the relative composition of the diet at the chick level. Relative read abundance was used in tandem with frequency of occurrence based on presence/absence during analysis.

Although it would have been possible to calculate diet diversity per chick from diet data using a single diversity metric from an index such as Shannon's or Simpson's, this would have been hard to interpret, as these values are influenced by richness and evenness (i.e. an intermediate diversity index value could have been driven by high richness with low evenness or by low richness with high evenness). On this basis, it was decided to calculate and analyses richness and evenness separately. Species richness was calculated as a simple count of the number of species detected in each chick faecal sample. Species evenness, i.e. the extent to which relative abundance of each taxon is similar (uniform distribution) or dissimilar (skewed distribution), was calculated for all chick faecal samples containing > 1 invertebrate species using Shannon's Evenness (EH). This involved quantifying Shannon's Diversity Index (H) and then dividing this by the natural logarithm of the species richness for that sample to obtain the EH value. This metric was bounded between 0 and 1, with 1 indicating complete evenness with all taxa uniformly abundant (Legendre & Legendre 1998).

Data analysis

To test for any spatiotemporal differences in summary variables of species richness (the number of invertebrate species per chick faecal sample) and species evenness (Shannon's Evenness metric), we ran two generalized linear models using R version 4.4.1 (R Core Team 2024). The richness model used a Poisson distribution with log link function to ensure it was optimized for count data using the *glm()* function in the *stats* package; overdispersion was assessed first using the ratio of Pearson chi to degrees of freedom and second using the ratio of deviance to degrees of freedom, which were < 1 in both cases.

The evenness model used a beta distribution with logit link function to account for Shannon's Evenness being bounded between 0 and 1; this was run using the *betareg* and *emmeans* packages. In both models, site ($n = 3$) and sampling time ($n = 3$: early-season broods, mid-season broods and late-season broods) were entered as fixed factors, with the interaction also specified. Both models used data only from a single chick per nest ($n = 54$ cases); the second chick sampled in a small subset of double-sampled nests was excluded to avoid pseudoreplication.

To examine spatiotemporal patterns in dietary composition, we used the freeware statistical package PAST (PALeontological STatistics) (Hammer *et al.* 2001). We initially ran a chi square test for association to determine whether the frequency of occurrence of different invertebrate orders differed relative to site and time. This was followed by ordination analysis using species-level invertebrate data, using Jaccard distance for presence/absence data and Bray–Curtis distance for relative read abundance. For both types of data, non-metric multidimensional scaling plots were created to visualize spatiotemporal differences, and PERMANOVA was undertaken to test for significance using a critical significance value $\alpha = 0.05$ and 9,999 permutations (Anderson 2001, Taguchi & Oono 2005).

While PERMANOVA tested for differences in the metric centroids of the groups, it was also important to test for potential differences in the distribution of values around those centroids: PERMDISP (Anderson 2006) was thus used to analyse betadispersion. The decision to use Bray–Curtis (Bray & Curtis 1957) as the distance metric for relative read abundance was based on it being robust to outliers (Shaw 2003) and its use in previous DNA metabarcoding studies (Deagle *et al.* 2019, Ingala *et al.* 2021, Quin *et al.* 2025). It is also expressed as a percentage in the same way as Jaccard (Jaccard 1912), thereby aiding direct comparison between the approaches.

While the ordination approaches exclusively used data only from a single chick per breeding attempt (the second chick sampled in a small subset of nests was excluded to avoid pseudoreplication), the final stage of the analysis specifically compared the pairwise difference in the invertebrate assemblage between both chicks in the doubled-sampled nests, relative to the difference between the first/only chicks from all nests, to quantify within-nest and between-nest similarity in diet. Analysis again used frequency of occurrence (Jaccard on presence/absence data) and relative read abundance (Bray–Curtis on percentage data). It is recognized that

the sample size here is extremely low (10 chicks in five pairs) and thus these comparisons were descriptive rather than inferential and should be viewed as tentative.

Results

Of the 96 Tree Sparrow faecal samples collected, 59 were processed successfully through the molecular and bioinformatics pipelines to give taxonomic data summarizing invertebrate diet. Spatially, these samples were subdivided between three sites: Site 1 = 17, Site 2 = 14, Site 3 = 28. Temporally, samples were subdivided thus: early-season broods = 22, mid-season broods = 17, late-season broods = 30. Of the 59 samples, 54 were at the nest level (i.e. one chick faecal sample per nest); the five remaining samples were of a second chick in five double-sampled nests to allow within-nest as well as between-nest comparison.

Baseline patterns: invertebrate species detected across all faecal samples

In the full dataset from 59 chicks, 22 invertebrate taxa were recorded, all of which were identified to species level. These came from five orders: Coleoptera (12 species), Diptera (five species), Hymenoptera (three species), Hemiptera (one species) and Araneae (one species). The prevalence of each taxonomic order is shown in Figure 2a, while the prevalence of each species across all samples is shown in Figure 2b. In both cases, these show frequency of occurrence based on a presence/absence data matrix across all 59 samples.

Spatiotemporal trends in invertebrate species richness and evenness in per-chick faecal samples

Most commonly, per-chick faecal samples contained a single invertebrate species ($n = 33$; 56%). Of the multi-taxa faecal samples, most contained two species ($n = 19$; 32%), a few contained three species ($n = 6$; 10%), and a single sample contained four species ($n = 1$; 2%). The overall mean richness was 1.576 (± 0.097 SEM) invertebrate species per faecal sample. When considering data from the 54 nests represented by a single chick, a generalized linear model showed invertebrate richness per chick was not influenced by site, time, or the interaction term (Table 1; Figures 3a–c).

For the 26 samples that contained multiple invertebrate taxa, Shannon's Evenness was calculated using relative read abundance (i.e. using sequence counts to weight the taxa present within each sample).

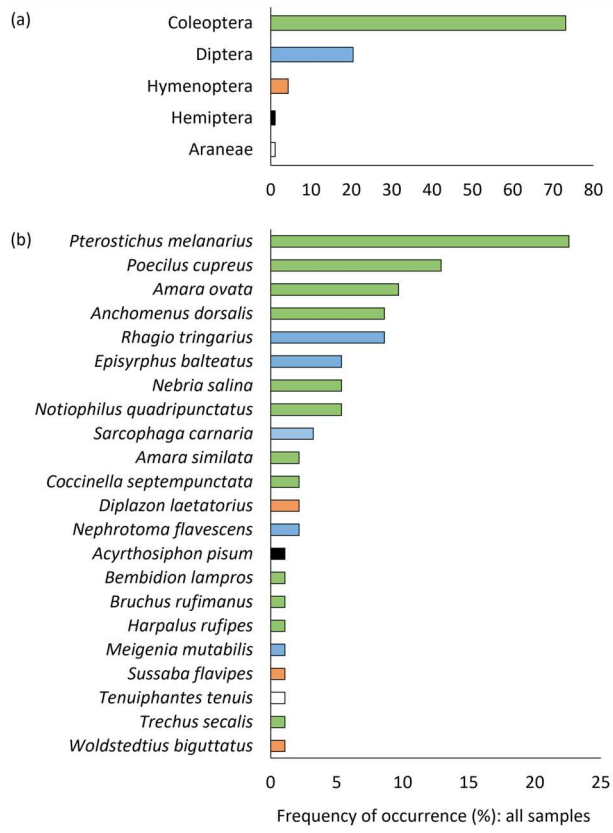

Figure 2. Frequency of occurrence of invertebrate taxa from 59 chicks showing: (a) order and (b) species. The shading of each bar is consistent between graphical elements, such that the order to which each species belongs can be visualized.

Mean evenness per chick was low, given the low species richness (mean = 0.744 ± 0.156 SEM), indicating that most multi-species samples were skewed rather than multiple invertebrate species being approximately equal within the sample. A generalized linear model showed that evenness was influenced by site and time; the interaction was not significant (Table 1). Post-hoc testing showed evenness was significantly higher at Site 1 compared to Sites 2 and 3, which did not differ (Figure 3d). There were significant differences in relation to time, with

Table 1. Generalized linear models for invertebrate species richness and evenness per-chick faecal sample relative to site ($n = 3$), time ($n = 3$: early-season broods, mid-season broods and late-season broods), and the site*time interaction. For post-hoc testing results for the evenness model, see Figure 3d-f.

	Richness			Evenness		
	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Overall Model	2.964	8	0.937	11.070	8	**< 0.001**
Site	0.268	2	0.874	8.305	2	**0.016**
Time	0.228	2	0.892	7.668	2	**0.022**
Site*Time	1.961	4	0.743	4.688	4	0.321

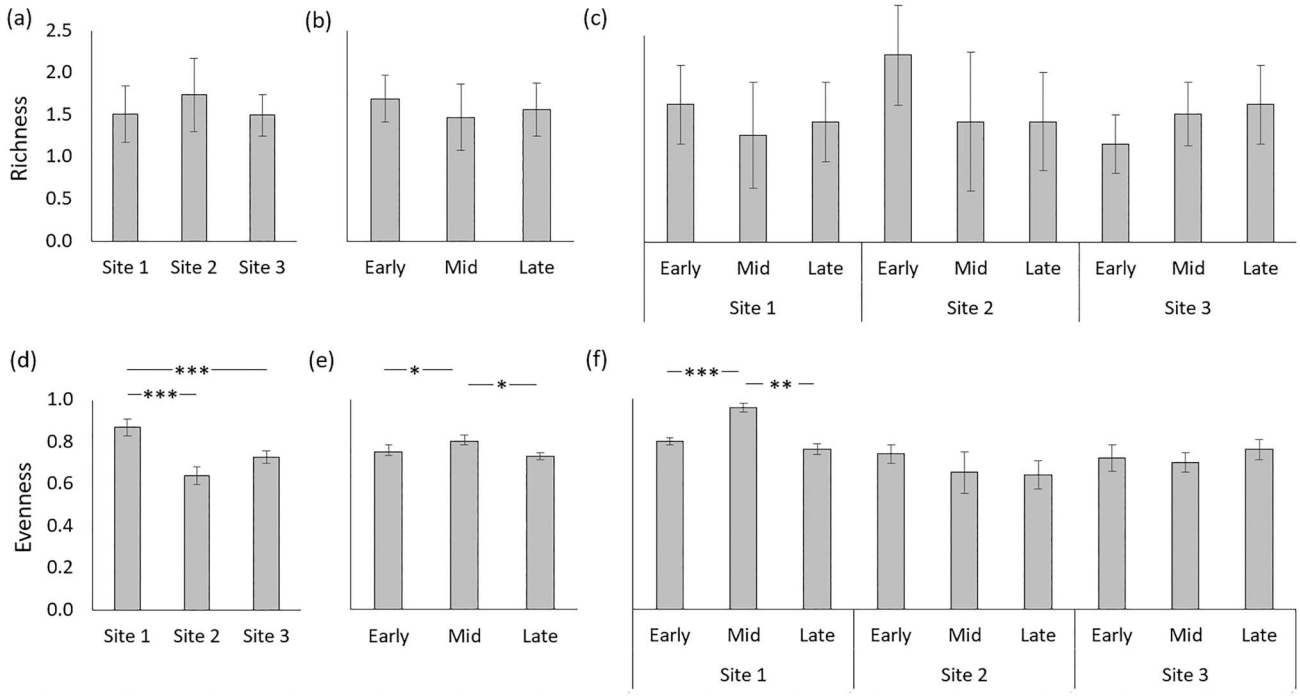

Figure 3. Invertebrate species identified in chick faecal samples showing: (a–c) richness and (d–f) evenness. Error bars show SE. For evenness, pairwise differences are shown using Tukey-corrected *P* values from post-hoc tests using asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

evenness being higher for mid-season broods than early-season or late-season broods (Figure 3e). There was no significant interaction overall but there were significant post-hoc contrasts at Site 1, where evenness was higher for mid-season broods than for early- or late-season broods (Figure 3f).

Spatiotemporal trends in invertebrate assemblage in per-chick faecal samples

There were clear spatiotemporal differences in the frequency of occurrence of each order using presence/absence data from the 54 nests, represented by a single chick only. These patterns, shown visually in Figure 4, were significant (chi square test for association: $\chi^2 = 47.476$, d.f. = 32, $P = 0.038$) and were driven by patterns in frequency of occurrence of individual invertebrate species (Table 2). Although these results are undeniibly a small snapshot of the breeding process (one chick from each nest sampled once), it was notable that Coleoptera were the only prey item recovered from chick faecal material for early-season broods at Site 1 and mid-season broods at Site 2, as well as accounting for > 80% of prey for two other site/time combinations (Figure 4).

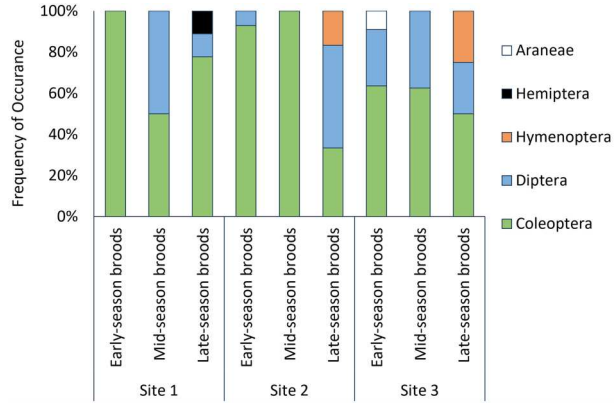

Figure 4. Spatio-temporal patterns in frequency of occurrence of each invertebrate order using presence/absence data.

Non-metric multidimensional scaling plots of the invertebrate species assemblage, firstly using frequency of occurrence (presence/absence data analysed using Jaccard's distance), and secondarily using relative read abundance (weighted percentage abundance of each invertebrate species within each sample using Bray–Curtis), showed visual differences in relation to site (Figure 5a and Figure 5c) and time (Figure 5b and Figure 5d). It should be noted that the high stress values associated with these analyses (Figures 5a–d), which indicate a relatively poor fit, mean these plots should be treated as indicative rather than definitive.

Table 2. Spatiotemporal patterns in frequency of occurrence of each invertebrate species in faecal samples, using presence/absence data. Order codes: Ara = Araneae, Col = Coleoptera, Dip = Diptera, Hem = Hemiptera, Hym = Hymenoptera.

Order		Site 1			Site 2			Site 3		
	Time of season	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late
Col	*Pterostichus melanarius*	–	25.0	33.3	7.2	33.3	16.7	9.1	31.3	33.3
Col	*Poecilus cupreus*	33.3	–	11.1	28.6	–	–	9.1	6.3	8.3
Col	*Amara ovata*	33.3	–	22.2	7.2	–	–	18.2	–	–
Col	*Anchomenus dorsalis*	–	–	–	21.4	33.3	–	9.1	18.8	–
Dip	*Rhagio tringarius*	–	–	–	–	–	–	18.2	31.3	8.3
Dip	*Episyrphus balteatus*	–	25.0	–	–	–	33.3	–	–	16.7
Col	*Nebria salina*	16.7	25.0	–	14.3	–	–	–	–	–
Col	*Notiophilus quadripunctatus*	–	–	–	14.3	–	–	18.2	–	–
Dip	*Sarcophaga carnaria*	–	25.0	11.1	7.2	–	–	–	–	–
Col	*Amara similata*	16.7	–	–	–	–	–	–	–	–
Col	*Coccinella septempunctata*	–	–	–	–	33.3	16.7	–	–	–
Hym	*Diplazon laetatorius*	–	–	–	–	–	–	–	–	16.7
Dip	*Nephrotoma flavescens*	–	–	–	–	–	–	9.1	6.3	–
Hem	*Acyrthosiphon pisum*	–	–	11.1	–	–	–	–	–	–
Col	*Bembidion lampros*	–	–	–	–	–	–	–	–	8.3
Col	*Bruchus rufimanus*	–	–	11.1	–	–	–	–	–	–
Dip	*Meigenia mutabilis*	–	–	–	–	–	16.7	–	–	–
Hym	*Sussaba flavipes*	–	–	–	–	–	–	–	–	8.3
Ara	*Tenuiphantes tenuis*	–	–	–	–	–	–	9.1	–	–
Col	*Trechus secalis*	–	–	–	–	–	–	–	6.3	–
Hym	*Woldstedtius biguttatus*	–	–	–	–	–	16.7	–	–	–

For frequency of occurrence, a subsequent two-way Jaccard's PERMANOVA with 9,999 permutations indicated the invertebrate species assemblage in the per-chick faecal samples was significantly related to site ($F_{pseudo} = 2.321$, d.f. = 2; $P = 0.002$) and time ($F_{pseudo} = 2.992$, d.f. = 2, $P < 0.001$), but the interaction term was non-significant ($F_{pseudo} = 0.862$, d.f. = 4, $P = 0.715$). For relative read abundance, the findings were similar with a two-way Bray–Curtis PERMANOVA indicating the invertebrate assemblage was significantly related to site ($F_{pseudo} = 2.310$, d.f. = 2; $P = 0.005$) and time ($F_{pseudo} = 3.058$, d.f. = 2, $P < 0.001$), with no interaction ($F_{pseudo} = 0.685$, d.f. = 4, $P = 0.917$).

All PERMDISP tests for possible significant differences in the distribution of the data were non-significant (site via Jaccard: $F_{pseudo} = 0.640$, d.f. = 2, $P = 0.529$; time via Jaccard: $F_{pseudo} = 0.388$, d.f. = 2, $P = 0.678$; site via Bray–Curtis: $F_{pseudo} = 0.696$, d.f. = 2, $P = 0.495$; time via Bray–Curtis: $F_{pseudo} = 0.267$, d.f. = 2, $P = 0.796$). This suggests that the significant PERMANOVA results were due to genuine differences in the metric centroids without being confounded by between-group heteroscedasticity.

Within-nest versus between-nest similarity in invertebrate assemblage in per-chick faecal samples

The ordination-based analyses exclusively used data from the 54 nests with a single chick sampled. Analysis of pairwise differences between chicks in the double-sampled nests, compared to the first/only chicks from single-sampled nests, revealed within-nest similarity was higher than between-nest similarity using frequency of occurrence and relative read abundance (Figure 6).

Discussion

Using DNA metabarcoding we have provided the first detailed description of the dietary composition, to species level, of Tree Sparrow chicks. The full dataset of 59 chicks revealed the invertebrates consumed by Tree Sparrow chicks: 22 invertebrate species from five orders were identified. Although these results are only a small snapshot of Tree Sparrow diet (59 chicks from 54 nests across three sites in one year), and should be considered baseline findings, there was a clear reliance by the birds on Coleoptera (overall prevalence of 73.11%) and, to a lesser extent, Diptera (prevalence of 20.43%), which were consistent spatially across three study sites, and across the breeding season in the early-, mid- and late-season broods.

These order-level findings are similar to those of McHugh *et al.* (2016), who assessed diet of Tree Sparrow chicks using fragment identification in faeces from mid-season broods at sites of similar habitat, in the same general study area (Marlborough and Pewsey Downs, UK). In those samples, Coleoptera was the most prevalent order (45.92%), followed by Diptera (33.33%). McHugh *et al.* (2016) also found low prevalence of Hemiptera, Hymenoptera and Araneae, as did we, but (surprisingly) we found no Lepidoptera,

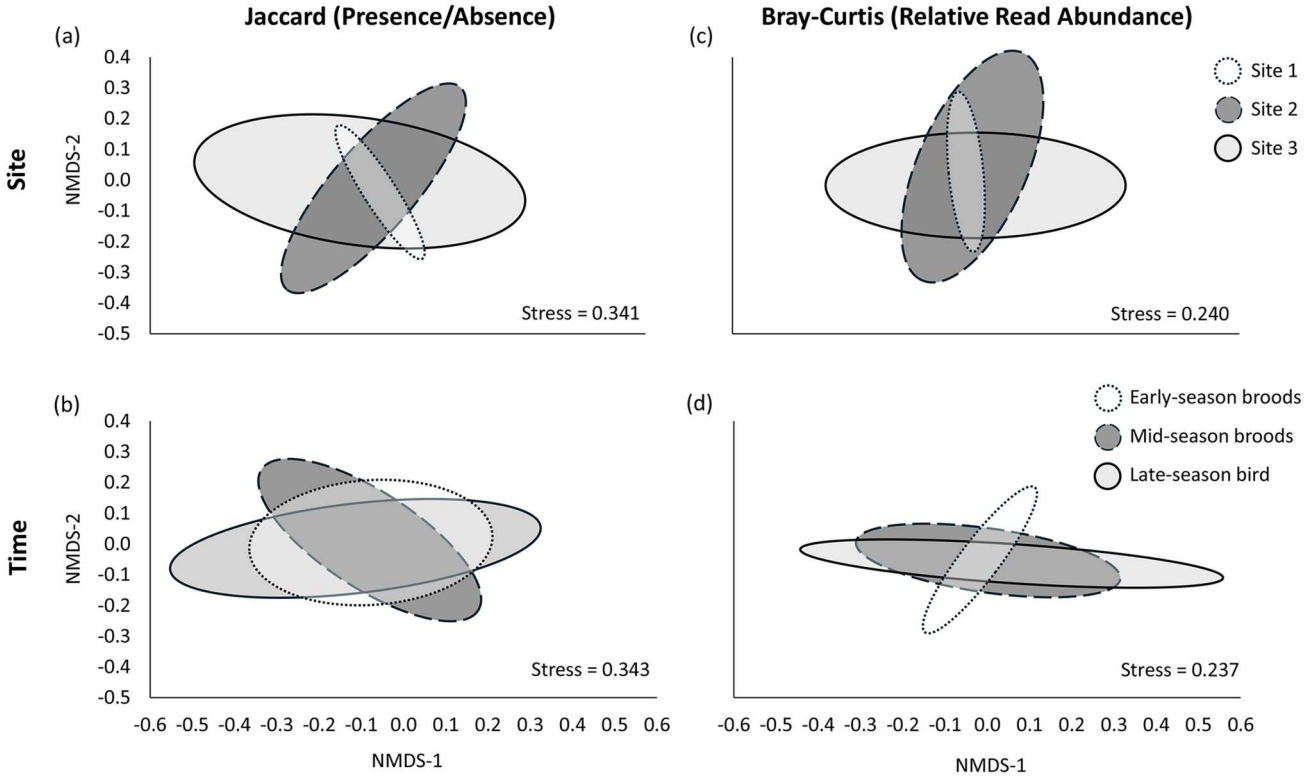

Figure 5. Non-metric multi-dimensional scaling of invertebrate diet of 54 chicks: (a–b) presence/absence data to summarize frequency of occurrence using Jaccard's distance; and (c–d) relative read abundance to weight species according to relative abundance using the Bray-Curtis distance. The ellipses show 95% confidence of each centroid. For clarity of display, datapoints are omitted. It should be noted that the stress values for each analysis are > 0.200, indicating a relatively poor fit, and should be regarded with caution.

which accounted for 6.29% of invertebrates in McHugh's results. However, 2024 was an adverse year for butterfly abundance locally (JNCC 2026), which may account for the lack of Lepidoptera within our results.

The three most prevalent species in dietary samples (> 10% each) were all ground beetles (Carabidae). The most prevalent species was *Pterostichus melanarius*, a predatory ground-dwelling beetle that is abundant in agricultural ecosystems (Helenius *et al.* 2001, Kinnunen *et al.* 2001, Eyre *et al.* 2013). This species breeds in autumn (Carmona & Landis 1999) and has an activity-density that peaks between June and August (Carmona & Landis 1999), suggesting that the adult life stage is consumed by Tree Sparrows; it inhabits cereal crops, refuge strips, short herbaceous boundaries, as well as being positively associated with tilled land and managed grassland (Carmona & Landis 1999, Eyre & Luff 2004, Eyre *et al.* 2013). This species was consumed at all site/time combinations other than early-season broods at Site 1, which was surrounded by spring cereals and Oil-Seed Rape.

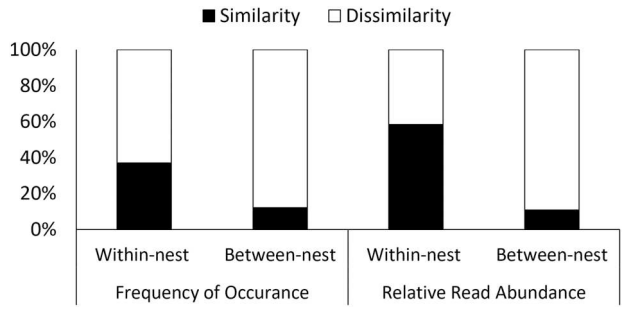

Figure 6. Balance of similarity and dissimilarity in chick diet within-nest ($n = 10$ chicks in five pairs from double-sampled nests) versus between nest ($n = 54$ chicks from 54 nests).

The second-most prevalent species, *Poecilus cupreus*, is an omnivorous ground beetle that is active during spring and summer (Labruyere *et al.* 2018, Sowa *et al.* 2022). It is positively associated with Oil-Seed Rape, where activity peaks from the end of May and decreases from mid-June (Marrec *et al.* 2015), with adults moving to other cereal fields after Oil-Seed Rape is harvested (Labruyere *et al.* 2018). In this study, *Poecilus cupreus* was found most often at sites with Oil-Seed Rape near to the Tree Sparrow colony, and in early-season broods, which accords with these habitat

preferences and activity patterns. The third-most prevalent species was *Amara ovata*, which is largely herbivorous and strongly associated with Oil-seed rape with spring activity peaks (Ribera *et al.* 1999, Eyre *et al.* 2013). It is notable that this species was primarily associated with early-season broods, which accords with known seasonality.

The key ecological information for these three beetle species, and all other species recorded on multiple occasions (and thus consumed by more than one chick in our sample), is given in Table 3. Of these other species, the most prevalent were Coleoptera with generalized habitat preferences for cultivated land and gardens, and activity peaks from spring through summer. *Notiophilus quadripunctatus* and *Anchomenus dorsalis* were consumed at Site 2 and Site 3 (arable farms planted with spring cereals). *Amara similata* was only consumed by early-season broods at Site 1 (garden surrounded by farmland), and *Coccinella septempunctata* was only consumed by mid-season and late-season broods at Site 2. Generally, the Diptera and Hymenoptera found are known to exhibit seasonal activity from May to September, with main habitat preferences across agricultural and grasslands. *Rhagio tringarius* was consumed at Site 3 throughout the breeding season, *Sarcophaga carnaria* was consumed at Site 1 and Site 2, while *Episyrphus balteatus* was consumed at all three sites.

The spatiotemporal patterns for individual invertebrate taxa were substantive enough that the dietary composition of sampled chicks differed significantly between sites, based on community-level analysis including NMDS and PERMANOVA. The pronounced effects of site and time on diet within the breeding season have been seen in other insectivorous farmland species, underlying the importance of local factors in diet and, ultimately, breeding success. For example, a study of breeding Yellowhammers and Grey Partridges *Perdix perdix* showed spatiotemporal variation in invertebrate abundance in relation to crop types and the extent of the field margin for species that formed the bulk of the chicks' diet (Holland *et al.* 2002).

In our study, invertebrate richness per chick was low and not influenced by site or time. This is likely because of short gut retention time, possibly influenced by the faecal samples in the study being collected during ringing, which might have been a stress response by the chick and thus an atypically short gut retention time. Alternatively, it may reflect repeat feeding to chicks of the same taxa, but it is not possible to distinguish between these possibilities using molecular techniques that quantify presence or relative abundance of prey in diet. Gut retention has not been quantified for Tree Sparrow chicks specifically, but Quan *et al.* (2015) found that passerine chicks did not always defaecate immediately after feeding, and that when feeding intervals are extended so too were defaecation intervals, resulting in larger faecal sacs. This is tangential evidence that sacs would likely contain more than one prey item. As 56% of our per-chick faecal samples contained a single invertebrate species, the results suggest that repeat feeding of the same species is occurring.

Adult Tree Sparrows are known to provision chicks with multiple prey items of the same species in succession if there is a plentiful supply (Deckert 1962), which can be attributed to optimal feeding and the search image hypothesis, where detection rates are increased by a focused search for specific prey (Dukas & Kamil 2001, Ishii & Shimada 2010). Repeated feeding of the same prey species is also indicated by the within-nest similarity, which was higher than between-nest similarity, and also the Shannon's Evenness scores for multi-taxa samples that were highly skewed to one species, rather than multiple invertebrate species being approximately equal within the sample. More generally, evenness was influenced by site, being highest at Site 1 (suggesting a more varied diet) and lower at Sites 2 and 3 (suggesting a less varied diet). Site 1, which combined residential and arable habitats, potentially supported more niches and thus a greater number of invertebrate taxa, leading to a more varied diet. Chicks from mid-season broods also had higher evenness than chicks from early-season or late-season broods, potentially indicating there was a greater prey diversity in the middle of the breeding season.

In addition to the importance of local factors and search images influencing what potential prey species were present and caught, several other factors likely affect parental provisioning of prey items. The age, and thus the gape size, of chicks is likely to affect their diet, with adult Tree Sparrows previously being shown to feed size-appropriate invertebrates to their chicks, beginning with aphids or Lepidoptera larvae on day one post-hatching (Deckert 1962), then Diptera, Coleoptera (up to 5 mm), Arachnida and Lepidoptera on days two to five (Orszácová *et al.* 2002), followed in later days by similar prey species but with item size increasing as gape continues to increase; our most prevalent species, *Pterostichus melanarius*, was the largest Coleopteran (at 13–17 mm) being consumed between days seven and 10. As the biomass of

Table 3. Summarized ecological parameters of species recorded as prey items from more than one chick in an analysis of Tree Sparrow diet. Size is body length unless otherwise specified. The stated ecological and agricultural role is based on the dominant effect within the ecosystem based on food source, pollinator status, and known agricultural pests. Information synthesized from Coe *et al*. (1950), Thiele (1977), Drake (1991), Stubbs (1992), Triltsch (1997), Luff (1998), Carmona & Landis (1999), Ribera *et al*. (1999), Helenius *et al*. (2001), Kinnunen *et al*. (2001), Oberholzer & Frank (2003), Eyre & Luff (2004), Luff (2007), Warner *et al*. (2008), Eyre *et al*. (2013), Klopfstein (2014), Marrec *et al*. (2015), Szpila *et al*. (2015), Symondson *et al*. (1996), Labruyere *et al*. (2018), Roy *et al*. (2018), Wotton *et al*. (2019), Brock (2021), Devlin *et al*. (2022), Sowa *et al*. (2022), Ball & Morris (2024), Sacco–Martret de Préville *et al*. (2024), NatureSpot (2025) and NBN Atlas Partnership (2025).

Species		Size (mm)	Habitat preference	Seasonality of activity	Ecological / agricultural role
Coleoptera	*Amara ovata*	8–9.5	Open habitats. Gardens, grassland, dry fields. Oil-Seed Rape.	Main adult activity and breeding occur in spring.	Neutral
	Amara similata	8–9.5	Open habitats, gardens and often near water. Oil-Seed Rape.	Activity density peaks in May–June, decreases after harvest, but more slowly if grassy field margins are nearby.	Neutral
	Anchomenus dorsalis	6–8	Arable crops, gardens, dry soils.	Breeds in spring, main adult activity in summer.	Beneficial
	Coccinella septempunctata	5–8	Generalist: grasslands, scrub, gardens, woodland (coniferous, deciduous, mixed) agricultural habitats (cereals, broad-leaved crops), hedgerows.	March–October.	Beneficial
	Nebria salina	11–14	Open countryside, cultivated fields.	Breeds in autumn, main adult activity in summer.	Beneficial
	Notiophilus quadripunctatus	5–7	Sandy heaths, barely vegetated ground such as gravel pits.	Breeds in spring.	Beneficial
	Poecilus cupreus	11–13	Grasslands, gardens and arable. Oil-Seed rape.	April–September. Peaks end of May and decreases from mid-June.	Beneficial
	Pterostichus melanarius	13–17	Gardens. Agroecosystems – wheat crops, refuge strips, cover crop, short herbaceous boundaries, tilled land, managed grassland.	Activity-density peaks in June–August. Breeds in autumn.	Beneficial
Diptera	*Episyrphus balteatus*	6–10.25	Woodlands, gardens, parks, grasslands.	June–August. Abundance rising through spring, peaking in late July.	Beneficial
	Nephrotoma flavescens	11–14 (wing length)	Grassland, agricultural.	June–July.	Agricultural pest
	Rhagio tringarius	8–14	Hedgerows, woods, riparian.	May–October.	Neutral
	Sarcophaga carnaria	10–15	Open habitats.	May–August.	Beneficial
Hymenoptera	*Diplazon laetatorius*	3.5–6 (wing length)	Cosmopolitan distribution.	June–September.	Beneficial

invertebrates increases, so does its potential nutritional value. Adult Coleoptera are high in crude fat and crude protein, with micronutrients also being present in substantial quantities (Razeng & Watson 2015). This means that feeding Coleoptera to older chicks would help meet energy intake requirements needed for growth.

The digestibility of chitin (contained within exoskeletons of invertebrate prey) is variable across avian taxa (Reeves *et al*. 2021), thus some birds prepare prey before consumption by the chicks (Kaspari 1991), with elytra of Coccinellidae observed in our Tree Sparrow nestboxes suggesting that some preparation may have occurred. The need for preparation of larger prey often necessitates longer handling, affecting optimal foraging times, although preparation reduces as the chicks grow older (Barba *et al*. 1996), as also seen in Whinchat *Saxicola rubetra* nestlings where older chicks received more chitinized prey (Orłowski *et al*. 2017). The ability of previous Tree Sparrow studies to identify invertebrate species from fragments in the faecal sacs (Cummins *et al*. 2000, Field *et al*. 2008, McHugh *et al*. 2016) suggests that chitin is not fully digested by Tree Sparrows.

Our data, while undoubtedly modest in sample size and limited in spatiotemporal extent, provide a valuable snapshot into patterns in the invertebrate diet of Tree Sparrow chicks. Identifying invertebrate prey to species level represents a notable increase in knowledge of the diet and, by extension, of Tree Sparrow breeding ecology. The spatiotemporal patterns for individual invertebrate taxa show the importance of local factors (bare ground and crop type) and seasonality on diet, with spatiotemporal differences in dietary composition of chicks, and more

detailed follow-up studies would be interesting to model these effects further.

The fact that ground-dwelling Coleoptera dominate implies that the foraging time of parent birds was primarily ground-based. This indicates the importance of bare ground, sparce and short vegetation in areas close to Tree Sparrow colonies during the breeding season. Refining conservation management practices through simple manipulations of habitat structure to increase bare ground, and thus invertebrate availability, may potentially increase optimal foraging and thus the condition and productivity of colonies. This is especially important in the simultaneous contexts of species-specific declines (DEFRA 2025) and reductions in invertebrate biomass (Wilson 1987, Conrad *et al.* 2006, Hallmann *et al.* 2017, Cardoso *et al.* 2020, Wagner 2020), which, based on studies of other bird species that are dependent for at least part of their life cycle, are likely to be causally linked (Rosenberg *et al.* 2019, Tallamy & Shriver 2021, Grames *et al.* 2023). Further investigation should focus on linking diet to measures of breeding productivity and chick growth, to gain a comprehensive picture of how to manage areas to support foraging during breeding for this declining species.

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Data availability statement

The data underpinning this research are available via the University of Gloucestershire Research Repository at https://eprints.glos.ac.uk/16406/.

ORCID

Hannah L. Baker https://orcid.org/0009-0002-0190-7547
Ewan H. Stenhouse http://orcid.org/0000-0002-2524-1914
Trudy Workman http://orcid.org/0000-0003-1687-1516

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