The Effects of Urban Living upon Diet Variation and Overall Condition of Blue Tit Chicks in Manipulated Broods

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ABSTRACT

Urbanisation is expanding rapidly and brings with it substantial threats to biodiversity. The milder climates of towns and cities, as well as the presence of nesting sites near to accessible food sources, can make them beneficial environments for many bird species. Unfortunately, much of the food found in urban environments may be of insufficient nutritional value, especially for the development of nestlings, and so urban populations of many species exhibit reduced reproductive success compared to forest populations. Whether this negative effect is consistent throughout urban areas is unclear; microhabitats within cities could host sufficient food, while some urban parents could be able to provide a better quality diet to their young. Here, the nestling diet variation of the blue tit, *Cyanistes caeruleus*, is assessed using diet metabarcoding of blue tit chick faecal samples from an urban and a forest population. This is combined with a brood manipulation experiment to explore the impact of increased parental stress upon nestling diet and condition. The impacts of enlarging broods - increased nestling mortality and worse quality fledglings - were consistent across the city and forest, which may be attributable to phenotypic plasticity in clutch size. Urban clutches averaged 2.5 fewer eggs than forest clutches, although its likely urban adults were physiologically constrained in egg production. Overall, city nestlings appeared to be in worse condition than their forest counterparts, and caterpillars, their preferred food, comprised just 39.36% of the insect food items found in their diet samples, compared to 69.24% in the forest. City diets were more diverse and exhibited greater variation both between and within nest boxes, with some urban chicks seemingly receiving as many caterpillars as their forest counterparts. This suggests that not only is it possible for blue tits to raise their chicks in urban habitats, but also that some of their offspring may be no worse off than forest nestlings. A greater understanding of the factors that could allow such success is needed, with improved and targeted management potentially helping towns and cities provide optimal breeding environments for blue tits and other bird species.

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ETHICS STATEMENT

Manipulations to brood sizes, the collection of faecal samples, and measurements of nestlings were all conducted under Home Office licence P6859F36E and Scottish Natural Heritage Licence 156597. Ethical approval was also received from Durham University's Animal Welfare and Ethical Review Body.

CHAPTER I. Background

1.1 Impacts of Urbanisation

Natural habitats throughout the world are under threat from the increasing rate of urbanisation. Fragmentation, environmental changes, pollution and shifts in community composition are prominent in most modified habitats (McDonald et al., 2013; Mackenzie et al., 2014). The responses of local species to these changes vary, and while a small number are able to successfully exploit the novel environment, most either have to adapt or are unfortunately forced to disperse to new habitats, as they otherwise face local extinction. This leads to an overall trend of reduced species richness in urban areas (Sol et al., 2014), which necessitates a greater understanding of the factors and mechanisms that influence species' success in these habitats, especially as the world continues to rapidly urbanise and the problem grows.

There are several features of urban areas that can benefit a variety of species, allowing them to adapt to the environment. Cities generally have milder climates, in part due to the urban heat island effect (Manley, 1958). This sees temperatures differ by up to 12°C between cities and the rural areas surrounding them, with the effect most pronounced on clear and calm nights (Parris & Hazell, 2005). Additionally, cities can provide birds with rich food sources. In the UK, nearly three quarters of households regularly feed birds (Cowie & Hinsely, 1988), while human foods that have been disposed of, dropped, or left unattended can often be more easily foraged than live prey, especially when wild resources are scarce in the winter (Robb et al., 2008). Meanwhile, parks and other green spaces can offer a safe nesting habitat in close proximity to the aforementioned food sources, especially as these are often supplied with nest boxes (Bańbura & Bańbura, 2012). Nest boxes are typically used by hole-nesting birds that would otherwise naturally breed in tree cavities, which are found in abundance in forests but not in towns and cities (Wesołowski, 2007). Unfortunately, artificial nest boxes are not always effective substitutes for natural cavities in terms of supporting reproductive success, although this varies between bird species (Sudyka et al., 2022). Furthermore, anthropogenic food can often lack the desired nutrients necessary for sustained health (Pollock et al., 2017). For example, the anthropogenic food waste that carnivorous mammals are able to scavenge is often low in protein and lacks fat reserves, and sometimes carries parasites (Murray et al., 2015). Despite the shortcomings of many anthropogenic food sources, their abundance and accessibility mean many individuals in urban environments become increasingly reliant upon their apparent benefit. Some urban areas may therefore act as ecological traps, with individuals choosing to live in a poor quality habitat that leads to a reduction in their survival or productivity due to a misguided view of its alleged advantages (Robertson & Hutto, 2006).

The species that typically inhabit towns and cities are birds and small mammals (Luniak, 2004). Detailed studies into the success of urban fauna regularly focus upon birds as they are more easily encountered in urban environments (Jarrett et al., 2020). For example, analysis of the European starling found that, despite behavioural adjustments by parents, clutch size, breeding success and nestling body mass fell as the level of urbanisation increased, while the proportion of nests which experienced total hatching failure was greatest in the more urbanised habitats (Mennechez & Clergeau, 2006). Similarly, a study

into the house sparrow found urban populations exhibited higher chick mortality and reduced post-fledging survival - inferred from pre-fledging body mass - to the extent that in two separate years reproductive output was below the estimated threshold for population stability (Peach et al., 2008). These findings were supported by a separate study, which also used cross fostering to demonstrate the growth rate of house sparrow nestlings was not influenced by their hatching environment, rather the habitat they grew in (Seress et al., 2012). Additionally, analysis of tit species in urban habitats found that clutch sizes were smaller for both blue and grey tits, with lower survival rates and reduced growth in the embryonic and nestling stages (Bailly et al., 2015). Further studies of the blue tit have also shown fledging success and nestling body mass are substantially reduced in more urbanised environments (Pollock et al., 2017). Ultimately, the most noticeable effects of urban living upon bird populations are seen in their reproductive success. Across several bird species, urban populations exhibit lower nestling weights and smaller clutch sizes, which result in smaller numbers of surviving and recruiting young than are seen in populations in more natural habitats (Chamberlain et al., 2009).

1.2 Study Species

The blue tit, *Cyanistes caeruleus*, is a small hole-nesting passerine, with adults weighing between 11 and 13 grams, that is common across most of Europe and generally found in deciduous forests (Andreasson et al., 2016), although it has increasingly expanded into towns and cities (Bańbura & Bańbura, 2012). Blue tits, especially those in Western Europe and the United Kingdom, usually have one brood per year (Nur, 1984). Their average clutch size is between ten and 13 eggs (Haftorn & Reinertsen, 1985). Eggs are incubated for up to 17 days in nests that are typically lined with soft foliage such as moss, with an inner layer of wool and feathers (Tomás et al., 2006). Blue tits are an altricial species. Thus, after hatching, nestlings are repeatedly brooded by the female for up to a week (Sasvari, 1986) and fed by both parents for up to three weeks before they fledge (Andreasson et al., 2016). Blue tits are generally socially monogamous, and both parents contribute extensively to the care of offspring (Lejeune et al., 2019).

Outside of their breeding season adult blue tits are opportunistic feeders, consuming plants and insects, as well as seeds, nuts, and even grains (Perrins, 1991). However, during the breeding season blue tits become highly selective in their food choices, providing their chicks with an almost entirely insectivorous diet (Bañbura et al., 1999). Like many birds, blue tit chicks have very specialist dietary needs early in their development, with leaf-eating caterpillars being the preferred food choice because of the ease with which they can be digested by nestlings, coupled with their high nutrient content (Bañbura et al., 1999). Micronutrients such as carotenoids, as well as essential amino acids, are particularly beneficial. Like other parids, they synchronise the provisioning of their young with the maximum availability of suitable food sources by attempting to exploit the considerable peak in caterpillars that occurs each spring (Perrins, 1991). The abruptness of this peak coupled with the obvious time constraint of daylight hours - like most birds, blue tits are diurnal and would struggle to find prey in the dark - necessitates a rapid provisioning rate that sees adults spend the entirety of the typical 16 hour days in the breeding season provisioning at an average rate of almost one diet item per minute (Perrins, 1991). Doing so at this rate enables blue tits to nurture sizable clutches. The act of food provisioning is a time

consuming and energy sapping responsibility and parents must balance it alongside other parental duties, such as nest sanitisation (Grieco, 2003).

1.3 Urban Blue Tits

Comparatively recent changes to the layout of towns and cities, with parks only becoming commonplace in the 18th century, have made urban areas somewhat suitable habitats for blue tits (Bańbura & Bańbura, 2012). As a species they have been relatively successful urban adapters, as demonstrated by their ability to establish and maintain new populations in towns and cities across Europe. An example of this success is in Barcelona, where blue tits have maintained a constant population in urban parks since the 1970s, despite previously only inhabiting the city in harsh winters (Senar & Björklund, 2021).

Yet while many of the food items consumed by blue tits outside of their breeding season can be foraged in towns and cities (Bańbura & Bańbura, 2012), the level of biodiversity supported by urban green spaces is extremely variable and depends on many other site-specific characteristics including management and connectivity (Lepczyk et al., 2017). This means that caterpillars can unfortunately often be a scarce food source in towns and cities. In forest habitats, the presence of leaf-eating caterpillars can be measured through the volume of frass fall. Frass is the waste passed by insects after digesting plant material, which typically falls from the canopy after excretion (Tinbergen, 1960). While there is limited evidence that environment type alone causes a reduction in frass fall, a study in Sweden found that there is significantly less frass fall from non-native tree species (Jensen et al., 2021), which are often found in urban parks. Additionally, vegetation composed mostly of exotic flora, as well as evergreen trees and shrubs, can host reduced levels of insects (Southwood, 1961). Indeed, urban habitats typically represent native trees poorly, and this likely impacts local invertebrate communities, including caterpillars (Glądalski et al., 2017; Pollock et al., 2017; Narango et al., 2018; Seress et al., 2018). Furthermore, as cities expand new developments often result in the loss of green spaces, with parks and gardens replaced with new builds and paved parking lots, increasing the scarcity of native vegetation (Shaw et al., 2008). However, it is worth noting that one study did find caterpillars to be more abundant in an urban environment, although their carotenoid concentrations were significantly lower, thus making them of less nutritional value (Isaksson & Andersson, 2007). Additionally, caterpillars in polluted areas can contain high concentrations of metallic trace elements (MTEs), making them a worse quality food item for nestlings as they risk exposure to potentially toxic materials (Dauwe et al., 2004; Chatelain et al., 2021). Furthermore, the densities of invertebrate populations in urban areas can be impacted by a variety of factors, including light pollution (Owens & Lewis, 2018) and traffic emissions (Summers-Smith, 2007). All these influences upon urban invertebrate populations are important, as the availability of caterpillars is often cited as the most critical factor in determining breeding success and chick quality in forest birds, like the blue tit (Tremblay et al., 2004).

It is important to note that blue tit parents are able to attempt to offset shortages of local caterpillars by increasing their search effort. However, tracking studies combined with chick diet analysis have shown that even when urban blue tit parents dedicate more time and effort into provisioning their young, demonstrated by increased foraging distances, the diets of their chicks do not contain significantly more caterpillars (Jarrett et al., 2020). Parents can also attempt to offset a lack of caterpillars by instead provisioning their chicks with

alternative food items. Notably, many of the items consumed by adults in urban areas would not be suitable for their young offspring, with certain anthropogenic foods potentially even causing chick mortality (Pollock et al., 2017). For example, attempting to consume peanuts, a food found commonly in bird feeders, may cause chicks to choke and die (Blake, 2014). Additionally, urban environments directly expose nestlings to MTEs such as copper, arsenic and lead, which have been shown to negatively affect fledging success and nestling mass (Chatelain et al., 2021).

Spiders are thought to be beneficial to nestlings, as, like caterpillars, they provide carotenoids and essential amino acids (Ramsay & Houston, 2003). However, aphids, which are typically found in abundance in towns and cities, are thought to be of limited nutritional value to blue tit chicks (Perrins, 1991). Ultimately, it has been shown that blue tit parents in urban habitats provide their chicks with fundamentally different diets to those in forest habitats (Pollock et al., 2017). Urban chicks are fed significantly fewer caterpillars, with this shortfall often compensated for with foods of insufficient nutritional value (Jarrett et al., 2020). One study showed blue tit reproductive success is positively associated with the volume of caterpillars provisioned, and as a result is lower at urban sites (Pollock et al., 2017). The impacts of the worse quality alternative urban diet upon blue tit chicks are seen across various studies that have found brood size and average chick mass to be lower for urban blue tit populations than forest populations (Pollock et al., 2017; Jarrett et al., 2020). Importantly, research has demonstrated that nestling weight plays a crucial role in influencing the survival rates in the following year across multiple similar bird species (e.g. Tinbergen & Boerlijst, 1990; Magrath, 1991; Monrós et al., 2002).

1.4 Diet Analysis

When assessing the impacts of an urban habitat upon the food items consumed by blue tit nestlings, accurate diet analysis is crucial. The diet of birds can be assessed through simple visual observations of adults and their nests. Monitoring foraging behaviour can be challenging, as birds can travel relatively large distances when finding food for their young. However, infrared cameras positioned beside nests, or even inside nest boxes, can be a useful tool in assessing the food items delivered to chicks. Unfortunately, foods typically can only be coarsely identified and categorised by type and sized (Pollock et al., 2017). Additionally, some food items are difficult to accurately distinguish from each other (Jarrett et al., 2020). Yet nest box observations have still successfully identified patterns in blue tit nestling diets. One study established urban nestlings are provisioned almost twice as often as their forest counterparts, while the proportion of caterpillars provisioned varied considerably between urban, suburban and forest nest boxes (Pollock et al., 2017).

An alternative, more refined, approach to diet analysis beyond simple visual observations involves the microscopic examination of faecal samples. Although the segregation and identification of prey remains from such samples is time consuming and labour intensive, research has shown it to be a feasible method to perform a rough assessment of diet composition, including the relative proportions of prey items, in insectivorous birds like the blue tit. This is because distinct insect structures, including the clypeus and chelicerae, remain identifiable, and these can be attributed to caterpillars and spiders respectively (Michalski et al., 2011). Another approach assessing bird diets is stable isotope analysis. This method analyses isotopic signatures within tissues to uncover their dietary origins in

order to gain insights into their dietary patterns over time. A study analysing blood samples from blue tit nestlings using stable isotope analysis showed nestling diets differed fundamentally between urban, suburban and forest environments (Pollock et al., 2017). While this approach effectively facilitates comparisons between different groups, by establishing and defining isotopic niches, it generally lacks the specificity to identify individual dietary items.

An approach to diet analysis that is capable of identifying specific dietary items whilst enabling group comparisons is DNA metabarcoding. This is a non-invasive method that identifies taxonomic groups through the use of specific primers, which are designed to amplify taxonomically informative genetic markers across a wide array of target species (Bohmann et al., 2022). DNA is extracted from heterogeneous samples and amplified with these primers using PCR. Sample-specific nucleotide identifiers are also used so that metabarcoding sequences can be assigned back to their original sample, allowing hundreds of samples to be pooled and sequenced simultaneously (Bohmann et al., 2022). Metabarcoding offers several advantages over more traditional diet analysis methods, including the ability to identify diet items to the species level rather than broad morphological categories. It also eliminates potential errors brought about by confusing different life stages as separate species, and it can also detect diet items that are more fully digested than those able to be identified through other methods (Hoenig et al., 2022). Perhaps its most significant advantage is that it allows the simultaneous identification to species level of multiple taxa from a single sample.

In fact, a great variety of taxa can be identified by DNA metabarcoding, from plants and animals to bacteria and fungi, while the types of samples that can be used are also diverse. Usually DNA metabarcoding uses environmental DNA, or eDNA, which can be derived from soil and water extracts, and this is helpful when assessing the species present in an area (Deiner et al., 2017). Additionally, faecal samples can be used to assess diets, with DNA extracted from faecal matter, or even directly from the gut, referred to as dietary DNA (de Sousa et al., 2019). Knowledge of the diets of species is important in understanding food webs and trophic niches, and dietary DNA metabarcoding provides a more accurate technique than previous methodologies, such as the simple visual identification of partially digested prey (de Sousa et al., 2019). In fact, dietary DNA metabarcoding can also reveal information about secondary consumption, potentially offering insights into additional links within the food web (Sheppard et al., 2005). While diet metabarcoding is particularly beneficial in aquatic ecosystems, where direct observations are generally infrequent, it is also a very advantageous method in identifying prey taxa in terrestrial habitats, especially for nocturnal animals, as well as those that feed in dense vegetation and those that are too fast or too small to easily observe. As a result, the popularity and usage of dietary DNA metabarcoding has increased dramatically in recent years, with it being both applicable in a wide range of scenarios and relatively cost-effective (Ando et al., 2020). Notably, dietary DNA metabarcoding has already been used to explore the differences in diet between blue tit chicks in urban and rural environments. Using faecal DNA a study by Jarrett et al. (2020) revealed the diets of urban blue tit nestlings contained a significantly lower proportion of caterpillars than the diets of nestlings in a nearby forest habitat.

Nethertheless, dietary DNA metabarcoding does have some potential issues. Degraded DNA from faeces is usually low in quality and quantity, which means that any contaminants

may be preferentially amplified, although this can be detected with negative PCR controls, and once identified contaminated replicates can be filtered out (e.g. Shirazi et al., 2021). PCR amplification also introduces errors such as nucleotide substitutions and chimaeras, as well as biases and false negatives (Bohmann et al., 2021). Biomass bias is where specimens of greater size are typically amplified more, which can cause smaller specimens to be lost during bioinformatic filtering owing to their low read coverage. As no primers are truly universal, they are also subject to bias. Primer bias can cause specific species to be amplified with greater efficiency than others, artificially inflating their sequence read abundance in comparison to the actual biomass present in the sample (Elbrecht & Leese, 2015). Primer bias can also cause some taxa to not be amplified sufficiently to be detected, leading to false negatives, where dietary DNA metabarcoding results may omit certain components of the diet, especially as the removal of false positives tends to be prioritised over false negatives (Drake et al., 2021). Digestion bias is where the DNA from different prey items is broken down unevenly as it is digested by the predator. For example, the faeces of insectivorous animals will often contain hard body parts such as exoskeletons which have been relatively undigested and thus likely contain undigested DNA (Deagle et al., 2019) Similarly, read abundances can also differ between specimens because of copy number variations of the target loci. Mitochondrial copy number can vary between different organs in a single organism (Wiesner et al., 1992), and so would differ significantly between species with different tissue type ratios. For example, a flying species would likely have a greater abundance of tissue with a high mitochondrial content than non-flying species (Krehenwinkel et al., 2017). Discrepancies in read abundances cause noise in DNA metabarcoding data and this can complicate the accurate assessment of species abundance and biomass in a sample. Because of this, presence-absence metrics are generally used to analyse dietary DNA metabarcoding findings, despite their own shortcomings. However, if biases are sufficiently understood and accounted for, more complex methodologies can be used when interpreting DNA metabarcoding results, and thus it may be possible to estimate the relative abundance of species (Thomas et al., 2016).

The use of eDNA brings its own issues, as it is shed from different organisms at varied rates and in several forms. These include faeces, mucus, and gametes, which will all behave differently in the environment (Barnes & Turner, 2015). The rate of eDNA shedding varies between organisms and is also affected by external environmental factors (Andruszkiewicz et al., 2020). Tissue shedding rates can increase by up to 100 times due to stress (Jo et al., 2019). After being shed, eDNA starts to decay immediately. This is due to a combination of enzymatic breakdown, microbial grazing, and exposure to ultraviolet light (Andruszkiewicz et al., 2020). The rate of decay varies and impacts how well and for how long eDNA can be detected in a sample. Additionally, eDNA can become diluted, either due to rainfall or if it is directly shed into water, and this can further impact how well it is detected (Staley et al., 2018). The variations in both shed and decay rate means that the amount of eDNA that can be collected is unlikely to accurately correlate directly to organism abundance.

Despite these limitations, the diets of songbirds, including blue tits, can be analysed in fine resolution by faecal DNA metabarcoding (Trevelline et al., 2016). The presence of both arthropod prey taxa, including caterpillars, and plant material can be identified in a faecal sample, which is important as blue tits consume seeds and nuts, as well as insects. Importantly, when considering the quality of blue tit chick diet, food items that may have been rarely provisioned remain relevant as, independent of the quantity at which they are

delivered, their nutritional value can be very impactful (Catoni et al., 2008). This is because these diet items are potential sources of rare nutrients, which while necessary for successful chick development, are not required in great abundance (Arnold et al., 2007). Thus, even if DNA metabarcoding provides noisy or inaccurate estimates of the relative abundance of each species' DNA in a sample, their presences alone in the chick's faecal sample remain an important indicator of the quality of the chick's diet.

1.5 Introduction to the Study

In this study, faecal DNA metabarcoding is employed to assess and compare the quality and composition of blue tit chick diets in both a forest and city habitat. These are Kelvingrove Park, an urban park near the centre of Glasgow; and The Scottish Centre for Ecology and the Natural Environment (SCENE), an undisturbed oak forested research station situated beside Loch Lomond, 22 miles northwest of Glasgow. Blue tits are an ideal species for this study because, as a relatively successful urban adapter, they are able to maintain stable urban populations, yet these populations also demonstrate reduced reproductive success. Improved knowledge of the blue tit may also enhance understanding of how similar species adapt to urban environments, as well as the factors that limit their success in doing so. Additionally, the prominence of blue tits in both urban and rural habitats enables the collection of a relatively large sample size, enhancing the robustness of this study's findings.

DNA metabarcoding is performed here using a macroinvertebrate primer which targets the cytochrome c oxidase subunit 1 (*COI*) mitochondrial gene (Vamos et al., 2017). DNA barcoding using the *COI* gene has been proposed as a standardised single molecular marker for the classification of animal species (Hebert et al., 2003), and as a result there is a large reference database available for the gene (Stoeckle & Hebert, 2008). The gene's phylogenetic signal appears more robust compared to the other mitochondrial genes (Strueder-Kypke & Lynn, 2010), and DNA barcoding using the *COI* gene is very efficient at discriminating between vertebrate and invertebrate species (Rodrigues et al., 2017), allowing the host DNA from the blue tit chicks in this study to be identified easily and discarded.

This study is split into two distinct sections. The first focuses upon the differences in blue tit chick diet between the forest and city habitats, whilst experimentally altering brood size. Diet analysis is combined with measurements of chick size and hatching and fledging success rates to gauge the impact of urbanisation upon chick consumption and body condition, as well as overall reproductive success. This is coupled with brood manipulations, which see the number of chicks in a nest either artificially increased or decreased, allowing the effects of brood size upon blue tit chick diet to be analysed. The ultimate aim is to ascertain whether the blue tit's response to alterations in brood size, encompassing both changes in foraging and provisioning, and the resultant reproductive success, varies between habitats of differing urbanisation levels. It is predicted that nestling diets in the city will be more diverse and contain fewer caterpillars and that reproductive success will be lower in the artificially enlarged broods than the reduced broods, with this difference more pronounced in the city habitat.

The second part of this study delves into the intricacies of blue tit chick diets, exploring variations both between and within nests and comparing these variations between the forest and city populations. By scrutinising diet variation at a fine scale, this part of the study aims

to contribute to the basis of an understanding of how nestling diets are able to be influenced by factors beyond overarching habitat type. Even though these factors, such as microenvironmental condition, parental foraging ability and capacity, and sibling dynamics, are not directly analysed in this study, establishing how consistent nestling diets are across each site provides insight into how much impact they may have. As with the first section, a key aim of this analysis is to establish whether any notable findings vary between the city and forest populations, given the well documented differences in blue tit reproductive success between the two environments (Pollock et al., 2017). It is hypothesised that within the forest habitat there will be minimal variation in chick diets both within and between nests due to the anticipated higher availability of caterpillars. Conversely, greater variability in chick diets is anticipated within the urban habitat.

Analysing the dietary variation of blue tit chicks at a nest box level is important because it allows for a more detailed understanding of whether diet consistency is influenced by local factors and whether it is possible for nestlings in urban areas to receive sufficient food. This is particularly relevant as urbanisation continues to expand. This analysis is separate because it focuses specifically on within-nest and between-nest dietary differences, rather than the broader impacts of urbanisation and brood size manipulation on overall reproductive success, as in the first section, although these factors are still considered. While site differences remain a key consideration, the second part of this study focuses upon diet, with less emphasis on reproductive outcomes. By isolating diet from reproductive success, this section enables a more targeted examination of how various factors influence chick consumption patterns, complementing the broader analysis of the first section. Ultimately, dividing the analyses into two sections allows for a more comprehensive investigation, where the more general findings from the first section can remain broadly applicable across urban bird species, whilst developing the fundamental principles upon which the more focused dietary analysis in the second section builds.

CHAPTER II. Brood Manipulation Study

2.1 Introduction

Studies of blue tit nestling diets across rural and urban environments have revealed notable differences in dietary provisioning by parents between city and forest habitats (Pollock et al., 2017). Chicks in urban settings generally receive significantly fewer caterpillars, which are typically seen as their preferred food item (Bañbura et al., 1999), and this deficit is typically inadequately offset by the addition of new food sources that are likely nutritionally insufficient (Jarrett et al., 2020), and may even expose nestlings to toxic MTEs (Dauwe et al., 2004; Chatelain et al., 2021). The poorer quality diets of blue tit chicks in more urbanised environments have been postulated to contribute to lower average chick mass and reduced nest success (Pollock et al., 2017). The use of artificial nest boxes, as opposed to natural cavities, has also been shown to reduce fledging success in blue tits (Sudyka et al., 2022), but that is not relevant here as identical nest boxes were used at each site. This part of the study compares multiple measures of reproductive success and chick condition including clutch size, hatching success, fledging success, and average body mass, between Kelvingrove Park in the centre of Glasgow, and SCENE, an undisturbed oak forest in the southwest of Scotland. Chick diets are also compared between the two sites, using faecal DNA metabarcoding. Reproductive success measures provide an overview of the overall success of blue tit populations in different environments, while diet metabarcoding analysis offers detailed insights into the diversity and quality of the chicks' food intake. As faecal samples typically overrepresent the last food item consumed, diet analysis data is better suited for population-level analysis, with a sizable dataset enabling robust comparisons. Individual analyses, such as comparing a single diet sample to a chick’s body condition, can introduce significant variability and inaccuracy, as they fail to account for broader dietary patterns or the cumulative nutritional intake. Thus, this study focuses on population-level trends in diet, while reproductive success and chick condition are analysed as separate, but complementary, measures of urbanisation's impact on blue tit populations.

A further important aspect to consider is how blue tits may be able to overcome the issues surrounding raising chicks in urban areas, especially with urbanisation on the rise. Perhaps the most obvious factor that could impact the diet and condition of a blue tit nestling, beyond habitat type and quality, is the size of the brood it is in. Blue tit broods can vary greatly, with natural broods observed with as few as two chicks, and as many as 15 (Nur, 1986). Observations of single populations have shown ranges of brood sizes to be as broad as between eight to 14 chicks within one breeding season (Fresneau et al., 2018). Brood size could potentially have an impact on the quality of diet for chicks, particularly for urban populations where the desired food items are a scarce and limited resource. For example, in urban habitats, chicks in smaller broods may receive higher quality diets, as the limited number of caterpillars found by their parents would be shared between fewer nestlings. Meanwhile, provisioning for a larger brood likely increases parental stress and fatigue, impacting the ability of the parents to meet the energy requirements and specific dietary needs of all their chicks (Thomas et al., 2001). The number of chicks in a nest influences the amount of attention each nestling receives from its parents, with those in larger nests facing more competition from their siblings, and this may affect feeding frequency and quality (Fresneau et al., 2018). In fact, sibling competition causes nestlings in large broods to

receive less food per capita, even in forest habitats (Stjernman et al., 2004). This would likely be exacerbated in areas with limited quality food sources, such as towns and cities. Additionally, larger broods, especially those in urban environments, may have increased susceptibility to parasites and diseases, with overcrowded nests potentially more conducive to the spread of infections (Allander, 1997).

While blue tits typically raise large broods, they are limited by parental energy requirements (Thomas et al., 2001). Brood size can be reduced, either strategically or due to poor health (Jarrett et al., 2020). This is important because if blue tit chicks in urban areas in larger nests are repeatedly in a significantly worse condition than those in smaller nests, reduced brood size may be selected for, and this could have significant knock-on effects in terms of population dynamics. Hence this part of the study also uses brood manipulations, where the brood size of a nest is either artificially increased or decreased shortly after hatching, to assess whether the number of chicks in a nest influences the diet, size, and survival of blue tit chicks, and whether any variation in these factors differs between the city and forest sites. Brood manipulation experiments are considered effective as blue tits are thought to treat fostered young as their own, meaning that artificial changes in brood size affect the upbringing of the entire brood equally. A three year study found no significant differences in the final masses of fostered and host chicks prior to fledging, indicating a similar quality of upbringing (Pettifor, 1993). Such experiments have been used to demonstrate flexibility in the foraging strategies of blue tits: provisioning rate has been shown to be greater in enlarged than in reduced blue tit broods, while larger larger prey items are delivered to smaller broods (García-Navas & Sanz, 2010). These findings suggest that in order to meet the energy needs of a greater number of chicks, parents may be forced to be less specific in the diet items they provision their offspring, with the focus potentially shifting from quality to quantity. Thus, especially in an environment with limited resources, prey selection may vary with brood size, and this could have implications for chick condition and survival.

In summary, this section of the study aims to assess the impact of urbanisation upon blue tit chick diets as well as upon various measures of reproductive success, whilst establishing whether diet and reproductive success vary with brood size, and determine if any effects of brood size differ between an urban and forest site. Specifically, the following were tested:

1. Blue tit clutches will likely be smaller in the city, either as a strategic response to limited resources or due to poor parental health caused by the lower quality habitat. Other measures of pre-manipulation reproductive success, namely egg hatch success, hatchling survival, and hatchling body mass, may be comparable between the two sites. This depends on whether a clutch size reduction is strategic, with parents adjusting the number of offspring to match their ability to provide in a more challenging environment, potentially mitigating the negative effects of the urban habitat on egg quality and chick condition. However, if urban clutches are smaller due to poor parental health, both egg quality and chick health may also suffer, leading to lower reproductive success, even prior to the experimental manipulation.
2. Following the brood manipulation, chicks in artificially enlarged broods are predicted to be more likely to die, while those that survive are expected to be in a worse condition, determined by several chick trait measures including body mass and wing length. This is because their parents will struggle to meet the increased demands of raising more chicks, and this is expected to be more pronounced at the city, as the limited supply of quality food resources exacerbates the challenges of raising enlarged broods. Cross

fostered chicks are not expected to be any worse off than those raised by their original parents, as previous studies indicate that blue tits raise young indiscriminately.

3. The diets of city blue tit chicks are expected to contain fewer caterpillars than those of forest chicks, due to the urban environment providing a poorer habitat for caterpillars. Consequently, city chicks are anticipated to consume a broader range of food items, including smaller invertebrates and anthropogenic items, resulting in a more diverse diet. In artificially reduced nests, parents may have more time to locate desired food items, and so diet samples from those nests may potentially contain more caterpillars. Nestling diets are not expected to differ between cross fostered chicks and those raised by their original parents, as adults are not thought to discriminate when raising young.

2.2 Materials & Methods

Data Collection

Field Sites

The diets, as well as physical characteristics and survival rates, of blue tits chicks were compared between a city site and at a forest site. The city site used was Kelvingrove Park in Glasgow (55°52′ N, 4°17′ W). This park is an urban green space beside the river Kelvin. It consists of managed lawns, unmanaged riverbank vegetation, sports fields, and trees. The trees are mostly scattered or in stands and consist of a mix of native and introduced species including low proportions of oak and birch. The forest habitat used was the woodland surrounding the Scottish Centre for Ecology and the Natural Environment, on Loch Lomond (56°7.5′ N, 4°37′ W). This woodland is mixed deciduous and is dominated by oak trees. Over 40 woodcrete nest boxes with 32 mm entrance holes were installed at each site as part of a previous study by Pollock et al. (2017), and for this study, samples and measurements were collected from broods in these boxes during the 2021 blue tit nesting season.

Brood Manipulation

In order to assess the impact of brood size on blue tit chick provisioning at both sites, broods were artificially manipulated. Nest boxes were paired based on the hatch date of their first egg, identified by checking the nests every other day, and their initial clutch size was recorded. The hatch dates in this study were from 13th May to 31st May 2021. Two days after the matched hatch date of each nest box pair, the number of unhatched eggs remaining in each box was recorded, and the surviving hatchlings were counted and ringed with a unique reference number. The chicks were also weighed at this stage, using a spring balance with a precision of 0.1g, but were too small to have any other measurements taken.

The broods were then manipulated by swapping nestlings within each nest box pair. A net change in brood size of three nestlings was instigated in experimental pairs, while no net change was implemented in control pairs. Following this manipulation, each brood consisted of approximately half original and half cross fostered nestlings, although this balance was skewed if the paired broods had significantly different original sizes. The identity of cross fostered nestlings was recorded, and they remained distinguishable for the duration of the study. The direction of the change in brood size within each experimental pair was randomised and was not influenced by the initial number of nestlings in each brood. A net addition or subtraction of three chicks intended to induce a sufficiently large change in brood

size to detect meaningful differences in chick diet, size, and survival, while remaining within the natural range of brood sizes to avoid unrealistic and extreme outcomes. This approach also ensured consistency and standardisation across experimental groups, thereby enhancing the reliability and interpretability of the results.

The forest site featured 30 nest boxes, which were equally divided into three groups of ten whose broods were either artificially enlarged, artificially reduced, or kept at their original sizes, in order to serve as controls. The city site had 27 nest boxes, with eight “control”, ten “decrease” and nine “increase” nest boxes. The additional “decrease” nest box was paired with a nest box at a different park site within Glasgow that was not analysed in this study. Ten days later, on the twelfth day of the experiment the fate of the individual nestlings and the total number of survivors in each nest box were recorded. Body mass was taken again, and tarsus and wing lengths were measured using a sliding calliper. Wing measurements were taken to the nearest 1 mm and tarsus measurements to the nearest 0.1 mm.

Faecal Samples

Between 19th May and 12th June, faecal samples were collected from 236 blue tit chicks, from 50 unique nest boxes. To avoid contamination, the faecal samples were collected by holding a sterile tube containing Longmire buffer directly below the chick’s cloaca when possible, although on occasion the sample was collected via the ringer‘s hand or trouser leg. Samples were frozen as soon as possible after collection, and stored at -20°C. The samples were collected on the sixth and twelfth days of the brood manipulation experiment, although only the latter were analysed in this study. There were 169 of these; 73 from SCENE and 96 from Kelvingrove Park. Approximately 60% were from nests that were manipulated, by either artificially increasing or decreasing the brood size. A full breakdown of the samples and their manipulation group is displayed in Table 1.

Table 1: Summary of the blue tit chick faecal samples selected to be sequenced from each site and manipulation group.

	Control	Decrease	Increase	Total
City	31	28	37	96
Forest	36	14	23	73
Total	67	42	60	169

Laboratory Work

DNA Extraction

DNA extraction was conducted in a laboratory dedicated to handling low quality DNA samples. All surfaces were bleached to minimise external contamination and filter tips were used to prevent cross contamination between samples. DNA was extracted in eight batches of up to 23 samples, each with an extraction control (ECL). Between 60 and 80 mg of wet weight faecal matter was taken from each sample, taking care to avoid any uric acid where possible. When the faecal sample was of insufficient size, the weight was boosted using the buffer the sample had previously been stored in. This was required for 28 of the samples, four of which had no solid faecal matter in the original sample. For each sample the extracted faecal matter was combined with 500 mg of 0.5 mm silica-zirconia beads, and then

650 µL of Gordon's buffer (0.1 M pH 8 Tris-HCl, 0.1 M pH 8 EDTA, 0.01 M NaCl and 0.01 M N-lauroylsarcosine). The samples were then vigorously homogenised for five minutes in a Qiagen Tissuelyser II at 25 Hz and put in a water bath at 56°C for at least 20 hours. They were then centrifuged to separate the beads and solid faecal matter from the buffer. 500 µL of this supernatant was removed, and DNA was then extracted from it using an EZNA Tissue Extraction Kit (Omega Biotek), adhering to the manufacturer's instructions with the following modifications: first, once the proteinase K solution and BL buffer had been added, the supernatant was incubated at 70°C for 45 minutes rather than just for 10 minutes; and second, only 50 µL of the elution buffer was used for each sample, and both elution steps were carried out using the same elution buffer, as this allowed more DNA to be eluted without increasing the final volume and thereby maintaining a greater DNA concentration. The eluted DNA was stored at -20°C.

PCR Amplification

PCR preparation and set up was also performed in the low quality DNA laboratory, while all subsequent steps were carried out in a different laboratory, which was instead dedicated to higher quality DNA samples. A fragment of the *COI* mitochondrial gene was amplified using the fwh2 primer set (Vamos et al., 2017) and the QIAGEN Multiplex PCR Kit. The PCR recipe is shown in Table 2, alongside the thermocycler program. Triplicate PCRs were completed for each sample, including the eight ECLs, and each PCR run also included positive and negative controls. After each run, 5 µL of PCR product from each sample was run on an ethidium bromide stained agarose gel and imaged in order to assess amplification success. Depending upon the success of prior PCR rounds, the amount of DNA used in the PCR was adjusted, from an original volume of 4 µL, to as low as 2 µL or as high as 5 µL. The volume of mastermix was adjusted from 11 µL accordingly, to ensure a total reaction volume of 15 µL. Additional repeats of PCRs were completed in an attempt to get three successful amplifications per sample per primer, although this was not always possible. PCR products for successful replicates were pooled in equal volumes for each sample.

Table 2: PCR recipe and thermocycler program settings for amplifying dietary DNA using a QIAGEN Multiplex PCR Kit with an insect (fwh2) primer.

PCR Amplification Recipe

Reagent	Reaction Volume (µL)	
Qiagen Multiplex PCR Master Mix	7.5	Master Mix
dH_2O	3.1	
Forward fwh2 Primer (10µM)	0.2	
Reverse fwh2 Primer (10µM)	0.2	
DNA	4.0	
Total	15.0	

Amplification Thermocycler Program

Process	Temperature (°C)	Time (minutes)	
Polymerase Activation	95	15:00	
Denaturarion	94	00:30	x40 cycles
Annealing	52	00:30	
Extension	72	00:30	
Extension	72	10:00	
Holding Temperature	5	∞	

Purification & Quantification

For each sample, and each ECL, the pooled PCR products were purified using 0.9x carboxyl paramagnetic beads, following the protocol described by Rohland & Reich (2012), and using 80% ethanol for washes. The cleaned products were stored at -20°C. The amount of DNA in each of the cleaned products was quantified using a Qubit Fluorometer and a high sensitivity double stranded DNA assay. This, combined with an estimate of the length of the DNA strands in each sample, measured in base pairs, allowed the nanomolar DNA concentrations

of each product to be calculated. The concentrations varied from 0.7 nM to 121 nM, although a small number of samples and most of the ECLs contained too little DNA to be detected.

Indexing & Sequencing

Unique indexing combinations were assigned to each sample and each ECL and recorded. The combinations were made up of two indexing primers, and were, in a second PCR step that also extended the sequencing adapters to full length, added to the cleaned and pooled product for each sample. The PCR recipe is shown in Table 3, as is the thermocycler program. After the indexing PCR, the products were once again cleaned using speed beads, and then quantified with a Qubit Fluorometer and a broad range double stranded DNA assay due to the expected increase in DNA concentration as a result of another round of amplification. The concentrations varied from 12.5 nM to 1140 nM. The products from all the samples were pooled in equimolar ratios, at a standardised concentration of 15 nM. 2.5 µL of diluted product was pooled from each sample, with the exception of three samples that had lower concentrations than 15 nM, so for which 2.5 µL of undiluted product was added. Quantitative polymerase chain reaction (qPCR) was then employed to assess the final pool concentration using the Kapa Universal Illumina Standards. The size distribution of the sequencing pool was also assessed using a TapeStation system with a D1000 high sensitivity screen tape. Finally, the pooled samples were sequenced on the Illumina MiSeq platform to produce 150 bp paired-end sequences.

Table 3: PCR recipe and thermocycler program settings for indexing dietary DNA using KAPA HiFi HotStart ReadyMix.

Indexing PCR Recipe

Reagent	Reaction Volume (µL)	
Kapa HiFi HotStart ReadyMix	25.0	Master Mix
dH_2O	10.0	
Forward Indexing Primer (10µM)	2.5	
Reverse Indexing Primer (10µM)	2.5	
DNA	10.0	
Total	50.0	

Indexing Thermocycler Program

Process	Temperature (°C)	Time (minutes)	
Polymerase Activation	95	03:00	
Denaturarion	98	00:30	
Denaturarion	98	00:10	x12 cycles
Annealing	63	00:30	
Extension	72	03:00	
Holding Temperature	5	∞	

Bioinformatics

Diet Analysis Pipeline

In accordance with the methodology outlined in Jarrett et al. (2020), raw sequencing read data from all samples was processed for trimming and error correction, with the reads grouped into operational taxonomic units (OTUs), which were then assigned taxonomic ranks. Specifically, the adapters were trimmed from the raw sequences using CutAdapt v1.10 (Martin, 2011), while quality-based trimming was executed with Sickle v1.33 (Joshi & Fass, 2011). Error correction was conducted through a Bayesian Model implemented within BayesHammer (Nikolenko et al., 2013) through the SPAdes program v3.10.1 (Bankevich et al., 2012). Forward and reverse reads were then merged together using PEAR v0.9.6 (Zhang et al., 2014), and PCR primers were trimmed off using CutAdapt. Non-unique reads were collapsed whilst preserving their read counts using the obiuniq function in obitools (Boyer et al., 2016). To prepare for chimera searching, the format of the DNA sequences was converted using DAMe, and then de novo chimera identification was performed using

Vsearch, with all identified chimaeras filtered out. The remaining reads were clustered into OTUs at the 97% identity threshold using Sumaclust, and only OTUs with more than 10 sequences were retained. Taxonomy was assigned to each OTU based on identity following a BLAST search against the Genbank NT database. The best 20 matches for each OTU sequence, determined by bitscore, were retrieved, and those with the maximum bitscore were retained. The consensus taxonomy amongst these hits was determined. Taxonomy was then assigned according to sequence identity, with matches exhibiting greater than 95% identity assigned to the order level, while those above 96% were assigned to the family level, and finally identifications above 98% were designated genus and species level taxonomy. These thresholds were consistent with other studies, such as Jarrett et al. (2020), and were further validated by preliminary analyses. The analyses demonstrated that applying a 98% species-level threshold resulted in the identification of approximately 21,000 distinct species, compared to around 15,000 species when using a 96% threshold. In contrast, applying a strict 100% threshold inflated the number of species to over 445,000, likely due to over-splitting of taxa caused by minor sequencing errors and natural intra-species variation. The 98% threshold minimises the risk of conflating closely related species without identifying slight variations within a species to be separate taxa.

Clean Up & Quality Control

OTUs with blank and non-order level classifications were discarded, as were those not belonging to Annelida, Arthropoda, or Mollusca phyla. Further taxonomic filtering saw obviously erroneous taxa, such as humans and fish species also removed, as well as known lab contaminants, including Drosophila. Several mite taxa and tick taxa were also omitted, as they were likely to be ectoparasites rather than actively foraged prey (da Silva et al., 2019; Shutt et al., 2020). Sample proportion filtering was then carried out on all samples and ECLs. This involved removing all OTUs from each individual sample that made up less than 0.1% of the sample's total reads. Samples were then split into the eight ECL groups in order to assess and address potential contamination. 65 unique OTUs were present in at least one ECL, although seven of these were only found in the control samples. When the relevant ECL accounted for more than 1.5% of the total read count for an OTU within a group, the read counts for that OTU were reset to 0 across all samples in the group. Furthermore, if any OTUs had a read count in a sample that was lower than the read count in the applicable ECL, the OTU's read count was also reset to zero, but just for that specific sample. Of the 169 faecal samples, 23 had less than the minimum read count cut off of 5,000 total reads at this stage, and so were deemed to be of poor quality and discarded, while the eight ECLs were removed from the main dataset to be assessed separately.

Dietary DNA Measures

There are two common approaches for analysing diet metabarcoding data: relative read abundance and presence-absence, or frequency of occurrence (Deagle et al., 2019). In this study, relative read abundance is calculated as the number of reads for each OTU present in a sample divided by the total reads in the sample, giving a proportional value based on the read count. To calculate occurrence values each OTU that is present in a sample is assigned a binary value of one, with all OTUs that are not present given a value of zero. Some analyses performed here use taxonomic groups at the order level, rather than unique OTUs. In order to calculate the values to use in these analyses relative read abundance values are simply added together to give the total relative read abundance of an order. Occurrence

data, however, is transformed by dividing the number of OTUs in a sample belonging to a taxonomic group by the total number of OTUs in the sample, to give the total proportion of OTUs for said group.

There are several advantages and disadvantages to these approaches. Relative read abundance is a quantitative measure that can provide a more accurate analysis of population-level diet (Deagle et al., 2019). However, it can be highly sensitive to primer bias, especially when these biases impact common taxa, potentially distorting findings. The amplification efficiency of COI, the gene targeted during metabarcoding in this study, varies between species which may influence results (Piñol et al., 2015). However, in this study, the impact of this is mitigated as primer bias equally affects samples from all sites and manipulation groups. Thus, focusing on differences between groups, rather than absolute taxonomic composition or specific biomass consumption, helps alleviate this concern. Occurrence-based summaries of diet provide fuller coverage of consumption but are prone to overestimating the importance of food consumed in small quantities (Cavallo et al., 2018). By carefully integrating both relative read abundance and occurrence-based summaries, this study's ability to discern differences in diet composition between the two sites is enhanced, striking a balance between accuracy, reliability, and informativeness.

Statistical Approach

Data Overview

Clutch size, hatching success and pre-manipulation nestling survival was recorded for all 57 nest boxes, while post-manipulation nestling success was recorded for 56, as one nest box from Kelvingrove Park disappeared during the experiment. Body mass was recorded for 203 city and 289 forest chicks on the second day of the study. All surviving chicks had their body mass as well as wing and tarsus lengths measured on the twelfth day of the study. There were 165 surviving chicks in the city, although 17 did not have their wing length recorded, and 255 surviving chicks in the forest. Of the 146 chick diet samples that remained following the quality control of the diet analysis findings, 70 were from the forest site and 76 from the city, with roughly a third from each manipulation group. The full breakdown is shown in Table 4, alongside the average adjusted brood sizes for each site-manipulation combination. Following the experimental manipulations, forest broods were larger than city broods across all nest box categories,

Table 4: Summary of the successfully sequenced day 12 blue tit chick diet samples from each site and manipulation group, and average manipulated brood sizes for each site-manipulation combination.

	Control	Decrease	Increase	Total
City	27	19	30	76
Forest	36	13	21	70
Total	63	32	51	146

	Control	Decrease	Increase	Overall
City	6.75	4.70	10.90	7.37
Forest	9.50	6.40	13.00	9.63
Overall	8.28	5.55	12.00	8.56

While the initial sequencing results had revealed 9045 distinct OTUs, with a total read count of approximately 25.3 million reads, following taxonomic filtering just 725 unique OTUs remained, with a total read count of about 10.77 million reads. A further 66,000 reads and 308 OTUs were removed during sample proportion filtering, while approximately 230,000 reads were discarded to counteract potential contamination. Finally, the removal of the

samples with less than 5000 reads, as well as the ECLs, left almost exactly 10 million reads across the remaining 146 samples, with an average of approximately 70,000 reads each. These reads were split between 329 unique OTUs, and each sample contained an average of 12 different OTUs. Statistical analyses were conducted in R 4.3.1 (R Core Team, 2023) using the RStudio interface (Posit team, 2023).

Reproductive Outcomes

Pre-manipulation reproductive success was compared between the two sites. As this analysis utilised data taken prior to the nest box manipulations, data from all nest boxes at each site could be considered as a single group. Clutch size was analysed with a Mann-Whitney U test, after Levene's and Shapiro-Wilk tests had indicated non-normal distribution in the dataset. Hatching success was derived from the hatch failure rate, which was calculated by dividing the number of eggs that remained unhatched two days after the first hatching event by the original clutch size. Pre-manipulation hatchling survival was calculated by dividing the number of living nestlings on the second day of the experiment by the number of successfully hatched eggs. As survival data is binary in nature, comparing hatch success and hatchling survival rates between sites required generalised linear models (GLM) with a binomial family. This approach is appropriate for binary outcomes, such as survival or death, and accounts for the non-normal error structures that are typical of such data. Hatchling body mass was compared between sites using a linear mixed-effects model (LMM) that accounted for random effects caused by nest box differences, as chicks raised in the same nest box cannot be assumed to be independent.

Post-manipulation nestling survival rate was calculated for each nest box by dividing the number of living nestlings in the nest on the twelfth day of the manipulation experiment by the artificially adjusted brood size, which was recorded on the second day of the experiment following experimental manipulation. A GLM with a binomial family was used to compare the post-manipulation survival data between the sites and manipulation groups, as this method suits binary data. As well as nest box survival rates, individual nestling fates were analysed. This allowed the impact of whether chicks were cross fostered or raised by their biological parents in their original nest - more simply their parental status - to also be assessed. A generalised linear mixed-effects model with a binomial family was used to analyse the survival outcomes in relation to site, manipulation group, and parental status, while accounting for nest box as a random effect. This approach suited the binary structure of the data and handled the non-independence of individuals raised within the same nest box.

Four measures of chick morphology, all also measured on the twelfth day of the study, were compared between sites and manipulation groups using separate LMMs that took into account the non-independence of data brought about by random nest box effects and considered the nestlings' parental status. The measures were wing length, tarsus length and chick body mass, as well as body mass index (BMI), calculated by dividing body mass by tarsus length (Butler & Davis, 2010). As well as simply considering the manipulation group, the effects of the actual manipulated brood size upon the mean values of the four chick traits for each nest box were tested using analysis of variance (ANOVA) based linear regressions.

Nestling Diet Diversity

In order to examine the diversity of each nestling's diet, relative read abundance data was used to calculate Shannon Diversity Index values for each diet sample. These were compared between sites and manipulations groups, whilst considering the parental status of the nestlings, using a LMM that accounted for random effects due to nest box variability. To explore dietary diversity across all samples, two distance matrices were constructed. Dissimilarity values were determined using two methods: first, by applying the Bray-Curtis index to relative read abundance data, and second, by using the Jaccard index on presence-absence data. These distance matrices were then subjected to non-metric multidimensional scaling (NMDS), a rank-based ordination method that evaluates the similarity between data points. NMDS is well-suited for analysing diet data from DNA metabarcoding because it handles complex, high-dimensional data without assuming linearity or normality. It reduces dimensionality while still preserving dietary composition patterns, even with the large number of taxa and varying abundance levels typical in dietary datasets. Permutational multivariate analyses of variance (PERMANOVA) were performed to test for differences in diets between sites and manipulation groups, whilst taking into account whether chicks were raised in their original nests. These were constructed with a nested design in order to also consider the non-independence of samples taken from within the same nest box. Specifically, nest box was nested within the factors of site, manipulation group and parental status. Separate tests were carried out using both the Bray-Curtis and Jaccard dissimilarity values. In order to establish the significance of any relevant differences in diets dissimilarities, homogeneity of multivariate dispersions analyses were also carried out. Essentially, these established the mean distance of all samples within each group from that group's median and compared this mean between groups.

Nestling Diet Composition

Diet composition was assessed by splitting OTUs into the most common orders: Lepidoptera, Diptera, Hemiptera, Coleoptera, Araneae, and Hymenoptera. The OTUs not belonging to any of these orders were disregarded. Although there were 41 of these OTUs, they only comprised 0.3% of the total reads and 3.5% of the occurrences. Total diet composition was compared between both site and manipulation group by conducting principal component analysis (PCA) upon all six common orders. This analysis was performed twice, once with the relative read abundances and once with the proportion data. For both PCAs, the principal component scores were extracted and combined with the experimental factors. LMMs were fitted to assess the effects of site and manipulation group, as well as parental status, whilst accounting for nest boxes as a random effect. Diets were also compared with a sole focus upon sites, using all samples regardless of manipulation treatment or parental status. LMMs were developed for both the relative read abundances and the OTU proportion of each of the six major orders, resulting in a total of 12 separate models. In each model, the site was assessed as a fixed effect to determine its impact, while nest boxes were once again considered as a random effect.

2.3 Results

Reproductive Outcomes

Mean clutch size was larger in the forest nest boxes than the city nest boxes by 2.50 eggs ($n = 57$, $W = 108.5$, $p < 0.0001$; Fig. 1a, pg. 24). Mean hatching success was 0.941 for the

forest nest boxes, compared to 0.909 for the city nest boxes, although this difference was not significant ($z = 0.3252$, $p = 0.167$; Fig. 1b, pg. 24). The mean pre-manipulation nestling survival rate was 0.951 for the forest nest boxes and 0.986 for the city nest boxes, and this difference was significant ($z = -2.064$, $p = 0.039$; Fig. 1c, pg. 24). Mean hatchling body mass was also greater in the city, at 1.991 g compared to 1.926 g in the forest, although this difference was not significant ($n = 492$, $t = -1.0$, $p = 0.322$; Fig. 1d, pg. 24), and, as a substantial amount of the variance was attributed to nest box effects (var = 0.1145, residual = 0.1091; 51.21%), much of the differences in hatchling body mass may be attributable to differences between nest boxes.

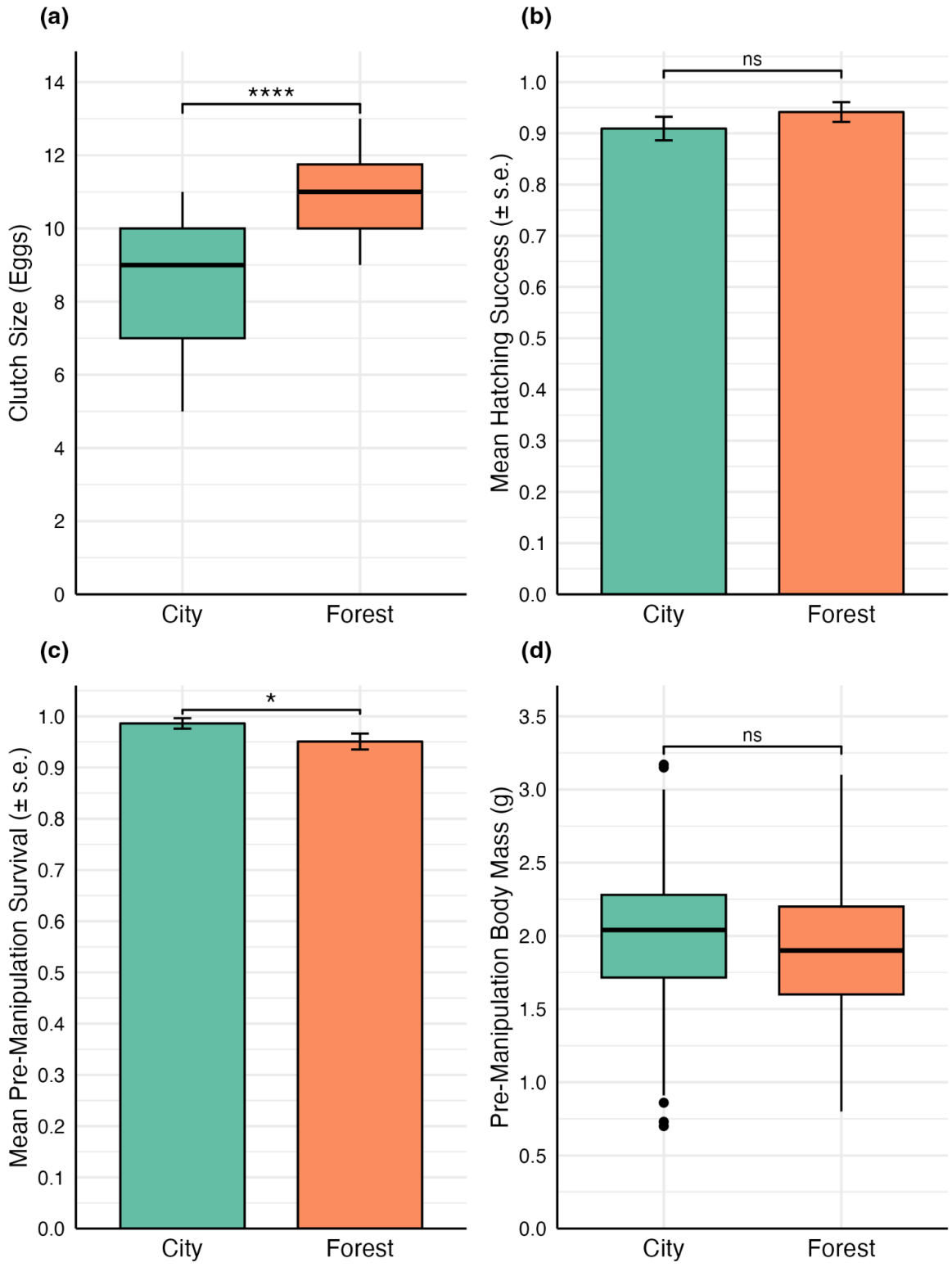

Fig. 1: Pre-manipulation reproductive success at the city (teal) and forest (orange) sites (n = 57 nest boxes unless stated otherwise): (**a**) clutch size, (**b**) hatching success rate, (**c**) pre-manipulation survival rate, and (**d**) hatchling body mass, taken on the second day of the experiment. (n = 492 hatchlings). Box plots display the median values, and upper and lower quartiles. Lines extend to outliers within 1.5 times the interquartile range, with further outliers plotted as individual points. Bar charts show mean values with standard error. Significance codes: '****' < 0.0001, '***' < 0.001, '**' < 0.01, '*' < 0.05, '.' < 0.1.

The lowest levels of post-manipulation nestling survival were seen in “increase” nest boxes, with nest box means of 0.762 and 0.776 at the city and forest sites respectively, while “control” and “decrease” nest boxes at both sites recorded mean nest box survival rates above 92% (Fig. 2). The post-manipulation survival rate did not vary significantly between the forest and city nest boxes (n = 56, z = 0.410, p = 0.6821), or between the “control” and “decrease” nest boxes (z = -0.099, p = 0.921). However, the survival rate was significantly lower in the “increase” nest boxes compared to the “control” nest boxes (z = -2.809, p = 0.005). There were no significant interaction effects between the site and artificially enlarging (z = -0.297, p = 0.7664) or artificially reducing (z = -0.936, p = 0.3495) the brood size. At an individual level, nestling fate did not vary between the sites (n = 491, z = 0.231, p = 0.8171). Nor did it vary between the “control” and “decrease” manipulation groups (z = 1.002, p = 0.3164), although death was significantly more common in the “increase” manipulation compared to the “control” manipulation (z = 2.721, p = 0.0065). Parental status had no effect upon nestling fate (z = -1.812, p = 0.07). The level of variance attributed to nest box effects was sizable (variance = 11.34), suggesting differences between individual nest boxes contributed to a considerable amount of the variability in nestling fate. This was likely due to whole nest failures, which can account for a significant proportion of nestling deaths, and amplify variation between nest boxes. Indeed, four such failures were observed in this study, three of which were in artificially enlarged boxes, with two nests failing at each site, and these failures led to 45 of the 62 observed nestling deaths.

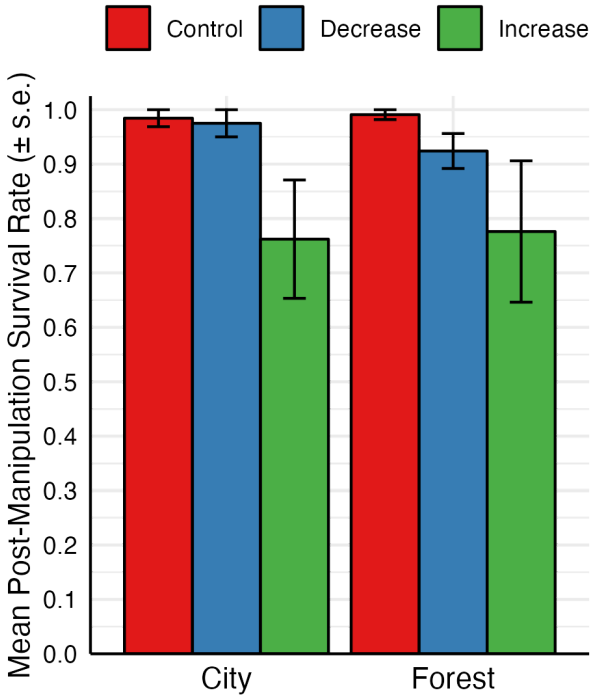

Fig. 2: Post-manipulation survival rate mean and standard error for all nest boxes at the city and forest sites, split into control (red), decrease (blue), and increase (green) manipulation groups (n = 56 nest boxes).

Nestling wing measurements were significantly longer at the forest site than the city (n = 403, t = 2.381, p = 0.0213; Fig. 3a, pg. 27). Wing length did not vary significantly between the “control” and “decrease” nest boxes (t = -0.105, p = 0.9172), nor between the “control” and “increase” nest boxes (t = -0.903, p = 0.3717). Tarsus measurements were significantly longer at the forest site (n = 420, t = 2.201, p = 0.0325; Fig. 3b, pg. 27). Tarsus length did not vary significantly between the “control” and “decrease” nest boxes (t = 0.191, p = 0.8492), but was significantly shorter in the “increase” nest boxes compared to the “control” nest boxes (t = -2.019, p = 0.05). Body mass did not differ significantly between the sites (n = 420, t = -1.140, p = 0.2594; Fig. 3c, pg. 27), or between the “control” and the “decrease” nest boxes (t = 1.636, p = 0.1076), but was significantly lower in the “increase” nest boxes compared to the “control” nest boxes (t = -2.192, p = 0.0333). Finally, BMI scores were significantly lower at the forest site (n = 420, t = -2.259, p = 0.0282; Fig. 3d, pg. 27), and showed marginally significant variation between manipulation groups, being higher in “decrease” nest boxes (t = 1.956, p = 0.0557), and lower in the “increase” nest boxes (t = -1.948, p = 0.0573), both in comparison to the “control” nest boxes.

Parental status had no effect upon any of the four traits (wing: t = 0.543, p = 0.5877; tarsus: t = 1.556, p = 0.1206; mass: t = 1.299, p = 0.1947; BMI: t = 0.895, p = 0.3712). Although nest box effects only contributed to minimal amount of the variation in tarsus length, they contributed to a substantial amount in the other three traits (wing: var = 5.599, residual = 8.172; 40.66%; tarsus: var = 0.0811, residual = 0.4126; 16.43%; mass: var = 0.4397, residual = 0.6062; 42.04%; BMI: var = 0.00116, residual = 0.00129; 47.35%). Thus, some of the differences in these traits may be attributable to differences between nest boxes, with the most obvious difference between nest boxes being brood size. Indeed, post-manipulation brood size correlated negatively with the nest box means for all four chick trait measures, although this was only significant for body mass (n = 53, t = -4.516, p < 0.0001; Fig. 4a, pg. 28) and BMI scores (t = -5.61, p < 0.0001; Fig. 4b, pg. 28). The strength of the negative correlation for both these traits was slightly greater at the city (n = 25; body mass: t = -3.459, p = 0.0021; BMI: t = -3.735, p = 0.0011) than at the forest (n = 28; body mass: t = -2.599, p = 0.0152; BMI: t = -3.213, p = 0.0035).

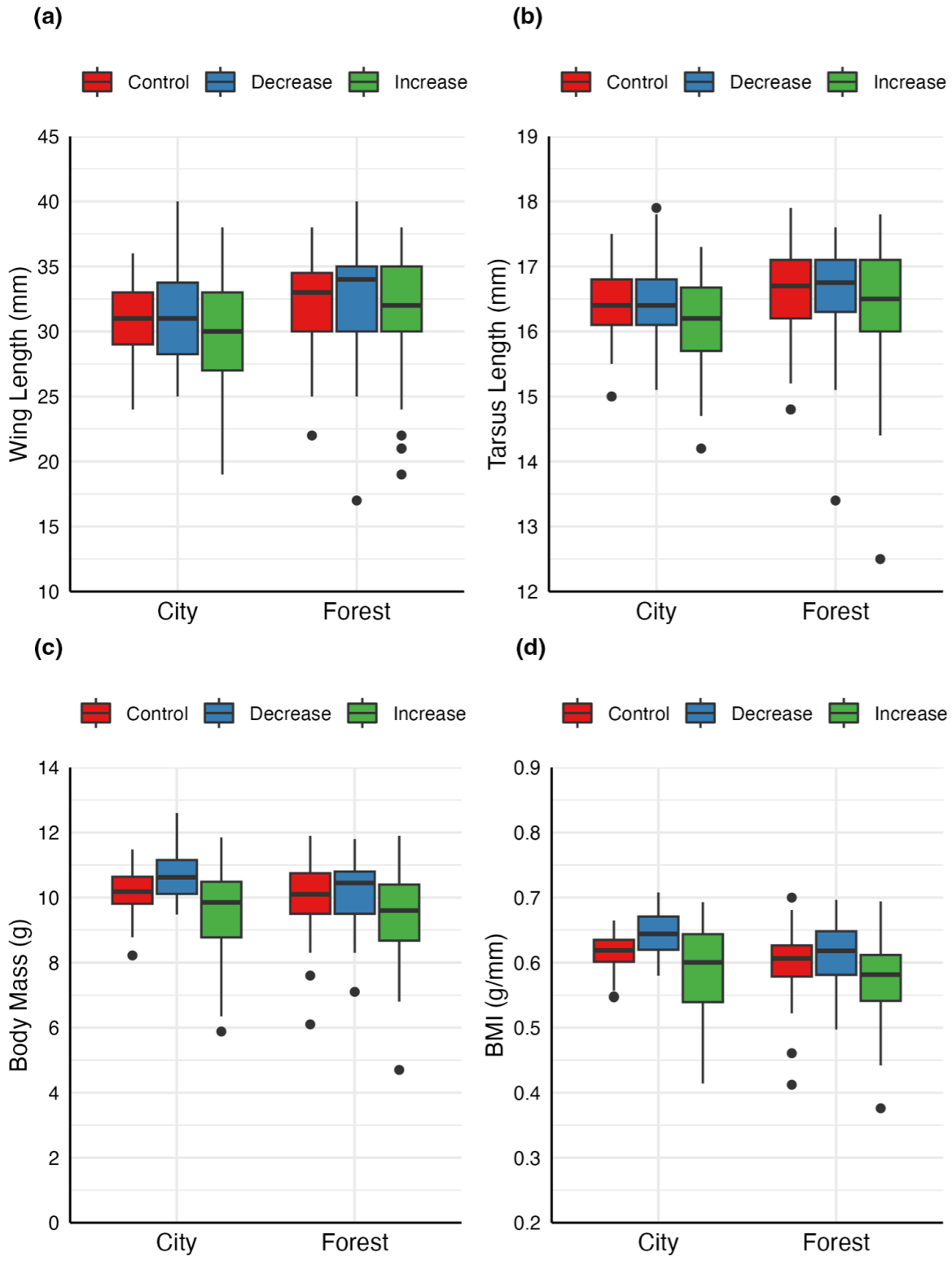

Fig. 3: Post-manipulation nestling traits for all nest boxes at the city and forest sites, split by manipulation group (n = 420 nestlings unless stated otherwise): (**a**) wing length (n = 403 nestlings), (**b**) tarsus length, (**c**) body mass, and (**d**) body mass index (BMI).

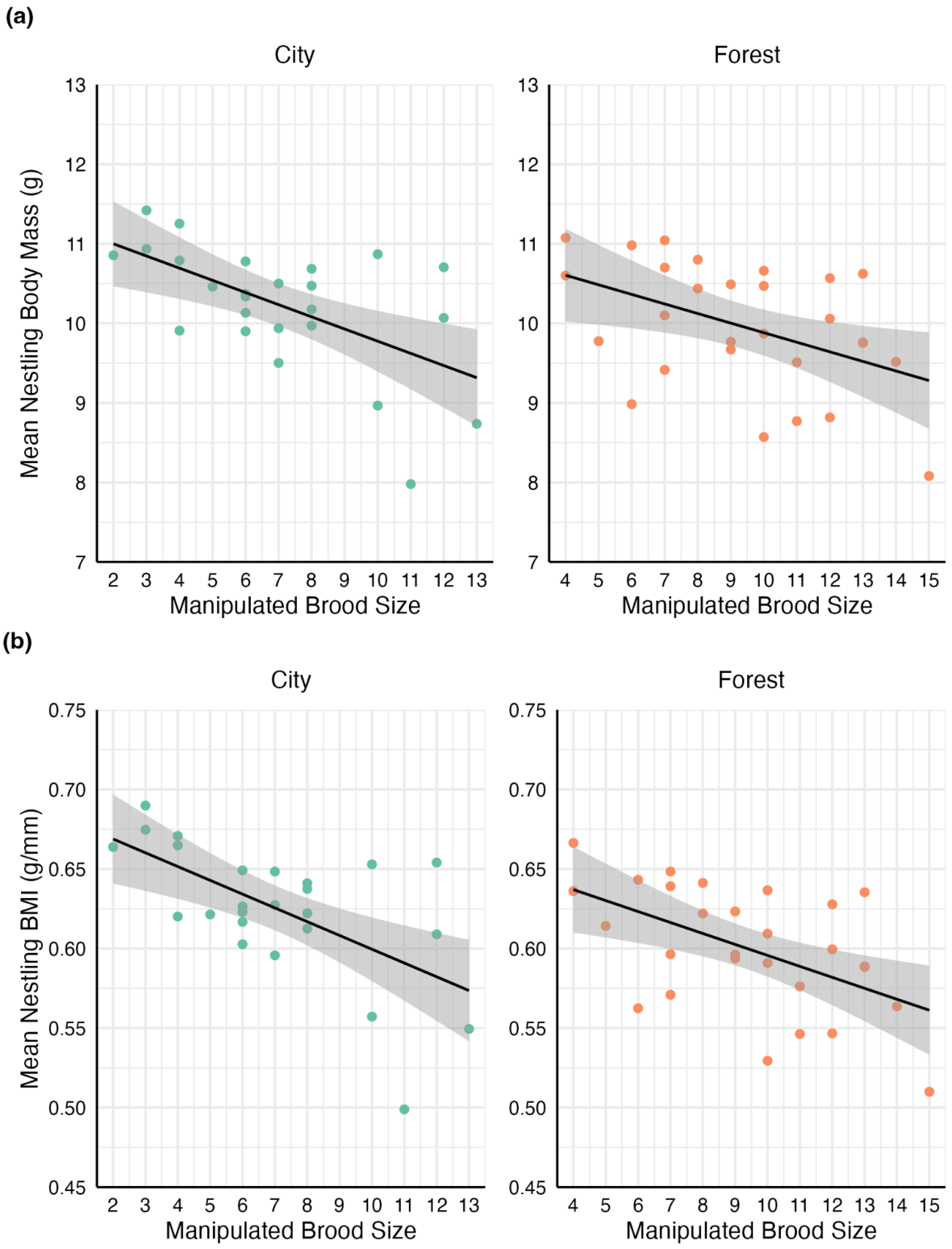

Fig. 4: Relationship at the city (n = 25 nest boxes) the forest (n = 28 nest boxes) between post-manipulation brood size and (**a**) average nestling body mass, or (**b**) average nestling body mass index (BMI), for each nest box. Linear regression lines are fitted to each data set, with the shaded areas indicating the standard error of the fitted values.

Nestling Diet Diversity

Shannon diversity values for the diet samples did not vary significantly with site (n = 146, t = 0.012, p = 0.990; Fig. 5). There was also no variation between control nest boxes and decrease nest boxes (t = 0.771, p = 0.445) or between control nest boxes and increase nest boxes (t = -0.35, p = 0.729). Parental status also had no effect upon the diet sample diversity values (t = 0.257, p = 0.798), and the variance attributed to nest box effects was minimal (var = 0.0404; residual = 0.2823; 12.52%). Mean Shannon diversity values ranged from 1.07 (city-increase) to 1.28 (forest-decrease), demonstrating that for the majority of diet samples a single OTU dominated, with additional OTUs only present in smaller proportions.

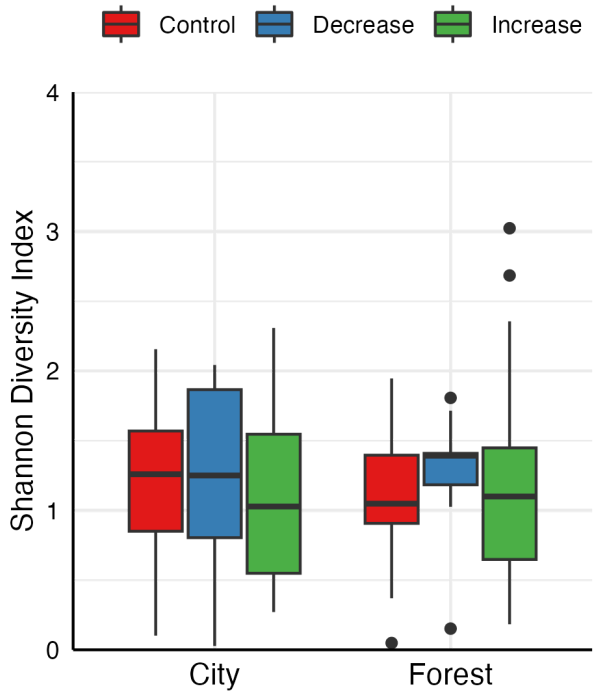

Fig. 5: Shannon diet diversity for all nestlings at the city and forest sites, split by manipulation group (n = 146 nestlings).

When using Bray-Curtis dissimilarity values, the nested PERMANOVA indicated that site (n = 146, f = 13.5559, p < 0.0001; Fig. 6a) and manipulation group (f = 2.2652, p < 0.0001), contributed significantly to the observed dissimilarity in the data. This suggests that diets differed significantly between the city and forest, and that the different manipulation treatments affected diet composition. Further, the interaction between site and manipulation group was significant (f = 2.3386, p < 0.0001), indicating that the effect of manipulation on diet varied between the two sites. Parental status did not have a significant effect (f = 1.0459, p = 0.3528). However, the interaction between site, manipulation group, parental status and nest box was significant (f = 1.1119, p = 0.0024), demonstrating that differences between individual nest boxes likely contributed to some of the observed dissimilarities. Importantly, there was a significant difference in dissimilarity between diets across the two sites (diff = 0.1827, p < 0.0001), with the average distance of the diets from the site's median value being almost 50% greater at the city site than the average at the forest site. This suggests nestling diets in the city show far greater variation than those in the forest.

When using Jaccard dissimilarity values, the nested PERMANOVA showed similar trends. Site contributed significantly to the observed dissimilarity in the data (n = 146, f = 13.5559, p < 0.0001; Fig. 6b), with significantly larger dissimilarity at the city (diff = 0.0279, p = 0.0009). Manipulation group also contributed significantly (f = 2.2652, p < 0.0001), as did the combined effect of site and manipulation group (f = 2.3386, p < 0.0001), while parental status did not (f = 1.0459, p = 0.3480). The interaction between site, manipulation group, parental status and nest box was again significant (f = 1.1119, p = 0.0019).

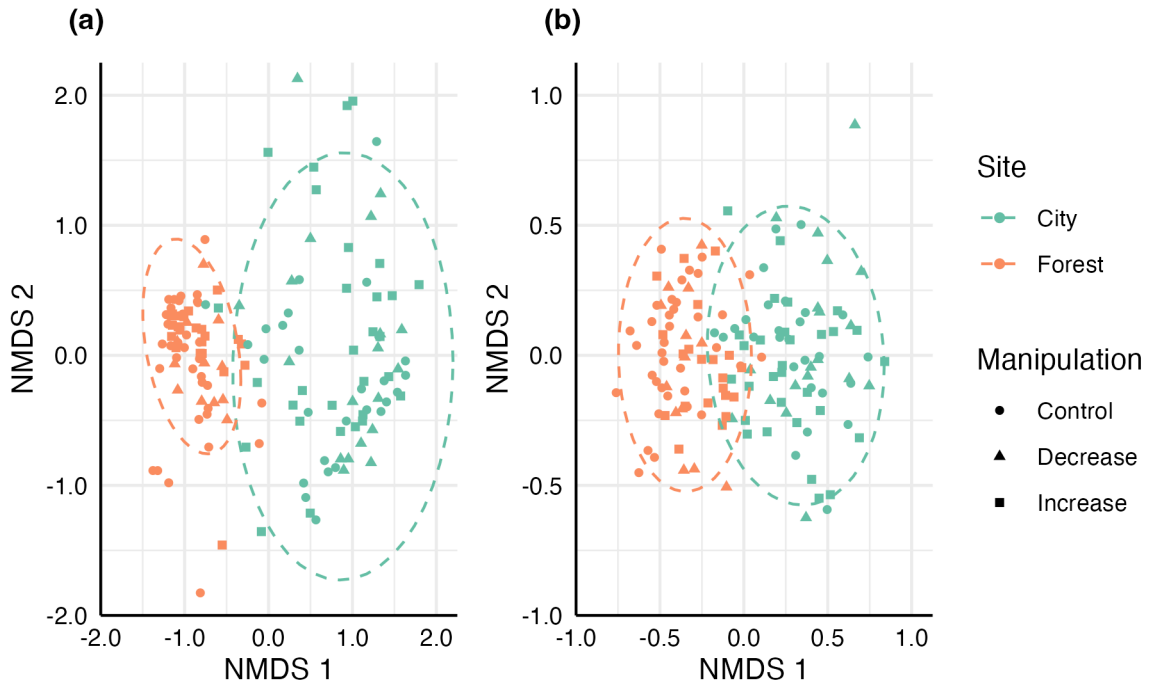

Fig. 6: Non-metric multidimensional scaling (NMDS) ordination plots of diet samples for all nestlings (n = 146), measured with (**a**) Bray-Curtis and (**b**) Jaccard dissimilarity values. Site and manipulation group are denoted by colour and shape respectively. The ellipses represent a 95% confidence interval based on a t-distribution for each site.

Nestling Diet Composition

Diet composition, when assessed with the mean relative read abundance of the major orders in the nestling diet samples, differed significantly between the sites ($n = 146$, $t = 8.512$, $p < 0.0001$; Fig. 7a, pg. 32), but not between the manipulation groups (Decrease: $t = -0.650$, $p = 0.5189$; Increase: $t = -0.074$, $p = 0.9411$), or by parental status ($t = -0.582$, $p = 0.5615$). A moderate amount of variance was attributed to nest box effects (var = 0.256; residual = 0.8116; 23.98%). When diet composition was analysed using the mean proportion of OTUs of major orders instead, the findings were the same, with significant differences between sites ($n = 146$, $t = 9.288$, $p < 0.0001$; Fig. 7b, pg. 32) but not between manipulation groups (Decrease: $t = -0.394$, $p = 0.6953$; Increase: $t = 0.03$, $p = 0.9759$), or by parental status ($t = -1.919$, $p = 0.0573$). The variance attributed to nest box effects was also moderate (var = 0.2861; residual = 0.8559; 25.05%).

Mean Lepidoptera relative read abundance was significantly greater at the forest site ($n = 146$, $t = 8.676$, $p < 0.0001$; Fig. 8a, pg. 33), with Lepidoptera OTUs making up an average of 90.78% of total reads in a sample, compared to just 42.43% at the city site. The variance due to nest box effect was minimal (var = 0.0144; residual = 0.0597; 19.43%). The mean proportion of Lepidoptera OTUs was also significantly greater in the forest ($n = 146$, $t = 8.789$, $p < 0.0001$; Fig. 8b, pg. 33), making up an average of 69.24% of OTUs in a sample compared to 39.36% in the city, and the variation attributable to nest boxes was again minimal (var = 0.0044; residual = 0.0255; 14.72%). Both mean Diptera ($t = -7.571$, $p < 0.0001$) and mean Hemiptera ($t = -3.876$, $p = 0.0003$) relative read abundances were significantly lower at the forest site, making up an average of 2.33% and 1.16% of reads respectively, compared to 28.83% and 20.32% at the city. The mean proportions of OTUs for both of these orders also differed significantly, with Diptera making up an average 25.93% of OTUs in the city compared to 7.89% in the forest ($t = -9.273$, $p < 0.0001$), and Hemiptera on average comprising 20.35% of city and 4.72% of forest OTUs. ($t = -6.322$, $p < 0.0001$). The mean relative read abundance of Coleoptera, Araneae and Hymenoptera OTUs did not vary significantly between the sites, nor did the mean proportions of OTUs for these orders. The results of the statistical tests for each order, as well as the level of variation attributable to nest box effects, are shown in Table 5 (pg. 34).

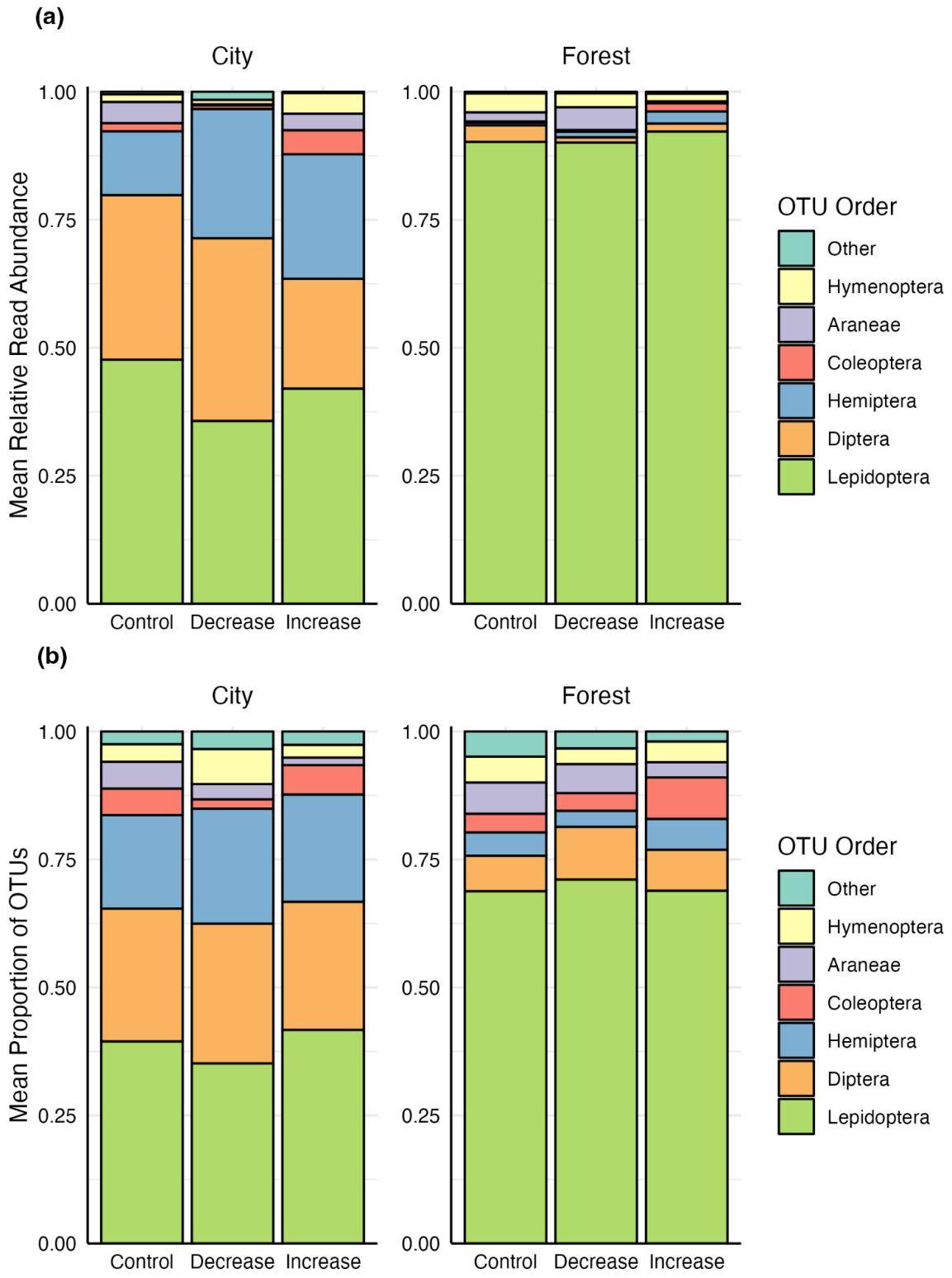

Fig. 7: Average diet composition, defined by (**a**) the mean relative read abundance and (**b**) mean proportion of OTUs of major orders in the nestling diet samples, split by manipulation group, for the city (n = 76) and forest (n = 70).

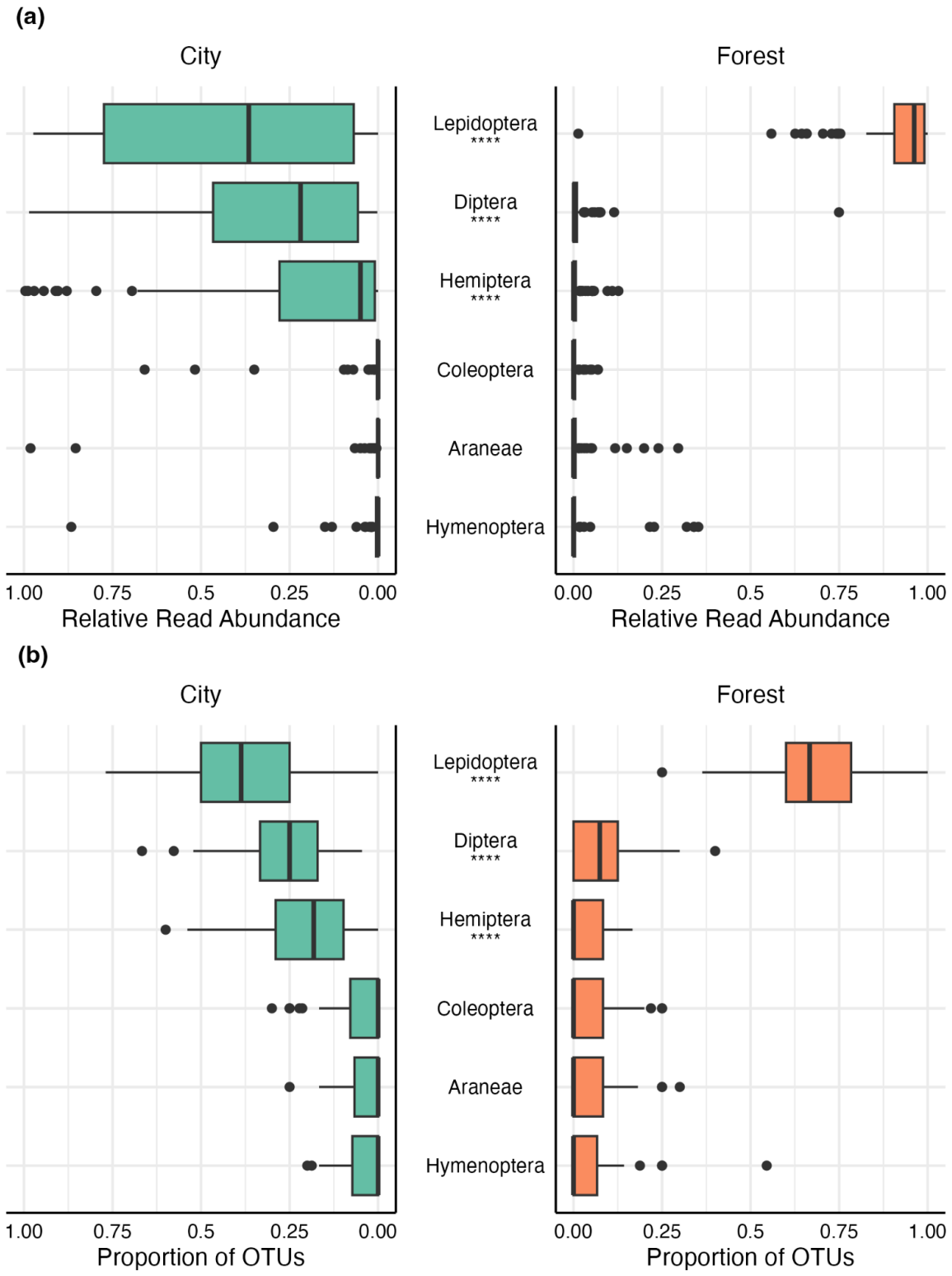

Fig. 8: (**a**) Relative read abundance and (**b**) proportion of OTUs of each major order for all diet samples, regardless of manipulation group, at the city (n = 76) and forest sites (n = 70). The orientation of x-axes is reversed between the two sites. Significance codes: '****' < 0.0001, '***' < 0.001, '**' < 0.01, '*' < 0.05, '.' < 0.1.

Table 5: Mean relative read abundance and mean proportion of OTUs of the six major orders at the city and forest sites, with t and p-values from the LMMs, as well as the variance attributable to both nest box effects and residuals. Significant p-values are underlined.

	Order	City Mean	Forest Mean	t-value	p-value	Nest Box Variation	Residual Variation
Relative Read Abundance	Lepidoptera	0.4243	0.9078	8.676	< 0.0001	0.0144	0.0597
	Diptera	0.2883	0.0233	-7.571	< 0.0001	0.0012	0.0400
	Hemiptera	0.2032	0.0116	-3.876	0.0003	0.0129	0.0349
	Coleoptera	0.0260	0.0067	-1.508	0.1432	0.0002	0.0055
	Araneae	0.0282	0.0188	-0.492	0.6256	0.0003	0.0126
	Hymenoptera	0.0234	0.0286	0.273	0.7891	0.0003	0.0091
Proportion of OTUs	Lepidoptera	0.3926	0.6924	8.789	< 0.0001	0.0044	0.0255
	Diptera	0.2593	0.0789	-9.273	< 0.0001	0.0005	0.0118
	Hemiptera	0.2036	0.0472	-6.322	< 0.0001	0.0040	0.0067
	Coleoptera	0.0456	0.0495	0.447	0.6582	0.0011	0.0038
	Araneae	0.0320	0.0510	1.305	0.1991	0.0008	0.0029
	Hymenoptera	0.0392	0.0438	0.276	0.7840	0.0002	0.0047

At the forest site, nine of the ten most common OTUs in terms of total read counts belonged to the order Lepidoptera, with the other being a Dipteran species only present in 4.29% of the diet samples, as is displayed in Table 6. This contrasts with the city site, where only half of the ten most common OTUs belonged to the order Lepidoptera, and the most common OTU, found in 97.37% of the samples, was a Dipteran species belonging to the Syrphidae family. The remainder of the ten most common OTUs at the city included another Dipteran species, and three Hemiptera species.

Table 6: Taxonomic classification and observation frequency of the 10 most common OTUs, ranked by total number of reads.

Rank	Total Reads	Observation Frequency	Order	Family	Genus	Species	
1	358,404	97.37	Diptera	Syrphidae	Unassigned	Unassigned	City
2	286,822	50.00	Lepidoptera	Noctuidae	Cosmia	*C. trapezina*	
3	155,977	67.11	Hemiptera	Aphididae	Drepanosiphum	Unassigned	
4	133,763	23.68	Lepidoptera	Noctuidae	Amphipyra	*A. pyramidea*	
5	116,567	13.16	Hemiptera	Aphididae	Cavariella	*C. pastinacae*	
6	114,721	14.47	Hemiptera	Miridae	Harpocera	*H. thoracica*	
7	80,548	22.37	Lepidoptera	Tortricidae	Ptycholoma	*P. lecheana*	
8	79,418	3.95	Diptera	Tipulidae	Tipula	*T. oleracea*	
9	78,796	31.58	Lepidoptera	Geometridae	Operophtera	Unassigned	
10	73,437	46.05	Lepidoptera	Geometridae	Agriopis	*A. aurantiaria*	
1	3,933,653	98.57	Lepidoptera	Geometridae	Agriopis	*A. aurantiaria*	Forest
2	505,980	85.71	Lepidoptera	Geometridae	Erannis	*E. defoliaria*	
3	449,361	45.71	Lepidoptera	Tortricidae	Tortrix	*T. viridana*	
4	358,870	41.43	Lepidoptera	Geometridae	Epirrita	*E. dilutata*	
5	313,682	67.14	Lepidoptera	Noctuidae	Cosmia	*C. trapezina*	
6	247,540	51.43	Lepidoptera	Elachistidae	Phigalia	*P. pilosaria*	
7	201,369	24.29	Lepidoptera	Noctuidae	Amphipyra	*A. berbera*	
8	195,054	34.29	Lepidoptera	Unassigned	Unassigned	Unassigned	
9	188,398	4.29	Diptera	Tipulidae	Tipula	*T. flavolineata*	
10	180,729	50.00	Lepidoptera	Tortricidae	Pandemis	*P. cerasana*	

2.4 Discussion

Reproductive Outcomes

In summary, the pre-manipulation reproductive outcome findings show that blue tit clutches were larger in the forest population than in the city population, although the proportion of eggs that hatched at each site was very similar. Prior to the brood size manipulation, the hatchlings were of similar weights and survived at comparable rates across both sites, albeit with a slight advantage in favour of the city chicks, some of which may be attributable to nest box differences. The post-manipulation reproductive outcome results, measured at the end of the manipulation experiment, showed nestling survival to be lower in the artificially enlarged nest boxes and unaffected by parental status, although differences between nest boxes likely contributed to the variation in nestling fate. The chicks from the enlarged nest boxes were smaller than the chicks from the control nest boxes for most measures. City chicks had shorter tarsi and wings than forest chicks, yet they were of comparable weights, resulting in higher BMI scores. As with nestling fate, parental status did not have an impact upon any nestling traits, while some variance may be attributable to nest box effects. Across all manipulation groups, both body mass and BMI scores were lower for chicks in larger broods. This trend was more prominent in the city blue tit population.

These findings support the hypothesis that pre-manipulation reproductive success would be greatest at the forest site. As predicted, forest clutches are larger than city clutches, and this difference in clutch size comfortably outweighs, in terms of the net number of living chicks, the slightly higher hatchling survival rate observed in the city. Furthermore, the larger forest clutches may well explain the similarities between the two sites in hatching rate and hatchling body mass. There are several potential reasons for the smaller clutches observed in the urban blue tit population. Firstly, clutches may be physiologically constrained by the diet and health of adult blue tits. Egg production has very high energy and nutrient requirements (Perrins, 1996). This is because the yolk must be rich in lipids and proteins for the embryo to develop, and variation in yolk mass can in fact affect offspring performance (Williams, 1994). Offspring performance can also be improved by the increased presence of carotenoids and vitamin E in the yolk (McGraw et al., 2005). While energy supply is not thought to usually limit egg production (Plummer et al., 2013), these antioxidants are maternally derived, and so the availability of antioxidants in the mother's diet could limit egg production, especially as there are physiological trade-offs in their usage (Blount et al., 2000). The food available to adult blue tits varies between urban and forest habitats. Reduced arthropod communities in towns and cities can limit access to crucial nutrients (Patten, 2007). Additionally, for bird species that consume supplementary food in the UK, including the blue tit, there is approximately one bird feeder for every nine individuals, with these typically found in urban areas, in parks and gardens (Davies et al., 2009). While some foods found in feeders, like seeds and nuts, contain antioxidants, many contain large quantities of fat, the consumption of which not only fails to provide a long term benefit as blue tits cannot store significant quantities of macronutrients (Drent & Daan, 1980), but can also lead to smaller relative yolk mass in larger eggs and diminished yolk carotenoid concentrations among early breeders in the subsequent breeding season (Plummer et al., 2013). Furthermore, food supplementation can enable lower quality individuals to recruit into breeding populations. Easily accessible food reduces competition for resources that would otherwise be scarce, allowing these individuals to achieve reproductive success when they

typically may not. However, this can have unintended consequences, including potential reductions in overall breeding success, as evidenced by smaller clutches (Plummer et al., 2013). In summary, urban clutches might be physiologically constrained by the fitness of adult blue tits, as the poorer but more accessible nutrient supply in towns and cities may impair the condition of breeding adults, limiting their ability to produce clutches of comparable size to those in forest environments.

In addition to potentially being physiologically constrained by the diet and health of adult blue tits, urban clutches could also be smaller due to phenotypic plasticity. The lower availability of caterpillars in urban environments might lead blue tits to lay fewer eggs, as adults may adjust offspring numbers plastically in response to prey availability and quality at the provisioning stage (Nur, 1986). This would help mitigate the negative effects of food limitations upon nestling development and survival (Sinkovics et al., 2021). Deliberately laying a size of clutch to suit the capacity of the habitat, as well as the health and fitness of the parents, is referred to as the individual optimization hypothesis, or IOH (Perrins & Moss, 1975). Phenotypic flexibility in regulating clutch size would also explain why the sites show similarities in the other pre-manipulation reproductive success measures, including hatching rate and hatchling survival. A successful strategic reduction in clutch size would allow the urban population to overcome the shortcomings of their environment, and achieve egg hatch and hatchling survival rates, as well as hatchling body masses, that are comparable to, or even slightly better than, those of the forest population, as this study has demonstrated. A possible explanation beyond phenotypic plasticity and physiological constraints is adaptive evolution (Nur, 1986), which could also account for the observed differences in clutch size, as well as the similarities in other reproductive measures. Over time, natural selection may have favoured smaller clutches in urban environments, if smaller broods enhanced overall reproductive success, which is plausible given the context of limited resources. This potential evolutionary adaptation would have enabled urban blue tits to optimise their reproductive strategies for the specific challenges of city habitats, allowing them to achieve egg hatch and hatchling survival rates comparable to those in forest environments.

However, adaptive evolution is perhaps a less plausible explanation than phenotypic plasticity and physiological constraints, as the relatively recent establishment of urban blue tit populations is unlikely to have allowed sufficient time for such evolutionary changes to take hold. Furthermore, a recent study by Pitt et al. (2024) indicated that blue tit egg production is almost certainly constrained in urban environments. The study investigated the response of forest and urban blue tits to the removal of the first four eggs laid and found that forest blue tits laid significantly more replacement eggs than their urban counterparts. This suggests that either urban adults are unable to lay sufficient replacement eggs to return to the original, optimal clutch size, or their original clutch size was not strategically optimised but rather constrained by environment specific factors. In either case, egg production appears to be restricted in urban environments, likely due to the impact of environmental factors on the health of the adults. In addition to worse quality food sources, the abundance of MTEs in urban areas (Chatelain et al., 2021) likely affects the fitness of adult blue tits, potentially impairing their ability to produce eggs. Indeed, high levels of MTE exposure have been linked to reduced clutch sizes (Eeva et al., 2009). Ultimately, the findings of the current study demonstrate that both populations of blue tits avoid unnecessary energy expenditure by not laying a significant number of eggs that would not hatch, or would hatch chicks that cannot be adequately provisioned. This is almost certainly influenced by physiological

constraints imposed by the environment, especially in the city, though phenotypic plasticity may also contribute.

The findings also support the hypothesis that post-manipulation reproductive success would be greater in the artificially reduced broods than the artificially enlarged broods. Although this trend was not more pronounced at the city site, the negative correlation between chick size and brood size was stronger in the city than the forest. It is unsurprising that enlarged broods saw lower rates of post-manipulation nestling survival. This measure is equivalent to fledge rate, which has been found to be reduced in enlarged blue tit broods in multiple similar manipulation experiments (e.g. Pettifor, 1993). This has typically been attributed to the IOH, where female blue tits lay an optimal number of eggs to maximise the recruitment of their offspring into the breeding population. As the original clutch size reflects the ability of the parents, and the capacity of their habitat, to raise chicks (Perrins & Moss, 1975), the differences in clutch size between the two sites in the current study supports this hypothesis. Thus, any increase to the size of a brood likely exceeds the parent's capacity to care for all the chicks in their nest, resulting in the higher mortality rate as resources become stretched and the ability to provide sufficient care diminishes. This is unsurprising, as birds rarely sacrifice their own survival or future fecundity when rearing their young, and so usually it is the chicks that suffer in enlarged broods (Moreno et al., 1995). Furthermore, an artificial decrease in brood size is unlikely lead to higher chick survival rates, as regular broods already exhibit a relatively high survival rate (over 98% in this study), and any observed mortality in both regular and reduced broods could be attributed to external factors, such as predation, rather than solely to the capacity of parents to raise young.

The lack of any effect of parental status on chick survival or traits such as wing length, tarsus length, and body mass suggests that adult blue tits raise all nestlings indiscriminately, whether cross fostered or their own. This aligns with previous findings, such as Pettifor (1993), and demonstrates that the lower survival rates and smaller nestlings observed in artificially enlarged broods are not due to parents distinguishing between biological and fostered young. Instead, the differences in chick fitness between manipulation groups appear to be driven by brood size. The absence of an effect of parental status supports the idea that chicks in larger broods are less fit because of the greater demands of rearing more offspring, rather than selective provisioning by the parents.

Additionally, previous studies have also shown enlarged blue tit broods produce less healthy and worse quality fledglings, evident through lower mass and shorter tarsus (e.g. Blondel et al., 1998). This is also thought to be due to the size of the enlarged broods exceeding the capacity of the parents and their habitat. Even if parents of enlarged broods are able to allocate more time to foraging and provide their young with a sufficient volume of food, this would reduce the time available for brooding. Brooding is vital to the development of blue tit chicks, as immediately after hatching they are poikilothermic, so their body temperature fluctuates with the environment as they have limited capacity to maintain core body temperature (Baarendse et al., 2007). Brooding helps chicks maintain body temperature until they endothermy, and start to produce their own heat, at which point they become functionally homeothermic (Sasvari, 1986). As parental brooding is the primary thermoregulatory mechanism for chicks of altricial bird species like the blue tit, reduced brooding time could hinder nestling development (Choi & Bakken, 1990).

The fact the brood manipulations appear to have had an almost identical effect at both sites can be attributed to the methodology used in the procedure. Simply adding or removing three chicks from each experimental nest, rather than artificially creating broods of certain sizes, meant that, following manipulations, forest broods were larger than their city equivalents. This is due to the forest clutches already averaging more than two additional eggs each. Thus, despite the apparent quality of the habitat, the parents of enlarged forest broods were just as restricted as those in the city when rearing additional chicks, hence the observation of similar patterns in nestling quality and survival. Furthermore, the experimental design may have contributed to nest box effects seemingly accounting for substantial variation in multiple traits. Since all nestlings within each box were obviously subject to the same manipulation, this shared treatment may have inflated the perceived influence of nest box effects upon trait differences, as much of the variation may instead stem from the impact of brood size changes. When brood size, rather than manipulation group, is considered there is a difference between the sites, as brood size has a greater negative effect upon chick size in the city than in the forest. This indicates it is easier to sufficiently feed additional chicks in the forest habitat, suggesting either the required food sources are more abundant, or forest blue tit adults are more able to adequately provision larger broods.

The observation that city chicks are smaller than their forest counterparts but have similar body masses, and as a result higher BMI scores, suggests chick growth and development patterns may differ between the two sites. There are multiple potential explanations for this. Firstly, differences in chick diets between the two sites, as a result of different food sources, including the presence of anthropogenic foods in the city, may influence chick growth. Secondly, urban females may have to spend more time foraging due to the scarcity of certain foods, reducing the time available for brooding their chicks, which may lead to them being less warm, potentially hindering their development. Lastly, distinct selection pressures in the two habitats, combined with potentially reduced genetic connectivity between the separate populations, may contribute to these anatomical differences. It is worth noting that body mass is not the best measure of health and condition, as it can fluctuate hourly depending upon the time surpassed since feeding, the weather, and the level of activity (Freeman et al., 1990). This, in turn, affects the accuracy of BMI calculations, which are more reliable when based on the average of several mass measurements. However, this approach is impractical here, given the rapid weight gain of nestlings. While tarsus alone is also not a perfect measure of condition, male birds with greater tarsus lengths attract more females (Kempenaers et al. 1992), suggesting it is a desirable and relevant trait. Thus, it can be inferred, based on the measurements taken in this study, that the forest chicks are unlikely to be less healthy than their city counterparts, despite their worse BMI scores.

Nestling Diets

To summarise, the nestling diet diversity analyses indicated that although the Shannon diversity values did not vary between site or manipulation group, there was significant separation in diets between these categories. Most notably, chick diets in the city showed greater variation than those in the forest. The nestling diet composition analyses showed the makeup of chick diets, in terms of order as opposed to individual species, varied between the two sites but not by manipulation group, with lepidoptera taxa found more frequently in forest diet samples, while Diptera and Hemiptera taxa were more common in the city. Parental status had no impact upon nestling diet diversity or composition, which is further

evidence that blue tits raise biological and fostered nestlings indiscriminately. Differences between nest boxes appeared to contribute to the observed dissimilarities in overall diets, as well as the variation in the proportions of some OTU groups, although this contribution may have been inflated by the effects of brood size, which were caused by the experimental manipulations.

While the Shannon diversity index findings do not directly align with the hypothesis that the diet of city blue tit chicks will be more diverse than that of forest chicks, this is likely because each diet sample is representative of a single feeding event. With each diet sample only consisting of up to 80 mg of wet weight, it is likely that it is primarily composed of the most recently consumed food item, along with any remnants left in the gut from previous ingestions. This is supported by the mean Shannon diversity values for each site and manipulation combination being between 1.0 and 1.3, indicating that, in most samples, one OTU was highly dominant, with some additional OTUs present only in smaller proportions. Moreover, the Shannon diversity index considers only species richness and evenness, without assessing the relationships between those species. It cannot distinguish between a diet sample with DNA from several species belonging to the same family and a sample with DNA from several species across different orders. Therefore, the Shannon diversity index results do not disprove the hypothesis that the diet of city blue tit chicks will be more diverse; rather, they indicate that the diversity levels in the diet samples themselves at the two sites are similar, at least in terms of the number of individual OTUs. Furthermore, the greater dissimilarity observed between city diet samples suggests a higher degree of variation between the diets of city chicks compared to between forest chick diets. This would mirror findings in other papers where urban bird populations have exhibited a wider diversity in their diets (Branston et al., 2021). This is despite the fact that, in this study, the individual diet samples, characterised by a predominant presence of DNA from a single species, exhibit similar levels of limited diversity across both sites. Yet even solely considering the fraction of the blue tit chicks' diets represented by the diet samples used in this study, it is clear diets vary with site, as evidenced by the separations between the sites, and this is backed up by the diet composition findings.

The separation between the manipulation groups could also be explained by foraging preferences and patterns differing with nest size. Although previous brood manipulation studies have shown enlarged broods are visited more frequently by adults, this increase is not proportional, and so these broods receive a smaller number of visits per chick (Gibb, 1950). Not only does this mean chicks in enlarged broods likely receive a smaller volume of food, but also that the types of food they receive may vary. Parents provisioning at faster rates than they would under normal conditions may bring food items that are typically ignored because of their poor nutritional content because they are unable to spend as much time provisioning each individual food item, so cannot travel as far to find specific foods (Arnold et al., 2010). This could explain the observed separation in chick diets between the manipulation groups at both sites. Intriguingly, this separation was not substantiated by the diet composition findings. When analysed with OTUs grouped by order, no change in diet composition was observed among the manipulation groups at each site. This may be attributed to foraging behaviour being largely influenced by the availability of insects. In urban areas, the scarcity of caterpillars necessitates that blue tits provision their broods, whether enlarged or reduced, with whatever prey is available. Conversely, in forested

environments, the abundance of caterpillars means there is little need for them to alter their foraging preferences, as caterpillars are readily available.

Alternatively, blue tits may well adjust their foraging, but with these adjustments only occurring at the species level, and not eliciting substantial changes in the proportions of the major arthropod orders consumed. This would mean that, across each site, adults provisioned their young with a broadly similar distribution of the various arthropod orders, but that the species within these orders varied depending upon the adjustment to the size of their brood. This theory is plausible, as parents of reduced broods would have more time to cater to each chick and may use this time to seek better quality food items. It has been found that food-supplemented parents use the time they save by not having to forage for their own sustenance to increase their degree of food selectivity for their young, feeding them less often but with larger larvae (Grieco, 2003), which contain a proportionately greater quantity of nutrients (Lease & Wolf, 2011). Thus, it's conceivable that blue tit adults with fewer chicks than they have the capacity to raise, and consequently with more available time, might focus on finding superior food items for their young in order to benefit their health. In contrast, parents tending to enlarged broods, facing tighter constraints on their time, may be compelled to select the first available food items for their young, and in doing so may settle for smaller caterpillars. Importantly, a study into house sparrows found the delivery frequency of larger arthropod prey items was a better indicator of chick health than the feeding rate (Schwagmeyer & Mock, 2008). This nuanced selective behaviour would likely manifest in the specific species provisioned, such as preferring larger caterpillar species over smaller ones when foraging time allows, or settling for smaller caterpillar species when time is constrained, rather than reflecting in a shift in the proportions of the arthropod orders. Ultimately, while the observed variation in nestling diets between the manipulation groups is likely caused by parents foraging a range of quality of arthropods, the total volume of food provided to chicks could also be a strong factor in influencing the differences in chick health between the groups.

Meanwhile, the diet composition results thoroughly support the hypothesis that the diet of city blue tit chicks will contain fewer caterpillars than that of forest chicks. This was entirely to be expected as several studies have found caterpillar availability to be lower in urban areas, for a variety of reasons including the lack of green spaces (Shaw et al., 2008), fragmentation of potential insect habitats (Robinson, 2005), the replacement of native plants with non-native species (Narango et al., 2018), and the high levels of environmental pollution, particularly traffic emissions (Summers-Smith, 2007). Additionally, urban caterpillars, as with most arthropods, are generally smaller than those in forest habitats (Merckx et al., 2018). Spiders are a potential alternative to caterpillars, as they also provide carotenoids and essential amino acids, although at significantly lower levels (Arnold et al., 2010), and thus are known to be of good nutritional value to blue tit nestlings (Ramsay & Houston, 2003). However, the proportion of spiders in the blue tit chick diets was similar at both sites, despite the scarcity of caterpillars in the city, suggesting that blue tit adults do not provision more spiders as a substitute for caterpillars for their chicks, regardless of the fact they share some nutritional benefits. This observation could instead imply accessibility to spiders in urban areas is also limited. However, spiders have actually been consistently observed to make up only a small component of the diet of blue tit nestlings across a range of habitat types (Naef-Daenzer et al., 2000; Arnold et al., 2007). Additionally, the proportion of spiders in the diet of blue tit chicks has been shown to decrease as chicks grow older (García-Navas et al.,

2012). These findings suggest that the modest number of spiders observed in the urban nestling diets in this study may be a result of selective feeding, rather than being limited by availability. This could be because they provide additional nutrients that are absent in caterpillars and essential for early nestling development. For example, spiders are known to contain high levels of taurine, which is thought to be important at a critical stage of chick development (Ramsay & Houston, 2003). Alternatively, the lack of spiders in blue tit chick diets across multiple habitats could instead be because when caterpillars are abundant, there's less need to provision a large quantity of spiders, and when caterpillars are scarce, spiders typically are too. This is plausible, as although most spiders do not directly consume plant material, the prey species they target often do. Consequently, the availability of prey, and therefore the abundance of spider populations, could be influenced by the same factors that affect caterpillar populations. Either way, relatively infrequent prey items, like spiders in this study, are important as they can be sources of rare but limiting nutrients (Arnold et al., 2007), and even thus even in small quantities enhance offspring development (Catoni et al., 2008).

Ultimately, in this study, the urban blue tits appear to compensate for the lack of caterpillars in their habitat with alternative insects from the Diptera and Hemiptera orders, rather than arachnids. This is consistent with observations from previous studies that urban blue tits rely on other arthropods from these orders, as well as anthropogenic food, to provision their young (e.g. Jarrett et al., 2020). DNA belonging to hoverflies and crane flies were the most frequently identified Diptera OTUs in the city here, while the most common Hemiptera OTUs belonged to aphids. Notably, the larvae of hoverflies, which belong to the Syrphidae family, typically feed almost exclusively on aphids, and so the abundance of these two species is often intertwined (Chadwick & Goode, 1999; Jarrett et al., 2020). Many of these species are thought to be limited in the nutrition that they supply (Mackenzie et al., 2014; Jarrett et al., 2020), so it is likely because of abundance and accessibility that they are commonly provisioned to blue tit chicks in the city, rather than their quality. Additionally, many fly and true bug species, especially those prevalent in the diets of city blue tit chicks, are very small and so would be easily provisioned to chicks without the risk of choking, making them a better alternative to caterpillars than common anthropogenic foods like seeds and nuts.

The potentially suboptimal nutritional value of the Diptera and Hemiptera species provisioned to the urban blue tit chicks might account for the observed discrepancies in their growth compared to forest chicks, as city nestlings may be experiencing a nutrient deficiency rather than a caloric restriction. Urban chicks exhibited shorter tarsus and wing lengths, which could be attributed to the inadequate provision of essential nutrients, such as calcium and protein, which are required for bone growth, and are fundamental growth limiting factors for nestlings (Smith et al.1983). Additionally, the prevalence of aphids and flies in the urban environment could contribute to the higher weights observed in urban chicks. This is because their great abundance allows for rapid provisioning, potentially outpacing that of caterpillars in the forest. Such a steady supply of calories and energy could lead to increased weight gain, despite these food items containing less nutrients. A weight gain of this nature would not necessarily be of benefit to the nestlings, especially in the long term, as small passerines like the blue tit cannot store fat to any great extent (Drent & Daan, 1980). Despite the differences in physical development, the impact of the worse quality urban diet on the survival of blue tit chicks seems to have been minimal, with their survival rates aligning closely with those observed in forest environments.

CHAPTER III. Nest Box Diet Variation Study

3.1 Introduction

Understanding nestling diets in urban and forest habitats is crucial when assessing the survival and adaptation of blue tits in towns and cities, yet very little is known about how diets vary within these specific habitat types. While it is known that chicks in urban habitats generally receive significantly fewer caterpillars than their forest habitat counterparts, and that reproductive success is reduced as a result (Pollock et al., 2017), there is minimal understanding of how diets vary across within these sites. Similarly, little is known about whether and how diets differ between chicks in the same nests. Comparing nestling diets within a single habitat could provide insights into several factors beyond habitat type that influence the foods chicks are fed, yet this topic is only under-researched, and not only for blue tits; there is a wide knowledge gap in the understanding of nestling dietary variation within urban sites across all bird species. This part of the study focuses solely on diet analysis, performed with faecal DNA metabarcoding, to determine the extent of between and within nest variation in blue tit nestling diets at two sites. The first is an urban park in Glasgow, and the second an undisturbed oak forest in the southwest of Scotland.

Comparing diets between nests at the same site could give an indication of the effects of the specific location of the nest. Across a single habitat, microenvironmental conditions, including vegetation density and habitat structure, are likely to vary significantly, and may influence both insect abundance and adult blue tits' foraging behaviour, and subsequently impact chick diet (Robinson, 2005; Bańbura & Bańbura, 2012). Indeed, a recent study which examined the effect of impervious surfaces upon blue tit reproductive success found that the percentage of these surfaces not only varied around individual nest boxes but also influenced fledging success (Corsini et al., 2021). Surfaces such as concrete and tarmac were hypothesized to reduce habitats for invertebrates, including caterpillars. Such findings illustrate how conditions can vary across as single habitat and have a material impact. This is especially pertinent for blue tits, who rarely travel further than 50 metres from their nest each time they forage (Jarrett et al., 2020), and so are heavily affected by the food sources available in their nest’s microhabitat. In urban habitats, variations in the level of anthropogenic influence would also be expected. This could impact chick diets in a variety of ways, for example the presence of supplementary food sources from bird feeders may contribute to diet diversity (Bańbura & Bańbura, 2012). Alternatively, lower levels of pollution may promote greater abundances of arthropod communities, potentially facilitating sufficiently nutritious nestling diets. This is important in the context of increasing urbanisation, as it would suggest blue tits are able to adapt to urban environments and raise healthy chicks.

Differences in diets between nests at the same urban site may also indicate parental foraging ability has an impact upon chick diet. Foraging ability could be inhibited by several factors, from injury and death, to having to dedicate more time to other parental duties. For example, it has been shown that female blue tits whose nests had high parasite densities spent less time foraging, with this attributed to them spending additional time on nest sanitisation, and as a result delivered smaller prey items to their nestlings (Hurtrez-Boussès et al., 1998). The death, injury or illness of a parent may also result in a clutch receiving

fewer or different food items. There are several threats to the mortality of adult blue tits. Raptors, including the sparrowhawk, as well as domestic cats, predate both blue tit adults and blue tit nests, and when near roads, the risk of a collision with a car is not insignificant (Seress et al., 2012). Additionally, there are some factors that may enhance parental foraging ability. Although increasing foraging distance is unlikely to yield significant benefits, as it has been demonstrated that urban blue tits who travel further whilst foraging do not find more caterpillars (Jarrett et al., 2020), it is possible that parents with certain attributes could enhance their chicks' diets through capabilities beyond merely extending their foraging range. For instance, better eyesight or a more refined knowledge of the optimal foraging sites within the habitat could enable blue tits to gather more caterpillars. This would suggest parents with particular traits are in fact able to compensate for the limited caterpillar availability in the urban habitat, and add further weight to the argument that blue tits are able to raise healthy chicks in urban environments.

Comparing diets between chicks in the same nest may provide some insight into the consistency of food sources in their local habitat, and perhaps help establish whether parents show any bias when provisioning their chicks. Greater inconsistencies would be expected at the city site as the limited abundance of caterpillars forces adults to provision their offspring with alternative food items (Jarrett et al., 2020). Diet variation at this scale may be primarily caused by adults providing their chicks with whatever food is available at the time of foraging. However, a significant variation in the number of caterpillars consumed between chicks within a single nest could be an indication of biassed food distribution. Hierarchical dynamics within the nest, driven by competitive interactions between siblings, could result in preferential treatment from parents, both in terms of frequency and quality of feeding (Fresneau et al., 2018). This potential feeding strategy may serve as an adaptive response to environmental pressures, where parents optimise their reproductive success by concentrating resources on the offspring with the highest survival prospects. This could be particularly pronounced in habitats where food is scarce or of poor quality, prompting parents to make difficult choices that favour the health and development of the strongest chicks, in order to ensure that at least some of their offspring are able to fledge (Caro et al., 2016). In theory, preferential feeding would lead to fewer chicks fledging, but those that do would exhibit significantly better health, and this would be expected to affect local population dynamics. Therefore, it is important to understand whether blue tits treat their chicks differently when resources are limited, especially given that urbanisation is likely to further restrict access to crucial food sources.

In essence, this section of the study aims to assess the extent to which blue tit chick diets vary across individual sites. Variations in nestling diet, both within and between nest boxes, are analysed with a view to addressing the knowledge gap surrounding the roles of local habitat, parental foraging ability, and parental priorities in determining the quality of chick diet. Importantly, nestling diet variation is examined separately at a city and a forest site, in order to evaluate the effect of urbanisation upon any such variations. Specifically, the following were tested:

1. The diets of city blue tit chicks are expected to vary more between nest boxes than those of forest chicks, primarily due to the scarcity of caterpillars in urban areas, which forces parents to rely on alternative food sources. Additionally, the greater variability of urban habitats, with differences in factors like vegetation cover and proximity to human activity, creates a more heterogeneous environment, where potential variations in insect

species at the microhabitat level may result in nest boxes having access to different food sources. In contrast, forest environments are more uniform and provide a favourable habitat for caterpillars, likely resulting in less between-nest variation in chick diets.

2. The diets of blue tit chicks may vary between chicks within the same nest boxes, potential parental bias or simply because parents feed their young based on the availability of food at the time of foraging. Any variation is expected to be more pronounced in city nest boxes, where the limited supply of caterpillars could compel parents to distribute food unevenly. Additionally, any random dietary differences between chicks may be amplified by the greater variety of food items in urban areas. In contrast, forest blue tits likely have more consistent access to caterpillars, reducing variability in diet between nest-mates.

3.2 Materials & Methods

Data Collection

See Chapter II Materials and Methods.

Laboratory Work

See Chapter II Materials and Methods.

Bioinformatics

See Chapter II Materials and Methods.

Statistical Approach

Data Overview

The 146 successfully sequenced diet samples were from a total of 45 nest boxes, although 8 of these only had one sample, which was deemed insufficient for nest box comparison studies. This left 20 city and 17 forest nest boxes, with the full breakdown shown in Table 7. While the effect of brood manipulations was not the focus of this part of the study, all nest boxes with sufficient samples were used in this part of the study, regardless of treatment group. Manipulated nest boxes were included to increase the sample size and provide a more comprehensive portrayal of dietary composition at each site. As a result, the analyses in this section consider and assess any effects of the brood manipulations. However, given the findings in the Chapter II demonstrated minimal dietary variation between treatment groups, the effects are not anticipated to be a particularly relevant factor here. As in Chapter II, statistical analyses were conducted in R 4.3.1 (R Core Team, 2023) using the RStudio interface (Posit team, 2023).

Table 7: Summary of the successfully sequenced blue tit chick diet samples, and the nest boxes with at least two of these samples, from each site and manipulation group.

	Chick Diet DNA Samples			
	Control	Decrease	Increase	Total
City	27	19	30	76
Forest	36	13	21	70
Total	63	32	51	146

	Nest Boxes with >2 Samples			
	Control	Decrease	Increase	Total
City	7	6	7	20
Forest	8	4	5	17
Total	15	10	12	37

Between Nest Box Diet Variation

Overall diet composition was calculated for each nest box with at least two successfully sequenced diet samples. This was done using two methods. The first saw the read counts for every OTU aggregated for each nest box. These were then divided by the total read count for each nest box, giving a proportional value for every OTU. For the second method, each OTU was given a binary occurrence value of zero or one depending on whether it was present in any sample from the nest box. Using these overall diet composition datasets, two distance matrices were constructed. Dissimilarity values were calculated first by applying the Bray-Curtis index to the proportional OTU values, and then by applying the Jaccard index to the binary occurrence data. Non-metric multidimensional scaling (NMDS) was applied to these distance matrices. In order to assess the differentiation in overall nest box diets between the sites and manipulation groups, analyses of similarities (ANOSIM) and permutational multivariate analyses of variance (PERMANOVA) were carried out. Separate tests were carried out using both Bray-Curtis and Jaccard dissimilarity values. Homogeneity of multivariate dispersions analyses were also performed to assess any relevant differences in nest box average diet dissimilarities.

Linear mixed-effects models (LMM) were used to assess whether the Lepidoptera component of chick diet varied between nest boxes at either site. For each site, a random intercept model was fitted with nest box as a random effect, allowing the variance attributed to nest boxes to be estimated. In fact, two models were created for each site, in order to analyse the proportion of Lepidoptera in terms of both relative reads and proportion of OTUs. As above, only nestling samples from 37 nest boxes with at least two diet samples were evaluated. This gave a dataset of 74 city diet samples and 64 forest diet samples. To determine whether the level of variation between nest boxes differed between the sites, two values of mean Lepidoptera proportion were calculated for each of the nest boxes that had at least two diet samples, using relative reads and proportion of OTUs. Homogeneity of variance in both these values between the two sites was assessed using Levene's test.

Within Nest Box Diet Variation

In order to compare variation within nest boxes at each site, two distance matrices were constructed. Dissimilarity values were first calculated by applying the Bray-Curtis index to relative read abundance data, and second by using the Jaccard index based on presence-absence data. These distance matrices were then subjected to NMDS. Only diet samples from nest boxes with at least five successfully sequenced samples were used, in order to ensure a relatively uniform sample size across the nest boxes, and to improve the reliability of statistical analyses by reducing variance caused by small sample sizes. A total of 15 nest boxes met this criterion: seven city nest boxes with 38 diet samples and eight forest nest boxes with 45 samples. For both of the matrices, a series of tests were conducted to assess dietary differences: ANOSIM was used to test separation between nest boxes at each site; PERMANOVA was employed to evaluate whether the specific nest box had a significant effect on dietary dissimilarity among individuals; a Mann-Whitney U test was utilised to determine if there were significant differences between sites in the average distance of diet samples from their nest box’s median; and ANOVAs were used to assess the influence of the manipulation group on the distance from the nest box median at each site.

Variation in the proportion of Lepidoptera DNA within nest boxes, in terms of both relative reads and proportion of OTUs, was assessed by comparing the ranges of values within each of the 37 nest boxes with a minimum of two samples. The dataset used here matched that used in the analysis of Lepidoptera variation between nest boxes previously. For relative read abundance data, a Shapiro-Wilks test demonstrated that the values for the forest were non-normally distributed, so a Mann-Whitney U test was employed. In contrast, the proportion of OTU data exhibited normal distribution and equal variance at both sites, allowing for comparison with an independent samples t-test.

3.3 Results

Between Nest Box Diet Variation

When Bray-Curtis dissimilarity values were used to calculate average diets for nest boxes with at least two diet samples, separate ANOSIMs found there to be significant separation between the two sites ($n = 37$, $R = 0.6936$, $p < 0.0001$; Fig. 9a, pg. 47) but not between the manipulation groups ($R = 0.0346$, $p = 0.208$), although there was significant separation when combined effect of site and manipulation group was considered ($R = 0.4413$, $p < 0.0001$). Similarly, the PERMANOVA indicated that the site significantly contributes to the observed dissimilarity in the data ($f = 12.7685$, $p < 0.0001$) and manipulation group does not ($f = 1.0655$, $p = 0.319$), however it also showed no significant interaction effect between the two factors ($f = 0.9806$, $p = 0.440$). Importantly, there was a significant difference in dissimilarity between nest box average diets across the two sites ($diff = 0.1899$, $p < 0.0001$), with the average distance from the site's median value more than 50% greater at the city than at the forest, demonstrating significantly greater variation in diets between urban nest boxes.

When Jaccard dissimilarity values were used to calculate average diets for nest boxes with at least two diet samples, the ANOSIMs again showed significant separation between sites ($n = 37$, $R = 0.6022$, $p < 0.0001$; Fig. 9b, pg. 47) but not between manipulation groups ($R = 0.08279$, $p = 0.053$), and, when considering the combined effect of site and manipulation group, there was significant separation ($R = 0.4635$, $p < 0.0001$). The PERMANOVA carried out with Jaccard dissimilarity values showed both site ($f = 4.5300$, $p < 0.0001$) and manipulation ($f = 1.3296$, $p = 0.036$) contributed significantly to the dissimilarities in the data, while the interaction between both factors ($f = 1.1442$, $p = 0.137$) did not. There was also no significant difference in dissimilarity between nest box average diets across the sites ($diff = 0.0127$, $p = 0.2423$).

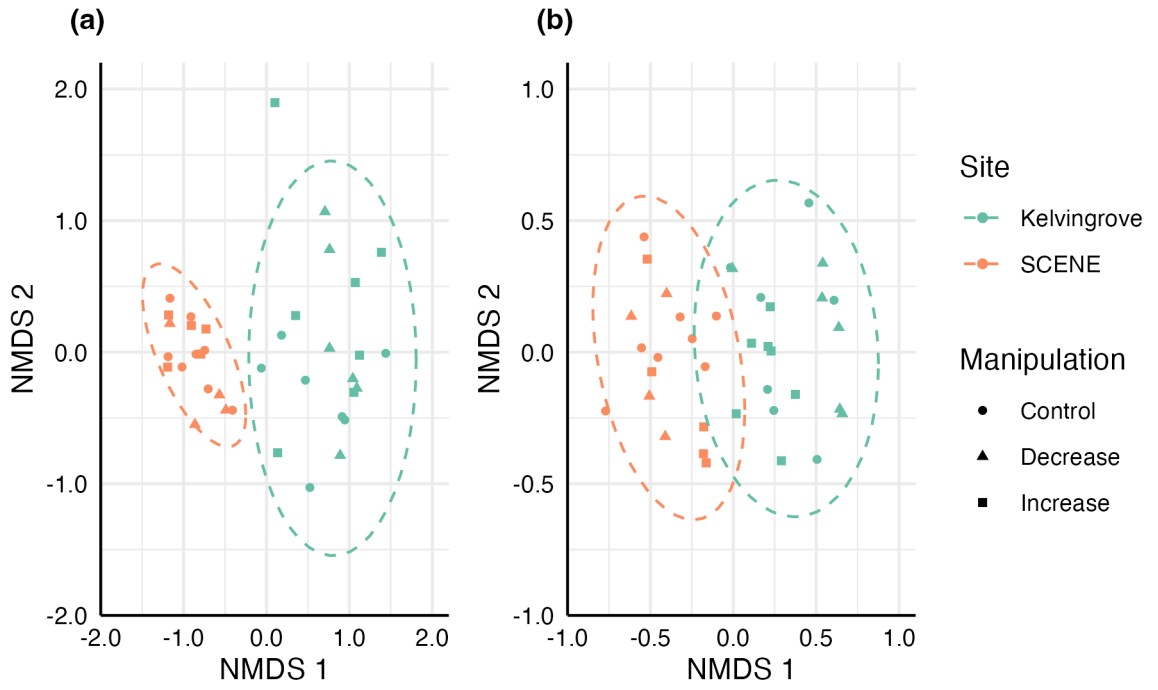

Fig. 9: Non-metric multidimensional scaling (NMDS) ordination plots of average diets for all nest boxes with at least two nestling diet samples (n = 37 nest boxes), measured with (**a**) Bray-Curtis dissimilarity, calculated using relative read abundance data, and (**b**) Jaccard dissimilarity, calculated using presence-absence data. Site and manipulation group are denoted by colour and shape respectively. The ellipses represent a 95% confidence interval based on a t-distribution for each site.

In the city, a moderate amount of the variation in Lepidoptera relative read abundance between diet samples from nest boxes with at least two samples was attributable to nest box effects (n = 74, var = 0.0272, residual = 0.094; 22.44%), compared with a minimal amount in the forest (n = 64, var = 0.0016, residual = 0.0233; 6.43%). There was also evidence for heterogeneity of variance in the nest box mean values of Lepidoptera relative read abundance between the two sites (n = 37, f = 16.982, p = 0.0002; Fig. 10a, pg. 48). A minimal amount of the variation in Lepidoptera OTU proportion between diet samples was attributable to nest box effects in both the city (n = 74, var = 0.0054, residual = 0.0269; 16.72%) and the forest (n = 64, var = 0.0037, residual = 0.024; 13.36%). The variances in nest box mean values of Lepidoptera OTU proportion did not differ significantly between the sites (n = 37, f = 1.2843, p = 0.2648; Fig. 10b, pg. 48).

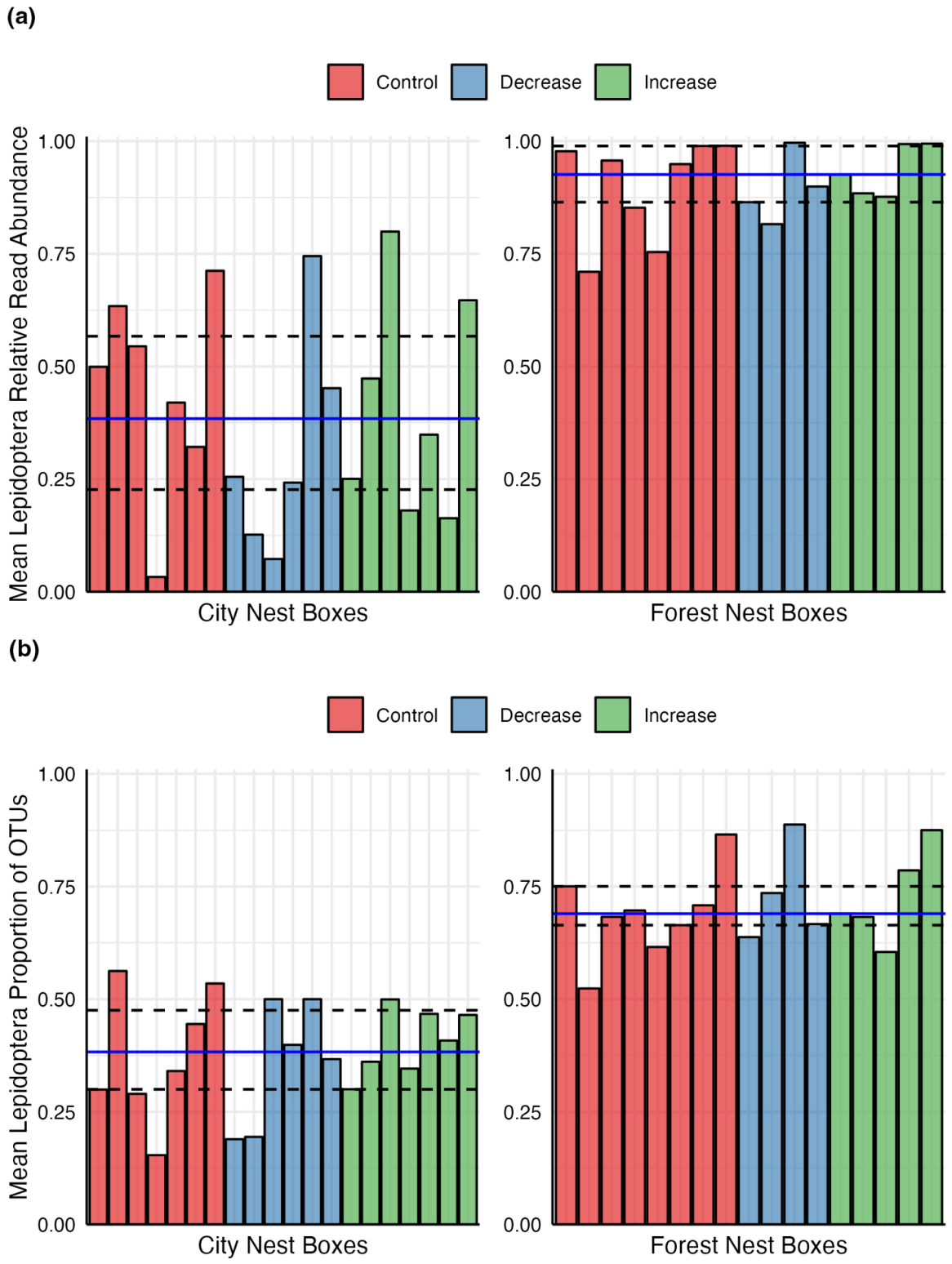

Fig. 10: (**a**) Mean Lepidoptera relative read abundance and (**b**) mean Lepidoptera proportion of OTUs for each nest box, split by manipulation group, at the city (n = 20 nest boxes) and forest (n = 17 nest boxes) sites. Only nest boxes with at least two nestling diet samples are plotted. The solid blue line is the median of the nest box mean abundance or proportion values at each site, with the dashed black lines showing the upper and lower quartiles.

Within Nest Box Diet Variation

Across both sites, when Bray-Curtis dissimilarity values were used, the ANOSIM showed there was significant separation in diets between the nest boxes with five or more diet samples ($n = 83$, $R = 0.532$, $p < 0.0001$; Fig. 11a, pg. 50), while the PERMANOVA found nest box significantly impacted dissimilarity of individuals ($f = 4.5601$, $p < 0.0001$). Nestling diet samples from the city were, on average, a distance of 0.4576 from the median of their nest box, which was significantly greater than the average of 0.3329 from the forest ($W = 1213$, $p = 0.0009$). There was no significant variation in the distance from nest box median for differing manipulation groups at either the city ($n = 38$, $F = 0.318$, $p = 0.73$) or the forest ($n = 45$, $F = 0.231$, $p = 0.794$).

When Jaccard dissimilarity values were used, significant separation in diets between the nest boxes with five or more diet samples ($n = 83$, $R = 0.6462$, $p < 0.0001$; Fig. 11b, pg. 50) was again observed, and nest box again significantly impacted dissimilarity of individuals ($f = 2.7375$, $p < 0.0001$), but there was no significant difference between the sites in terms the distance of samples from the median of their nest box ($W = 934$, $p = 0.4751$). There was also no significant variation in the distance from nest box median for differing manipulation groups at either the city ($n = 38$, $F = 0.657$, $p = 0.525$) or the forest ($n = 45$, $F = 0.508$, $p = 0.606$).

The variation in Lepidoptera component of diets within nest boxes with two or more diet samples, determined by the range of values, differed significantly between the two sites when compared using relative read abundance ($n = 37$, $W = 279$, $p = 0.0006$; Fig. 12a, pg. 51) but not when compared using occurrence data ($n = 37$, $t = -0.5295$, $p = 0.5998$; Fig. 12b, pg. 51). Variation in Lepidoptera relative read abundance was greater at the city, with a mean range of 0.5548, compared to 0.2064 in the forest.

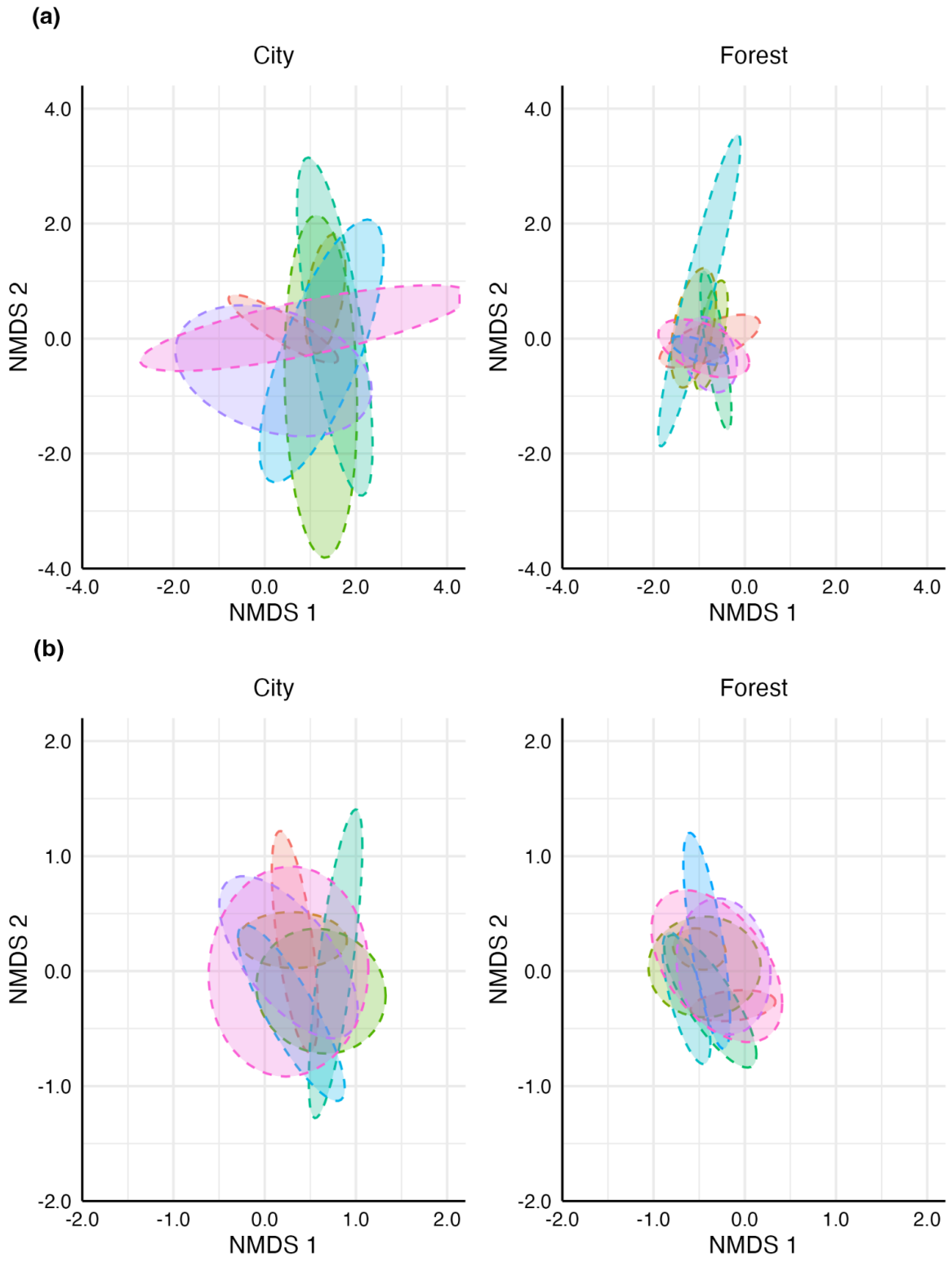

Fig. 11: Non-metric multidimensional scaling (NMDS) ordination plots displaying nest boxes with at least five diet samples as ellipses, with each representing a 95% confidence interval based on a t-distribution of the relative differentiation, measured with (**a**) Bray-Curtis dissimilarity and (**b**) Jaccard dissimilarity, of nestling diets within each nest, for the city (n = 7 nest boxes) and forest (n = 8 nest boxes).

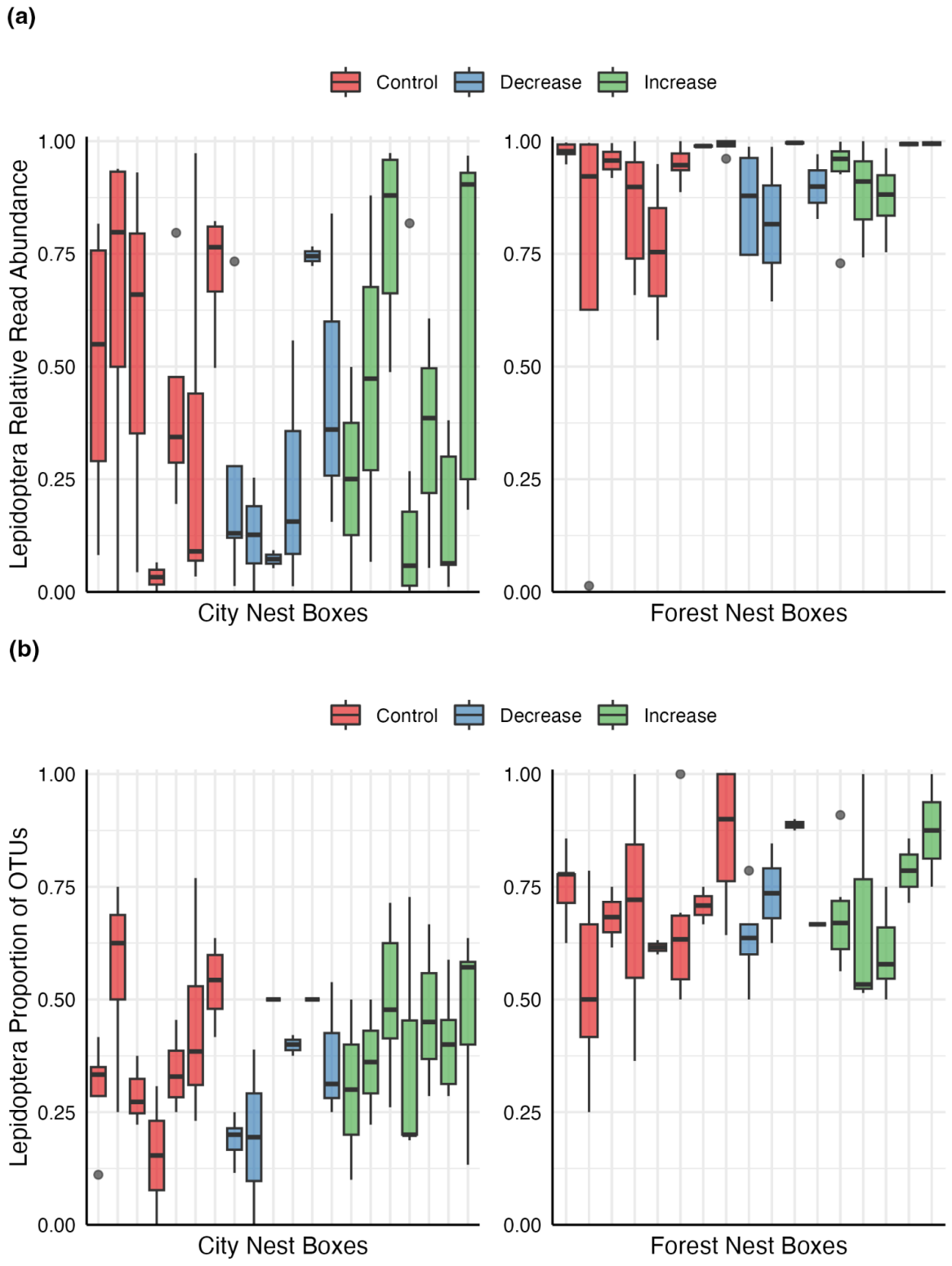

Fig. 12: Box-and-whisker plots showing the (**a**) Lepidoptera relative read abundance and (**b**) Lepidoptera proportion of OTUs in the diet samples from each nest box, split by manipulation group, at the city (n = 20 nest boxes) and forest (n = 17 nest boxes) sites. Only nest boxes with at least two nestling diet samples are plotted.

3.4 Discussion

Between Nest Box Diet Variation

In brief, the between nest box diet variation analysis revealed separation in average nest box diets between the two sites, a finding that mirrors the significant separation in individual diet samples between sites from the first part of this study, although it is unclear whether there is separation in nest averages between the manipulation groups beyond that caused by nest box effects. The more relevant finding to understanding the levels of variation between nest boxes is that, when relative read abundance is considered, there was significantly more variation in average nest box diets at the city than at the forest, echoing the greater variation observed at the city between individual diet samples. Additionally, only in the city did the nest box significantly influence the relative read abundance of Lepidoptera OTUs, with a more pronounced variability in mean Lepidoptera read abundance observed across city nest boxes than those in the forest. These results strongly support the hypothesis that the diets of city blue tit chicks vary more between nest boxes than the diets of forest chicks.

There are several potential reasons why diets varied more between city nest boxes than those in the forest. First, the scarcity of the caterpillars, assumed to be the preferred food choice for blue tit chicks (Bañbura et al., 1999), in the city likely plays a significant role in the dietary variation observed between urban nests. The lack of caterpillars forces parents to rely on a broad spectrum of alternative food items, which are not thought to be able to serve as direct replacements for the nutritional value of caterpillars (Perrins 1991; Mackenzie et al., 2014; Jarrett et al., 2020). As established in Chapter II, the substitute prey appears to be mostly made up of flies and aphids, although previous studies have also found evidence for human sourced foods, such as mealworms and sunflower seeds, being fed to urban blue tit chicks (Jarrett et al., 2020). These food items are commonly found in bird feeders. The availability of several alternative food sources to caterpillars in urban areas could explain the considerable dietary variation observed between nest boxes within the city site. In contrast, the abundance of caterpillars in natural environments (Seress et al., 2018) likely accounts for the minimal variation in chick diet, especially in terms of the Lepidoptera proportion, between the forest nest boxes.

Second, the microhabitats surrounding each city nest box could vary in terms of food accessibility and availability, thus influencing the diet received by chicks. Greater variability in the conditions of each microhabitat would be expected in the city compared to the forest, with this attributed to habitat fragmentation and the introduction of non-native plant species, leading to a more diverse environment (Narango et al., 2018). Across a single urban park, several distinct microhabitats would be expected. For example, Kelvingrove Park, used in this study, consists of managed lawns, unmanaged riverbank vegetation and sports fields, while its tree species are a mixture of native and introduced. This habitat heterogeneity likely influences the communities of arthropods present, and thus the provisioned food items could easily differ between nests at a single site, explaining the considerable blue tit chick diet variation observed between nest boxes. Microhabitats can also explain the separation in average nest box diets at the forest site. While this separation is far less pronounced than observed in the city, it still exists, and suggests that, even though the proportion of diet made up by Lepidoptera is consistent between nests, diets are still influenced by the location of the nest box. The species of caterpillar present can differ between tree species (Seress et al.,

2018), suggesting that the specific types of caterpillars consumed might vary slightly across different areas of the forest. Although this variation is likely to be minimal in the forest since it is dominated by oak, it could go some way to explaining the separation in average nest box diets in the forest. Meanwhile, the presence of both native and non-native tree species in the city will exacerbate this effect, further explaining the greater levels of chick diet variation between nests observed there.

Third, the condition, and even the presence, of the parents of the urban broods may be of more varied than those in the forest, impacting parental ability to provision their young. Adult birds may experience higher mortality rates in urban areas, primarily due to vehicle collisions and predation by domestic cats, threats that are significantly less common in forest environments (Seress et al., 2012). Single parents have to forage at a faster rate in order to sufficiently feed their young, and so they might provide food items usually overlooked due to their low nutritional value (Arnold et al., 2010). Similar effects would be expected if the condition of a parent was compromised, potentially by illness, injury, or parasitism, to the extent that provisioning effort was severely diminished. Diseases and parasites can be more prevalent in urban bird populations (Robb et al., 2008). The higher likelihood of death or a decline in health among parents of urban blue tit broods suggest parental condition and vigour could be a factor influencing the dietary variation observed between urban nest boxes, especially in terms of the proportions of Lepidoptera in the diet. A healthy parent would need to forage at a faster rate if its partner was compromised, and so would likely provision more abundant and accessible items, such as flies and aphids, rather than scarce items like caterpillars, as they may take too much time to find. The presence of predators can also induce sublethal impacts on birds, including reduced foraging efficiency (Beckerman et al., 2007). This occurs as birds may avoid areas with richer prey sources, or reduce their foraging time there, due to the risk of predation, and also may waste potential foraging time while hiding from, or monitoring for, predators. Predator density likely varies across urban habitats, influenced by factors such as cat ownership and the availability of suitable nesting areas for predatory birds. This potential variation in predator presence means the intensity of the sublethal effects likely also fluctuates, helping to explain the significant variations in chick diets between city nest boxes.

Within Nest Box Diet Variation

Analysis of diet variation within nest boxes indicates that, at both sites, nest box influences nestling diet, and, when relative read abundance is considered, there is greater variation in diets within each nest box at the city than the forest. Furthermore, the range between the highest and lowest relative read abundances of Lepidoptera OTUs in chick diet samples from each city nest box was, on average, larger compared to chick diet samples from the forest nest boxes, demonstrating that there was greater variation in the Lepidoptera proportion of blue tit chick diets in the city. This supports both the hypothesis that the diets of blue tit chicks would vary between siblings and within nest boxes, and the hypothesis that this variation would be more pronounced in the city nest boxes. Additionally, the previously discussed finding that blue tit chick diets also vary between nest boxes in the forest, although not originally predicted, is also supported by these results.

There are two primary explanations for why blue tit chick diets would vary within nest boxes, and why this variation would be more pronounced in the city habitat. Firstly, differences in

diet within the nest could be entirely random, attributed solely to the variation in the availability and accessibility of the food sources, which are likely to be more diverse in the city due to the heterogeneity of habitat types (Faeth et al., 2011). At the forest site, the abundance of the preferred food source of caterpillars means they are likely to be provisioned during most food visits (Perrins, 1991), resulting in a more uniform diet among nestlings, explaining the minimal variation observed. Conversely, the relative scarcity of caterpillars in the city could lead to the substitution of a variety of alternative food items, as explored in the Diet Composition section of Chapter II. This substitution could explain the greater observed chick diet variation within nest boxes observed in this habitat. The rarity of caterpillars can also explain the greater variation observed in the Lepidoptera proportion of the diet of city blue tit chicks compared to their forest counterparts, as it does not appear to be feasible for adults to provide all their chicks with caterpillars simultaneously. The diet samples analysed in this study were collected concurrently from all chicks in a nest box, and predominantly feature waste from a single feeding event, albeit with remnants from previous ingestions, evidenced by the low Shannon Diversity values across all samples, as discussed in Chapter II. This means the samples from each nest box likely just show a snapshot of a brief period of provisioning. During such a period, it may be particularly challenging for a city dwelling blue tit to find caterpillars for all its chicks, leading to the reported levels of dietary variation.

Secondly, the variations in diet could be as a result of potentially deliberate provisioning strategies by parents in response to the state of their young and the stresses of their environment (Grieco, 2003). There are several mechanisms through which adult birds may decide which chicks to preferentially feed. Across various species, preference may be given to either the nestlings that are closest to the parents in the nest, those that begin begging first upon the adult's arrival with food, or those that reach the highest to receive the food (Moreno-Rueda et al., 2007). Larger nestlings would be able to outcompete their siblings for privileged positions in the nest as well as when begging for their food. Additionally, larger chicks may have more capacity to be able to beg with greater intensity, given the associated fitness costs, including physiological deterioration and reduced development, tend to constrain begging effort (Fresneau et al., 2018). Alternatively, parents may simply prefer to feed their larger young, as they typically have a higher survival rate and a better chance of fledging (e.g. Monrós et al., 2002). This is a common strategy in species that exhibit natural brood reductions. When quality food is in short supply, and thus the survival of all nestlings is unlikely, some birds prioritise the health of their fittest chicks to ensure they reach fledgling, at the expense of other nestlings who may starve (Soler, 2001). This may be more likely to occur in urban habitats, where optimal food resources are limited. However, blue tits typically do not engage in natural brood reductions, which means that in most cases the overwhelming majority of their nestlings fledge successfully. That said, in adverse circumstances, for example following the death or injury of one or both parents, more nestling deaths may be expected. Indeed, in this study, the artificial inflation of broods, which also puts additional pressure upon adult blue tits, led to greater rates of chick mortality. These deaths could potentially suggest a deliberate brood reduction strategy under adverse conditions. In such cases, variation in diets within the nest would be anticipated, although this may be as much in regard to the quantity of food distributed to each chick as the specific items delivered. Given the impact of artificially enlarging blue tit broods upon nestling survival was found to be broadly consistent across both sites in this study, it is likely that any

potential brood reduction strategy would also be consistent, and therefore would not explain the increased levels of within nest box chick diet variation observed in the city.

Moreover, blue tits are not typically brood reducers, and instead exhibit the behaviour of clutch adjusters (Nur, 1986). This is evidenced in this study by both the significant variation in clutch sizes between the two sites and the very high nestling survival rates in unmanipulated broods. Clutch adjusters instead tend to distribute food equitably between their chicks, and any preferential feeding usually benefits their poorer quality offspring (Soler, 2001). In blue tits, this is likely to occur in response to amplified begging signals. A previous study has shown lighter blue tit chicks beg more persistently than their heavier siblings, with parents feeding the nestling they perceive to be hungriest first, based upon these begging cues (Fresneau et al., 2018). Size variation in chicks is primarily attributed to hatching asynchrony, where eggs hatch over a period of a couple of days (Slagsvold et al., 1995). Hatching asynchrony has been observed in many altricial bird species, including blue tits, where it is mostly explained by incubation asynchrony (Stenning, 2008). Essentially, eggs that are laid first, and consequently incubated earlier, tend to hatch sooner. Numerous hypotheses have been offered for the occurrence of hatching asynchrony, and these propose a variety of potential advantages (Slagsvold et al., 1995). Among these hypotheses, offspring quality assurance is perhaps the most probable among bird species that do not engage in strategic brood reductions. This theory suggests that by establishing a size hierarchy within the nest, parents can ensure that at least some of their offspring develop sufficiently and reach a high quality before they fledge from the nest (Lack, 1947). Additionally, hatching asynchrony may allow the first hatch to be sooner, helping parents best exploit potentially limited food resources whilst also spreading out the peak food demands from their chicks, and reducing the competition between them (Slagsvold et al.,1995). Hatching sooner could also maximise the father's investment into raising their young as, once their chicks are born, males are less likely to want to, or be able to, engage in further mate attraction. Ultimately, although preferential feeding based on chick size and begging may explain some variations in diet between chicks within the same nest, this does not account for the significantly greater dietary variation observed between siblings at the city site compared to the forest habitat. This suggests that other factors, such as availability and diversity of food resources in the habitats, play a more crucial role in influencing the extent of dietary variation between blue tit chicks in the same nests.

CHAPTER IV. Synthesis

4.1 Summary & Significance

In summary, this study has illustrated that urban blue tit adults lay smaller clutches than their forest counterparts, likely as a result of their own poor condition, although potentially amplified by a phenotypic plastic response to the lack of optimal food resources. In doing so, city blue tits raise nestlings that successfully fledge at an equitable rate to forest broods, despite providing their chicks a far more varied diet with significantly fewer caterpillars than their forest counterparts. Other studies have similarly demonstrated that blue tit clutches are smaller in urban populations than in non-urban ones, and that these smaller clutches may be selected for in response to reduced and irregular natural insect food availability in urban environments (Branston et al., 2021). The current study also demonstrates that the benefits of artificially reducing the size of a brood upon its remaining chicks are limited to a minor improvement in chick quality, as the vast majority of chicks successfully fledge in unmanipulated broods. Additionally, the impacts of artificially inflating a brood, which manifest in increased nestling mortality and smaller, lighter fledglings, are, despite the inherent shortcomings of the urban environment, no more prominent in the city than they are in the forest. In fact, the negative effects of the urban habitat upon the city nestlings are shown to be limited to their diet, and as a result their condition, rather than their overall survival.

Despite fledging at equitable rates to the forest chicks, city chicks are potentially less healthy, as demonstrated by their shorter wings and tarsi than those observed in the forest population, although their weights were comparable. This could be due to different selective pressures in the city combined with a lack of genetic connectivity between the two populations, or as a result of urban females spending less time brooding their young. However, it may instead be related to the poor nutritional value of the food items they consume, with this study demonstrating, as found in previously, that urban populations exhibit a wider diversity in their diets (Branston et al., 2021), and that the lower availability of caterpillars in urban habitats is compensated for with the provisioning of other insects including flies and aphids (Jarrett et al., 2020). The diversity of food resources in the city is the most likely factor to explain the observed high degree of variation in chick diets both between and within city nest boxes. However, differences in various characteristics, such as predator density and arthropod community presence, between microhabitats likely exacerbates diet differences between urban nest boxes. Preferential feeding strategies could contribute to the diet variation recorded within nest boxes, but this would likely only be in response to differences in chick size brought about by asynchronous hatching, and so whilst offering a potential explanation for some of the differences in diet within nest boxes seen at both sites, it probably does not account for the markedly higher variation observed in the city.

The comparable fledging success of city and forest blue tit broods appears to suggest that blue tits are able to adapt to, and flourish in, towns and cities, and that these do not act as ecological traps. However, this success appears to occur only as a result of the smaller clutches observed in the city, which themselves result from either physiological constraints due to the health of urban blue tit adults or as an example of phenotypic plasticity in response to limited urban resources. Consequently, despite the comparable fledging

success rates, fewer birds recruit into the blue tit breeding population in the city than do in the forest, potentially altering future population dynamics and leading to a smaller gene pool and reduced genetic diversity. This may leave urban populations vulnerable to diseases and environmental changes. It is important to consider the different selection pressures birds are exposed to in towns and cities, and how this can differ between species, when trying to understand how species adapt to urban life (Branston et al., 2021). Additionally, the long term effects upon blue tit nestlings of receiving a more diverse and less caterpillar heavy diet are unclear. Chick diet during early development not only dictates survival, but also influences various traits including fecundity, sexual ornamentation, and cognitive ability, all of which strongly relate to fitness and future reproductive success (Arnold et al., 2010). It is important to understand how blue tit populations adapt to urban environments, and the effects of this upon their offspring and their future reproductive success, given the rapid and ongoing expansion of urbanisation. As cities grow, an increasing number of blue tits, along with other species, will be compelled to live in, and adapt to, urban environments. Notably, the significant chick diet variation between urban nest boxes suggests there are microhabitats within the city within which blue tits can provide their young with quality food, which could mitigate some negative effects of expanding urban environments. An understanding of the factors, such as caterpillar availability, that can drive successful urban blue tit reproduction and improved nestling health is vital as it can facilitate the targeted management of urban environments in order to provide optimal breeding environments. For example, increasing native tree populations whilst enacting pollution reducing measures could bolster caterpillar populations. Such an approach would help counteract the adverse effects of urban environments and help prevent them from potentially acting as ecological traps.

4.2 Biases & Limitations

There are some potential biases and limitations to this study. The multifaceted approach restricted it to two sites, and thus the results and findings may not necessarily be applicable to blue tits in all urban habitats. Similarly, this study considered only a single bird species, and so the generalisability of the findings is unclear. Notbaly, blue tits share multiple characteristics with other urban birds, and so some comparisons can be made with them. For example, other species that span urban and forest habitats, and also provision their young with insects, would likely be affected by the differences in arthropod community composition between the sites.

The main limitations of this study surround the diet metabarcoding, both in terms of the samples analysed, and the interpretation of results. Firstly, only taking one faecal sample from each chick skews the diet analysis in favour of the most recent feeding event. It is important to note that the diet samples analysed in this study appear to predominantly feature waste from a single feeding event, albeit with remnants from previous ingestions, and as they were collected concurrently from all chicks in a nest box, they only show a snapshot of the nest box provisioning rather than a comprehensive overview of the entire dietary intake. Indeed, only analysing faecal samples taken on the twelfth day of the study also introduces survivorship bias to the results, as samples obviously could not be taken from nestlings who had died beforehand. As a result, diets linked to mortality may be underrepresented in the findings of this study, while the adequacy of the diets of surviving nestlings may be overestimated. Additionally, the level of dietary variation may also be

underestimated. Furthermore, for most nest boxes, the analysis of faecal samples was limited to only a subset of nestlings, potentially further restricting the comprehensive understanding of diet variation among siblings.

Secondly, many of the trends noted in this study only emerge, or only become significant, when analysis was performed using relative read abundance values for each OTU in each sample, rather than the more simplistic presence-absence data or occurrence-based summaries. Importantly, because the same primers were used for samples from both sites, any biases in amplification intensity would be consistent between them, thus making results comparable. Notably, none of the presence-absence based findings directly contradict the relative read abundance findings. The most contrasting findings relate to chick diet and nest box average diet dissimilarity values. While both methods of calculating these values found separation in diets between the two sites, only relative read abundance analysis found there to be greater separation between diets within the city than within the forest. This is not to say these results should be discounted. The comparable levels of separation between diet samples at both sites, observed when calculated using presence-absence data, suggest that, in terms of the number of unique insect species present, diet samples vary between chicks in a similar manner at each habitat. However, the greater separation between samples in the city, observed when calculated with relative read abundance data, suggests the city shows greater variation in the most recently consumed item. This is because the relative read abundance for this item will be greater, and this demonstrates how, at least across the period of the most recent feeding event for each chick, there is greater dietary variation in the city.

4.3 Future Questions in Health and Diet of Urban Blue Tit Chicks

This study opens several potential avenues for new research. While additional blue tit brood manipulation experiments seem futile, given the effects upon urban broods have been shown to be mitigated by adaptations to clutch size, repeated manipulations could prove to be insightful if additional variables are incorporated. For instance, employing video capture to monitor differences in provisioning rates between enlarged and reduced broods, whilst also assessing chick diet in depth, would help fully understand the impacts of brood manipulations. Furthermore, replicating the brood manipulation study across different breeding seasons would offer a greater breadth of understanding, and mitigate the risk that the current study's findings are exclusively pertinent to the conditions of the 2021 breeding season. Manipulating the city broods more extensively may also be insightful. A disparity in the impacts of enlarging broods would be expected between the two sites if urban broods were further inflated to be the same size as the enlarged forest broods. This is because the smaller clutch sizes observed in the city mean the stress of enlarging broods to the same size would be greater for the urban blue tit parents. Such an approach would help determine whether these urban clutch sizes match the capacity of urban parents to raise young in that environment.

The observation that city blue tit nestlings exhibit smaller wing and tarsus lengths than forest nestlings, despite having similar body masses and hence superior BMI scores, necessitates a greater understanding of how best to measure the condition and health of living chicks, as well as why blue tit growth patterns differ between sites, and whether they are impacted by the consumption of certain foods. Implementing cross fostering experiments between the city

and forest sites would help to clarify whether the observed anatomical disparities are a result of differing selective pressures and restricted gene flow between the two populations, while more detailed observations of nest boxes would determine whether nestling brooding time differs between sites. Should these factors be ruled out, the results from this study would suggest that a caterpillar heavy diet may promote bone growth, while the alternative city diet of smaller insects, potentially supplemented with anthropogenic foods, appears to instead facilitate weight gain, possibly at the detriment of skeletal development. To assess this hypothesis future studies should attempt to quantify the nutritional value of common blue tit prey items, including various species of caterpillars, aphids and flies.

In order to gain a more comprehensive understanding of blue tit chick diets, especially with regard to variation between and within nests, nestling faecal samples could be taken multiple times a day and over several days. This would provide a more complete picture of each chick's diet and reveal any shifts in the food items provided throughout the day, or as the chicks develop. This depth of analysis may not be possible however, as repeated disturbances to the nest could result in parents abandoning their broods. Finally, specific urban microhabitat analyses could be performed to assess how blue tit clutch sizes, as well as the diet and condition of their chicks vary with certain environmental characteristics such as arthropod abundance, proximity to traffic and predator density. This would help achieve an understanding of the extent to which blue tits, and potentially other species, can flourish in towns and cities, and how best to help them achieve this through targeted conservation management.

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