UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA, EVOLUÇÃO E BIODIVERSIDADE

SEED DISPERSAL AND PLANT DIET OF FRUIT-EATING BIRDS IN SECONDARY FOREST FRAGMENTS

CARINA ISABELLA MOTTA

Rio Claro – SP
2024

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Orientadora: Dra. Marina Corrêa Côrtes

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RESUMO

A mobilidade das aves frugívoras as torna dispersoras chave de sementes na paisagem fragmentada atual, impulsionando o estabelecimento e sucessão de novas florestas. Distúrbios antropogênicos circundantes moldam essas florestas em estágio inicial, resultando em comunidades florestais secundárias novas. Para entender melhor em quais condições a dispersão de sementes é mais eficaz, assim como as espécies de plantas na dieta das aves dessas comunidades, trabalhamos em dez parcelas de floresta em estágio inicial na bacia do rio Corumbataí no estado de São Paulo. Estabelecemos redes de neblina em cada parcela uma vez por mês ao longo de um ano para coletar fezes de indivíduos capturados. As sementes encontradas nas fezes foram usadas para testar o componente quantitativo da efetividade da dispersão de sementes (SDE) entre as parcelas e espécies de aves. Características do habitat tais como idade da floresta, cobertura florestal e comunidade de espécies de plantas lenhosas zoocóricas não explicaram as variações no componente quantitativo da SDE entre as parcelas. A espécie *Antilophia galeata* se destacou como a dispersora mais efetiva, seguida de *Turdus leucomelas*, *Ramphocelus carbo* e *Tachyphonus coronatus*. O grau de frugivoria das aves se relacionou positivamente com a SDE enquanto que a massa corporal não teve efeito. O sequenciamento de um mini-barcode de *rbcL* (Ribulose-1,5-Bifosfato Carboxilase), foi aplicado em DNA isolado das fezes de dez espécies de aves dispersoras de sementes. Dos gêneros de plantas detectados, quatro são exóticas e, embora a maioria tenha sido classificada como árvore ou arbusto, pelo menos dois são de hábito herbáceo. Embora tenhamos detectado variação na dispersão de sementes entre fragmentos, indicando heterogeneidade espacial de processos ecológicos em florestas jovens, essa variação não é explicada pelas características analisadas. Nossa análise da dieta indica que algumas espécies de aves quantitativamente eficientes na dispersão de sementes provavelmente forrageiam fora das florestas. Se por um lado a movimentação para além da floresta potencialmente aumenta a conectividade entre os fragmentos, também pode aumentar a introdução de espécies exóticas, contribuindo para a organização de novos ecossistemas na Mata Atlântica.

Palavras-chave: Restauração florestal; Frugivoria; Metabarcoding; Interação animal-planta; Fezes

ABSTRACT

The mobility of fruit-eating birds makes them key seed dispersers in today's fragmented landscape, ultimately driving the establishment and succession of new forests. Surrounding anthropogenic disturbances shape these early successional forests, favoring habitat generalist bird species and introducing exotic plants, resulting in novel, secondary forest communities. To better understand under which conditions seed dispersal is most effective, as well as the plant species important to bird diet within these new communities, we worked in ten plots of early successional forest throughout the Corumbataí river basin in the state of São Paulo. We mist netted in each plot once a month over the course of a year to collect droppings from captured individuals. Seeds found in droppings were used to calculate and test the quantitative component of seed dispersal effectiveness (SDE) among plots and bird species. Habitat feature differences in age, forest cover, and the zoochorous woody plant species community were not found to explain changes in the quantitative component of SDE among plots. We identified *Antilophia galeata* to be the most quantitatively effective seed disperser, followed by *Turdus leucomelas*, *Ramphocelus carbo*, and *Tachyphonus coronatus*. Bird species-level SDE was found to be explained by the degree of bird frugivory, but not bird body mass. Using metabarcoding sequencing of *rbcL* (Ribulose-1,5-Bisphosphate Carboxylase), a locus used to identify plants in the diet, we analyzed droppings from ten key, seed dispersing bird species to detect diet items beyond seeds present in droppings. Of the plant genera detected, four are exotic, and, while a majority were classified as trees or shrubs, at least two are herbaceous. Although we detected variation in seed dispersal among fragments, indicating heterogeneity in ecological functions, this variation is not explained by analyzed characteristics. Additionally, our diet analysis indicates that bird species we found to be highly efficient at dispersing seeds are foraging outside of forests and, while potentially increasing connectivity among fragments, can also increase the introduction of exotic species.

Key-words: Forest restoration; Frugivory; Metabarcoding; Plant-animal interaction; Feces

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1 INTRODUCTION

Mutualistic relationships between plants and their animal seed dispersers are keystone interactions that form the foundation of forest ecosystem structure (BASCOMPTE; JORDANO, 2007; CARLO *et al.*, 2011). The movement of seeds by animals is essential to the maintenance and formation of forests, with a majority of woody plants being dispersed by frugivores and granivores (HERRERA, 2002). Meanwhile, many animal dispersers depend on plants as an integral part of their diet, especially in the tropics, where the consumption of fruit by vertebrate species is proportionally higher than in other regions (FLEMING *et al.*, 1987; JORDANO, 2000). The increase of fruit in animal diet in the tropics is matched by an increase in animal-mediated seed dispersal, principally by birds (ESCRIBANO-AVILA *et al.*, 2018; WHEELWRIGHT, 1988). In Brazil's Atlantic Forest, 51% of animal-dispersed woody plant species are dispersed by birds (ALMEIDA-NETO *et al.*, 2008). Birds and plants depend on and benefit from one another for services and resources, especially in Atlantic Forest.

Today, less than 15% of the Atlantic Forest remains, with more than 80% of remaining forest consisting of scattered fragments (REZENDE *et al.*, 2018). The aggressive deforestation of this incredibly biodiverse ecosystem has created an urgency to not only conserve the forest that is left, but also to restore areas that were once forested (MELO *et al.*, 2013; ZWIENER *et al.*, 2017). A Brazilian law created in 1934 and amended in 2012 established incentives and obligations for private landowners to allocate a portion of their land as natural reserves (Law 12.651/2012, Brancalion et al. 2016). This law, in combination with other social and economic factors, led to the abandonment of areas previously deforested to make pasture lands, allowing the forest to naturally regenerate (CALABONI *et al.*, 2018; GOMES *et al.*, 2013). However, the forests that establish in these abandoned pasture lands are not the same as the forests that once occupied the space (CÉSAR *et al.*, 2021; ROZENDAAL *et al.*, 2019). Aside from being young, these forests are novel in the sense that they contain species that previously did not occur in the region, such as plant species introduced by humans for agricultural cultivation or unintentionally (PETRI *et al.*, 2018; SUGANUMA *et al.*, 2014). The exotic species within these reconstructed forests can establish interactions with seed dispersing bird species, creating new mutualisms.

The fragmented nature of these secondary forest fragments has not only resulted in a novel suite of species interactions, but has also further exaggerated the importance of, as well as modified, plant-bird mutualisms (EMER *et al.*, 2020). The surrounding matrix of these forests, as well as habitat features of the fragment itself, such as forest age, connectivity, and plant community, can determine habitat accessibility and quality for frugivores, impacting seed dispersal dynamics (GONZÁLEZ-VARO *et al.*, 2017; RIBEIRO DA SILVA *et al.*, 2015). The mobility of bird species has always made birds key players in long-distance seed dispersal, transporting seeds further away from the maternal plant and facilitating gene flow among plant populations (DICK *et al.*, 2008; JORDANO, 2017). Today's fragmented landscape, with urban areas and open pastures separating forests, has made habitat generalist bird species essential in the establishment of new forests (HAGEN *et al.*, 2012). The tolerance of these bird species to anthropogenic disturbance and dynamic environments allows them to move between fragments, consequently dispersing seeds in the open areas that separate the forests (CARLO; MORALES, 2016; HARRIS; PIMM, 2004). Simultaneously, these secondary forests are critical in providing food to fruit-eating species in a resource-limited landscape (QUITIÁN *et al.*, 2019; VLEUT *et al.*, 2013). Although fruit availability in secondary forests is known to be lower than that available in primary forest, the early successional forests are often the only refuge from the surrounding, human-impacted areas (BARLOW *et al.*, 2007; CHAZDON *et al.*, 2009). Therefore, just as birds are necessary for the establishment and maintenance of new forests, these forests provide essential resources for the remaining bird populations.

Insights can be gained into plant-bird mutualisms, especially how to facilitate and preserve these interactions in the context of active restoration efforts, by evaluating the plant perspective of seed dispersal. The function of plant species within seed dispersal networks can be measured both quantitatively and qualitatively using the seed dispersal effectiveness (SDE) framework (SCHUPP, 1993; SCHUPP *et al.*, 2010, 2017). Traditionally, the quantitative component of the SDE provides a standardized measure of seed dispersal effectiveness by frugivorous species by distilling the data collected into comparative metrics, like the frequency of visits and number of seeds dispersed (e.g., Godinez-Alvarez and Jordano 2007, Rother et al. 2016). While focal observations of disperser activity and feeding behavior are most

often used to assess the quantitative component of SDE, bird capture rate from mist netting sampling serves as a proxy of bird activity and can be used to evaluate visitation frequency (MONTAÑO-CENTELLAS, 2013). Similarly, seeds found in droppings collected from captured birds allows for the quantification of seeds being dispersed (NOGALES *et al.*, 2017). The quantitative component of SDE can be used to explore differences in seed dispersal effectiveness among habitat types, as well as bird species (BLENDINGER, 2017; HOHMANN *et al.*, 2023). Among bird species, characteristics such as body size and diet can heavily influence seed dispersal effectiveness (GODÍNEZ-ALVAREZ *et al.*, 2020). While the quantitative component of SDE is key in evaluating the plant perspective of bird-plant interactions, the equally important bird perspective, assessed through the resource-provisioning effectiveness (RPE), has only recently begun to be explored (QUINTERO *et al.*, 2020, 2023). The assessment of both plant and bird perspectives can deepen our understanding of plant-bird mutualisms essential to forest regeneration.

By complementing the quantitative component of SDE with knowledge of all plant species that comprise bird diet, a more comprehensive image of plant-bird mutualisms can be created (GONZÁLEZ-CASTRO *et al.*, 2022; QUINTERO *et al.*, 2020). Evaluating the effectiveness of plants as resources not only provides critical insights as to which plants may be dispersed, but can also indicate the quality of available resources, as well as foraging habits of species, both of which further determine seed dispersal potential (FONTÚRBEL *et al.*, 2017; MILESI *et al.*, 2008). While seeds found in bird droppings provide direct evidence of plant species consumption, in the case of large, pulpy fruits with small seeds, such as exotic *Psidium guajava* (Myrtaceae), birds may ingest the pulp while avoiding swallowing the seeds (FOSTER, 2016; LOPES *et al.*, 2016). In recent years, molecular techniques have begun to be developed in order to identify the diet items present in droppings of which seeds were not ingested (ANDO *et al.*, 2020; SHUTT *et al.*, 2021). While challenging to apply in tropical areas due to high plant diversity, the development of universal, mini-barcodes enable the identification of plant family and genera from bird droppings, even in the absence of a robust reference library (ROSEMARY *et al.*, 2018; VASCONCELOS *et al.*, 2021). The characterization of bird diet in conjunction with the quantification of SDE provides a more robust understanding of the plant-bird mutualisms that are so critical for future forests.

In this study, we explore both the plant community and bird perspective of bird-mediated seed dispersal in secondary Atlantic Forest fragments. We quantify SDE from the plant perspective to investigate differences in seed dispersal effectiveness across areas that vary in forest age, cover, and zoochorous woody tree species communities, as well among bird species and their species traits. Using molecular techniques, we evaluated the bird perspective of seed dispersal by identifying plant genera present in the diet of principal seed dispersers. We predicted an increase in the quantitative component of SDE with an increase in forest age, cover, and zoochorous woody plant species abundance, as well as richness. Additionally, we predicted an increase in SDE with an increase in bird body size and the percent of diet composed of frugivory. Due to the heavy anthropogenic impact on these secondary forests, we expected to detect exotic and weedy plant species in bird diets.

2 OBJECTIVES

We aim to investigate seed dispersal and bird diet in secondary forest fragment communities of the Atlantic Forest. In doing so, we evaluate the implications of plant consumption by birds from both the perspective of 1) zoochorous plant community, in which we explore which habitat conditions and what bird species result in the highest seed dispersal effectiveness, and 2) fruit-eating birds, in which we evaluate which and how many plant species are important to bird diet. Seed dispersal effectiveness is calculated and tested according to habitat and bird features, while plant diet profile is characterized by bird and plant features, as outlined below:

1. Characterize the quantitative component of seed dispersal effectiveness, based on bird activity (i.e., capture rate) and seed dispersal frequency (i.e., frequency of seeds in droppings). Evaluate how the seed dispersal effectiveness varies according to the following:
    a. Habitat features: how does forest age and cover, as well as the richness and abundance of zoochorous tree species influence seed dispersal effectiveness?
    b. Fruit-eating birds: how effective are bird species to seed dispersal? Do their contributions vary according to bird species traits (body size, % diet frugivory)?
2. Characterize the plant profile of bird diet by identifying how many (diet richness), which (diet content), and with what frequency plant species are consumed. Using these values, we will evaluate principal items of bird diet:
    a. Fruit-eating birds: how do diet richness, content, and consumption frequency vary among bird species?
    b. Plant features: how does plant life form and origin vary within bird plant diet?

3 MATERIALS & METHODS

3.1 STUDY AREA

Our study area is located in the Corumbataí river basin in the state of São Paulo, Brazil (*Fig. 1*). This region is an ecotone between the Atlantic Forest and the Cerrado biomes, consisting of seasonal, semi-deciduous forests. The NewFor team, led by Pedro Brancalion, has established a series of 20 x 45 m plots within naturally regenerating secondary forest throughout the region. The fragments in which these plots have been installed are located on private property, where landowners are obligated to conserve the forest that borders rivers and streams. Detailed survey data within these plots includes a record of forest age and cover, as well as the abundance and richness of woody tree species individuals with > 10 DBH (Brancalion et al., unpublished data). Forest fragments in which this study was conducted are characterized by a canopy height of approximately 15 – 30 meters (Motta, C.I., personal observation). Approximately half of the woody tree species present are dispersed by animals, including species from families Euphorbiaceae, Meliaceae, Mytraceae, Rubiaceae, and Salicaceae (*Supplementary Material 1*).

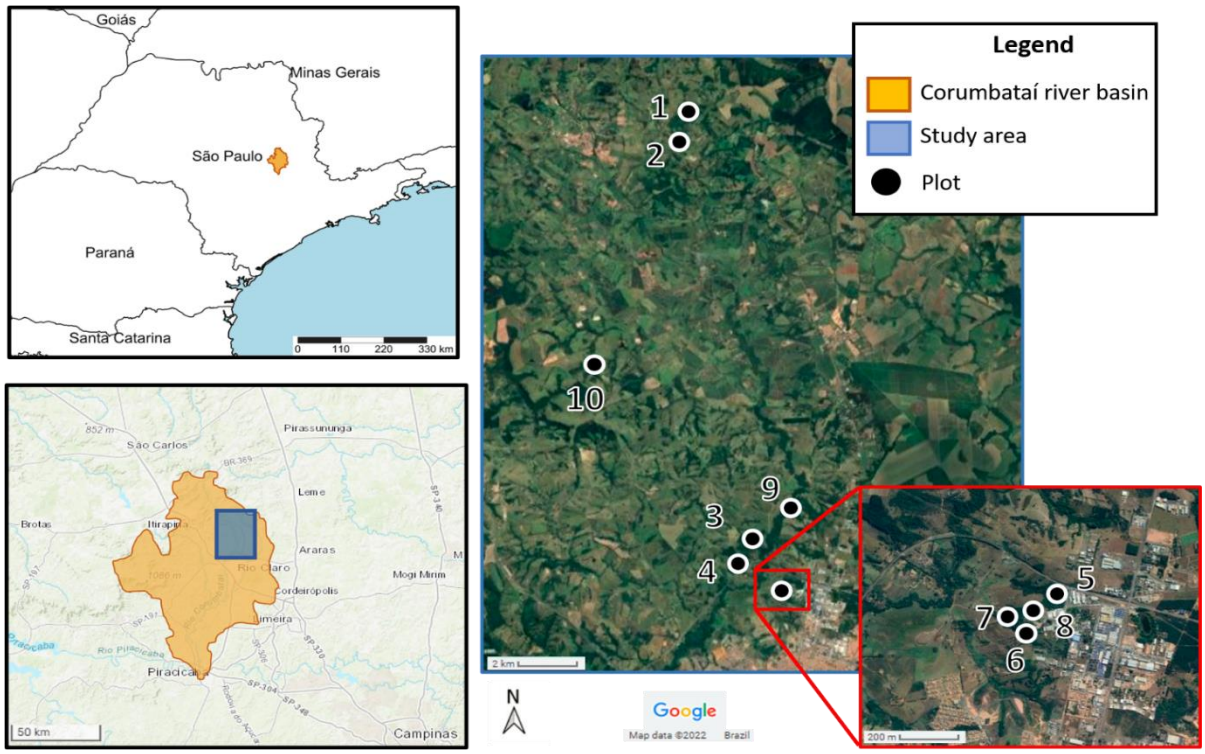

Fig. 1 Map of study area and ten plots located in the Corumbataí river basin in the state of São Paulo, Brazil.

These secondary forests accompany bodies of water, creating continuous fragments that are challenging to characterize by size. Rather than using fragment size, we calculate forest cover from MapBiomas satellite imagery using a 1 km-radius buffer centered in the plot (SOUZA *et al.*, 2020). We selected ten of the NewFor plots to conduct this project (*Table 1*).

Table 1 Plot age, forest cover, and abundance and richness of zoochorous woody plant species (DBH >10 cm) within the ten plots of naturally regenerating secondary forest former pasture land in the Corumbataí river basin, São Paulo State, Brazil.

Plot	Age	Forest Cover (1 km)	Zoochoric Woody Plant Species	
			Abundance	Richness
1	29	0.144	39	8
2	41	0.125	44	6
3	50	0.143	47	10
4	35	0.151	46	13
5	20	0.045	34	10
6	39	0.058	58	18
7	25	0.060	44	14
8	16	0.053	37	11
9	21	0.186	45	7
10	53	0.120	24	11

3.2 BIRD CAPTURES AND DROPPING COLLECTION

3.2.1 Bird captures

Birds were captured to collect droppings once per month at each of the ten plots from August 2022 - August 2023. A total of five mist nets (12 x 3 m, 15 mm mesh) were assembled in an “L” formation along the plot edge, with plastic sheets placed under the nets to catch any bird droppings that fell while the individual was tangled in the net (*Fig. 2*). The mist nets were opened at sunrise and checked every 30 minutes until the nets were closed at 11:00. Bird captures and dropping collection were authorized by the Comissão de Ética no Uso de Animal (Decisão CEAU N° 05/2022) and the Sistema de Autorização e Informação em Biodiversidade (Sisbio; N°81210-1).

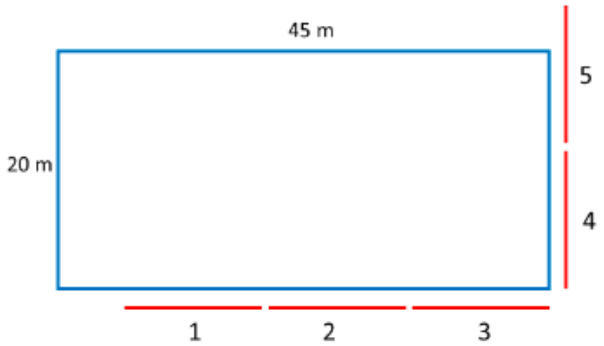

Fig. 2 Schematic of placement of 5 mist nets (12 x 3 m, 15 mm mesh) in an “L” formation along plot borders

Bird species captured were categorized in diet guilds based on EltonTraits (WILMAN *et al.*, 2014) and presence of seeds in feces. We define “fruit-eating” bird species as those that have a percent use of fruit in their diet greater than 0, which we consider as the potential to consume fruit (WILMAN *et al.*, 2014). This definition includes granivores, such as *Arremon flavirostris*. Because mist netting selectively captures species that occupy the understory, literature indicates that less than half of bird species present are usually captured using mist netting (DUNN; RALPH, 2002; MALLET-RODRIGUES; DE NORONHA, 2003). However, because these secondary forest fragments are relatively young, their canopy is lower, potentially increasing the proportion of bird species we capture using mist netting (DIDHAM; LAWTON, 1999). Species accumulation curve was created using the *vegan* package in R (Oksanen et al. 2016, R Core Team 2021).

3.2.2 Dropping collection

Droppings were collected using both plastic sheets under the mist net and by holding birds in a paper bag (*Fig. 3*). Upon capture, the plastic sheets under the mist net were inspected for droppings and if found, were collected (*Fig. 3C*). Regardless of collection from the plastic sheet, captured birds were placed in paper bags with a plastic liner and observed every 10 minutes for up to 30 minutes until the bird defecated or regurgitated (*Fig. 3B*). Bird droppings were placed in a 2 mL autoclaved Eppendorf tube with 99.5% alcohol and stored in a -20 °C freezer upon return from the field (*Fig. 3A*). After the bird has defecated (or 30 minutes after being extracted from the net), captured individuals were identified, weighed, and released.

Fig. 3 Bird dropping collection after mist netting in forest fragments in the Corumbataí river basin, Brazil. *A.* Bird droppings containing pulp and seeds collected from captured *Turdus leucomelas* individuals conserved in 99.5% alcohol in autoclaved 2 mL tubes *B.* Bird droppings in paper bag with plastic liner *C.* Bird droppings on the plastic sheet placed below the mist net.

3.3 SEED DISPERSAL

3.3.1 Quantification and identification of seeds found in droppings

Seeds were counted and identified by manually disassembling droppings in Petri dishes under a dissection scope to isolate the intact seeds. Seeds are still being identified using seed reference collections, including the seed reference collection being constructed from fruiting species within our plots and seed reference collections maintained by Dr. Marco Aurelio Pizo at UNESP and Dr. Wesley Silva at UNICAMP, in addition to other identification resources (Lorenzi, 1992). All intact seeds found will be photographed using a microscope camera, added to a digital reference collection, and deposited in Dr. Marco Aurelio Pizo's seed collection (UNESP).

3.3.2 Quantitative Component of Seed Dispersal Effectiveness

3.3.2.1 Habitat features

The quantitative component of seed dispersal effectiveness (SDE), based on bird activity and seed dispersal frequency, was calculated at the plot level. The quantity component represents a standardized measure of the average seed dispersal

per plot (SCHUPP *et al.*, 2010). The quantitative component of SDE takes into consideration the bird activity (which is a proxy of visitation frequency) and frequency of seed ingestion (which is a proxy of the quantity of seeds consumed). The first subcomponent was calculated by dividing the number of fruit-eating birds captured by the total number of net hours in the plot. Seed dispersal frequency was calculated by dividing the number of droppings found with seeds by the total number of droppings collected. We chose to calculate frequency using seed presence in droppings rather than the number of seeds in droppings due to the effect of seed size on the number of seeds consumed. While seed dispersal events were rare in some areas, consumption of small seeds (e.g. *Miconia* spp., Melastomataceae) inflated seed dispersal frequency. Additionally, we found calculations of seed dispersal frequency using presence of seeds in droppings and number of seeds in droppings to be significantly correlated (*Supplementary Material 2*). The SDE landscape was plotted to visualize the contribution of both quantitative subcomponents on plot-level SDE, using the *effect.lndscp* package in R (Schupp et al. 2010, R Core Team 2021).

Differences in SDE among plots were tested according to the following plot habitat features: forest cover, age, zoochoric tree species richness and abundance. Forest cover was calculated using a 1 km buffer of the plot with data from MapBiomas (Souza et al. 2020). Age of the forest was estimated using historical satellite imagery by the NewFor team, led by Pedro Brancalion (FAPESP/NWO #18/18416-2). Zoochoric tree species richness and abundance were quantified from detailed tree survey data, also collected by the NewFor team. We run separate linear models (LM) for each covariate using *stats* package in the R environment (Wilkinson and Rogers 1973, Chambers 1992). Plot-level SDE values were found to be normal according to a Shapiro-Wilks test ($p > 0.05$).

3.3.2.2 Fruit-eating birds

The quantitative component of seed dispersal effectiveness, based on bird activity and seed dispersal frequency was also calculated on a bird species level, pooling data from all plots. Bird activity was calculated by dividing the number of individuals of the species captured by the total number of net hours across all plots. Seed dispersal frequency was once again calculated by dividing the number of droppings found to contain seeds for each species by the total number of droppings

collected of that species. We modeled species-level SDE according to bird body mass and level of frugivory based on data from the literature (WILMAN *et al.*, 2014). Species-level SDE was not found to be normal according to a Shapiro-Wilks test ($p < 0.05$). We log-transformed species-level SDE and when tested again with a Shapiro-Wilks test, met the requirements of normality. We therefore run separate LMs for each of the two covariates. The SDE landscape was once again plotted (SCHUPP *et al.*, 2010) to visualize the contribution of both quantitative subcomponents on species-level SDE.

3.4 BIRD DIET

3.4.1 DNA metabarcoding of plant residue

While the presence of seeds in the droppings provides direct evidence of fruit consumption and seed dispersal, the use of DNA metabarcoding allowed us to evaluate if droppings contain additional vegetative material which could provide more complete information about seed dispersal and plant-based diet. We selected droppings from 32 individuals of 10 fruit-eating species (*Table 2*). Of the droppings selected, 29 contained seeds, while no seeds were found in three droppings. DNA extraction and library prep were conducted in the Laboratory of Molecular Ecology in the Department of Biodiversity at São Paulo State University (Universidade Estadual Paulista – UNESP), Rio Claro, São Paulo, Brazil.

3.4.1.1 DNA extraction protocol optimization

We tested a modified CTAB procedure on both fresh fruit material and droppings collected from birds in captivity to optimize our DNA extraction protocol. DNA was successfully extracted from guava (*Psidium guajava*), banana (*Musa* sp.), juçara (*Euterpe edulis*), and papaya (*Carica papaya*). Droppings were collected from Pale-breasted Thrush (*Turdus leucomelas*) in captivity offered guava fruit (*Psidium guajava*). Undergraduate student Vinicius Marciano De Godoy is evaluating the effect of collection time post-consumption and quantity of fruit consumed on the detection of plant DNA extracted from bird droppings using the same *T. leucomelas* individuals. The captive *T. leucomelas* used in this study are housed at UNESP, under the care of

post-doc Dr. Augusto Batisteli and Dr. Marco Pizo, and dropping collection was authorized by the Use of Animals Ethics Commission (Comissão de Ética no Uso de Animal; Decisão CEAU N° 05/2022).

Table 2 Captured bird individuals of which droppings were analyzed using DNA metabarcoding to detect plant residue

Family	Species	No. Individuals Sequenced
Pipridae	*Antilophia galeata*	11
	Manacus manacus	3
Thraupidae	*Eucometis penicillata*	2
	Ramphocelus carbo	1
	Tachyphonus coronatus	3
	Stilpnia cayana	1
	Thlypopsis sordida	1
Turdidae	*Turdus amaurochalinus*	2
	Turdus leucomelas	7
Tyrannidae	*Pitangus sulphuratus*	1
Total		32

3.4.1.2 DNA extraction

In order to preserve DNA integrity, a small amount of the sample (50 - 100 mg; 200 µL alcohol from sample) was reserved for DNA extraction prior to the manual removal of intact seeds. Samples were dried for two hours in a dry bath (65°C) under a fume hood before the extraction process. We used a modified CTAB protocol with increased lysis time and the use of sodium acetate and NaCl in the wash step. The full protocol can be found here.

3.4.1.3 Primer selection

We selected a universal plant mini-barcode within the *rbcLa* subregion of the *rbcL* (Ribulose-1,5-Bisphosphate Carboxylase) chloroplast gene (*rbc*L1 Forward – 5’ TTGGCAGCATTYCGAGTAACTCC 3’; rbcLB 5’ AACCYTCTTCAAAAAGGTC 3’) (LITTLE, 2014; PALMIERI *et al.*, 2009). This barcode results in a 184 bp fragment and

is therefore ideal for degraded plant DNA found in droppings (SHUTT *et al.*, 2021). We tested the selected primer on DNA from fresh fruit material and droppings collected from birds (*T. leucomelas*) in captivity extracted while testing DNA extraction protocol. Although species level identification is not guaranteed with a fragment this small, an additional barcode from another region (such as ITS) can be added to create a more robust analysis in the future, funding permitting. We selected this mini-barcode due to the development of a reference library of the species present in NewFor plots by post-doc Dr. Cássio Toledo using the *rbcL* region.

Six versions of the primer were fabricated with an Illumina sequencing overhang adapter and one of six 5 bp tags by Exxtend Biotecnologia, Paulinia, SP (AXTNER *et al.*, 2019) (*Fig. 4*). Utilizing tags, we economized by grouping samples, while still being able to separate samples according to tag in our bioinformatics pipeline and conduct analyses at an individual level (SARANHOLI *et al.*, 2023). Fragment length when accounting for the Illumina overhang adapter, tag, primer and target gene region (184 bp) was a total of 243 bp.

3.4.1.4 PCR protocol

PCR conditions were set according to post-doc Dr. Bruno Saranholi (UFSCar): 3 µL of the extracted DNA, 1.25 µL of 10 × reaction buffer, 1.25 µL of MgCl2 (25 mM), 0.8 µL of the dNTP mix (10 mM), 0.50 µL of each the forward and reverse primers (10 pmol), 0.2 µL of Taq polymerase (Taq Platinum) and 0.2 µL of BSA for a 12.5 µL total reaction volume. Cycling conditions consist of an initial denaturation at 94 °C for 10 min, followed by 30 cycles of 30 second denaturation at 94 °C, 30 second annealing at 56 °C and 40 second extension at 72 °C, and a final extension at 72 °C for 5 minutes. The same PCR conditions were used for all six versions of the primers. A blank sample was included for each of the six versions of the primers, where extracted DNA was replaced with water during PCR. Following amplification, PCR products were visualized using gel electrophoresis in a 1.5% agarose gel. Due to variation in amplification success within the same sample, each sample was amplified three times and 6 µL of each PCR product was combined into a single sample of 18 µL.

Tag	A	B	C	D	E	H
Sequence	TGCAT	TCAGC	AAGCG	ACAAG	AGTGG	GGATG

Fig. 4 Schematic demonstrating how multiple versions of the same primer were fabricated and the tags (AXTNER *et al.*, 2019) used to differentiate each of the six versions of the same primer are shown in the table.

3.4.1.5 Primer and sequencing protocol testing

In order to guarantee successful sequencing, we worked with EcoMol Consultoria (Piracicaba, SP) to test our methods in June 2023. DNA extracted from two samples (sample 027, *Turdus leucomelas*; sample 269, *Ramphocelus carbo*) was amplified using two, tagged versions of the selected primer (Tag A and B; *Fig. 4*). The two samples were grouped (combined into the same tube) and sent for sequencing (see sequencing details below). With the successful sequencing and bioinformatics analysis of these two grouped samples, we repeated our tested methods with an additional 30 samples, resulting in a total of 32 samples sent for sequencing.

3.4.1.6 Sample preparation and grouping

The additional 30 samples were grouped in sets of five based on bird body size and band intensity (*Fig. 5*). When possible, samples from birds of the same species were therefore grouped together. We avoided grouping samples that had strong bands with weakly amplified samples. A volume of 6 µL was taken from each of the five samples and combined in a single tube according to grouping, resulting in a final volume of 30 µL. A grouped sample of blanks was also combined using 5 µL of each tag associated blank, resulting in a final volume of 30 µL.

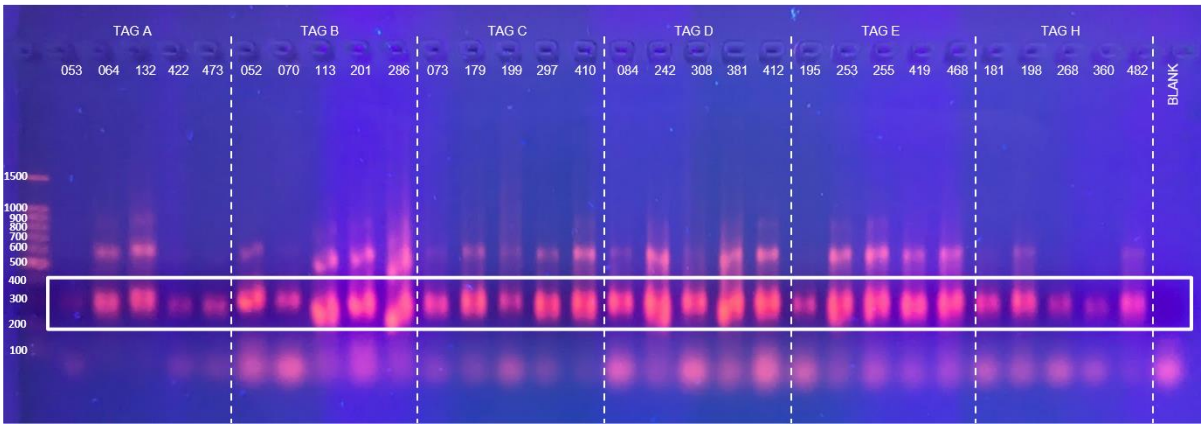

Fig. 5 Gel electrophoresis demonstrating the PCR product of a mini-barcode of the rbcL gene region amplified from DNA extracted from bird droppings. Each well contains PCR product from a unique dropping sample. Six versions of the same primer with unique tags (A, B, C, D, E, H; *Fig. 4*) are shown here, with five samples amplified with each primer version. The target region is highlighted in a white box, showing the final sequence length of 243 bp. A pooled sample of blanks is shown in the final well and did not amplify.

3.4.1.7 Sequencing

Sequencing was performed by EcoMol Consultoria. Grouped samples were purified using magnetic beads (Agencourt AMPure XP® – Beckman Coulter) and quantified using a Qubit fluorometer (ThermoFisher). Standardized samples (50 ng/µL) were indexed using a Nextera Index kit® (Illumina) and the pooled library was quantified using real-time PCR (Kapa Biosystems). Paired-end metabarcoding sequencing was performed on an Illumina iSeq® platform, using an iSeq 100 v2 300 Cycle Reagent kit (2 × 150 bp), for a total of 70,000 reads per each of the six sets of sequencing samples (five sets of samples and one set of blanks).

3.4.1.8 Bioinformatics pipeline

Bioinformatics processing was conducted following Saranholi et al. 2023. Obtained sequences were inspected using FastQC software to confirm sequence quality (> 33 Phred score) (ANDREWS, 2010). Sequences were then demultiplexed with the process_radtags program in Stacks v2.59 (CATCHEN *et al.*, 2013) in order to separate sequences by tag and retrieve individual-level sample information (*Fig. 4*). Using PEAR, corresponding forward and reverse sequences were merged and trimmed using the following conditions: a minimum overlap (−v) of 60 bp, a minimum length (−n) of 100 bp, and minimum quality score threshold (−q) of 15 (RODGERS *et al.*, 2017; ZHANG *et al.*, 2007). Singletons were discarded and Operational Taxonomic

Units (OTUs) were obtained by clustering unique sequences with at least 97% similarity using USEARCH v.11.0.667 (EDGAR, 2010).

3.4.1.9 Identification of Operational Taxonomic Units

Basic Local Alignment Search Tool (BLAST) was used to compare OTUs to reference sequences available from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and Barcode of Life Data Systems (BOLD; https://www.boldsystems.org/index.php). Due to the small fragment size of the amplified region (184 bp), a 100% match was achieved with multiple plant species. High plant diversity within the region resulted in multiple possible species and identifications were therefore maintained at a genus level. If genus-level assignment was not possible due to 100% match to two genera, both genera were indicated as possible identifications. Further filtering of identified genera was conducted by evaluating geographic distribution, cross-referencing species lists of our study area, and consulting local botanists (Juliano Zardetto). Geographic distribution was evaluated using the Global Biodiversity Information Facility (https://www.gbif.org/) and lists of woody tree species (individuals with > 10 DBH) present in NewFor plots throughout the region were consulted. OTUs with poor identity match to any species (< 97.99%) and below the 0.05% read threshold were removed. The read threshold was determined by calculating 0.05% of reads for each run following bioinformatic cleaning (23036 - 44492), with a resulting minimum number of reads ranging between 11 - 22 (N = 5, sequencing libraries). Read thresholds are often implemented to avoid including OTUs that result from sequencing artifacts (BROADHURST *et al.*, 2021; LOZANO MOJICA; CABALLERO, 2021; SALES *et al.*, 2020).

3.4.2 Plant diet profile

3.4.2.1 Fruit-eating birds

The frequency of consumption of plant genera was calculated by dividing the number of bird species in which a genera was detected by the total number of species sequenced (n = 10). Diet richness was calculated by dividing the number of

plant genera detected per species by the total number of individuals (droppings) sequenced.

3.4.2.2 Plant features

Plant genera were classified as one of four life forms: tree, shrub, climbing, herbaceous. Classifications were determined using plant life form described on Reflora (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, 2024). Climbing plants include lianas and vines and, to reduce the number of categories, we considered graminoids to be herbaceous.

4 RESULTS

4.1 BIRD CAPTURES

Birds were captured using mist netting in each of the ten selected plots from August 2022 to August 2023 for a total of 2758 net hours (*Table 3*). Between 12 and 25 bird species were captured in each plot, with a total of 58 species and 38 fruit-eating species captured across all plots. We consider species “fruit-eating” if the percent use of fruit in their diet defined by EltonTraits is greater than 0, or if seeds were found in droppings. A total of 443 individuals were captured, with 483 captures (40 recaptures) of all species and 273 captures (10 recaptures) of fruit-eating species (*Supplementary Material 3*). Capture rate per net hour averaged 0.175 ± 0.05 for all species and 0.099 ± 0.03 for fruit-eating species across all ten plots. Both species accumulation curves for all species and fruit-eating species approach an asymptote and the curve of fruit-eating species leveled off more than all species (*Fig. 6*).

Table 3 Summary of net hours, species and individuals captured, and capture rate of birds (all and fruit-eating only) captured from August 2022 - August 2023 across ten plots of secondary Atlantic Forest in Corumbataí river basin, São Paulo.

Plot	Net Hours	No. Species	No. Fruit-eating Species	No. Captures/ Recaptures	No. Fruit-eating Captures/ Recaptures	Capture Rate ± SD	Capture Rate of Fruit-eating Species ± SD
1	274	25	17	83/8	44/2	0.303	0.161
2	276	19	12	49/3	22/0	0.178	0.080
3	288	21	16	36/1	27/1	0.125	0.094
4	306	22	14	50/4	29/1	0.163	0.095
5	272	21	14	45/2	24/0	0.165	0.088
6	269	25	17	58/9	30/2	0.216	0.112
7	272	18	14	38/4	25/2	0.140	0.092
8	276	19	16	48/1	37/0	0.174	0.134
9	257	12	8	32/5	10/0	0.125	0.039
10	268	21	15	44/3	25/2	0.164	0.093
	2758	**58**	**38**	**483/40**	**273**	**0.175 ± 0.05**	**0.099 ± 0.03**

* We consider species “fruit-eating” if the percent use of fruit in their diet defined by EltonTraits is greater than 0, or if seeds were found in droppings.

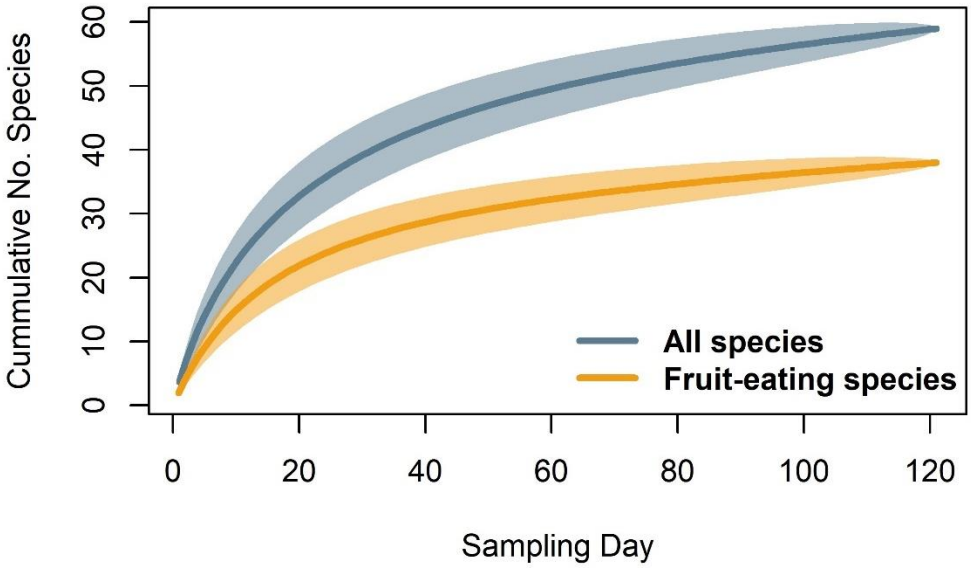

Fig. 6 Species accumulation curves of all and fruit-eating species of birds captured from August 2022 - August 2023 in plots of secondary Atlantic Forest in Corumbataí river basin, São Paulo. All species are shown in blue while exclusively fruit-eating species are shown in orange.

4.2 DROPPING COLLECTION

Droppings were collected from 417 of 483 captures, with between 31 and 66 droppings collected per plot (*Fig. 7*). Out of the 417 droppings collected across all plots, 65 droppings (15%) contained seeds, although an additional 212 dropping samples were collected from fruit-eating birds.

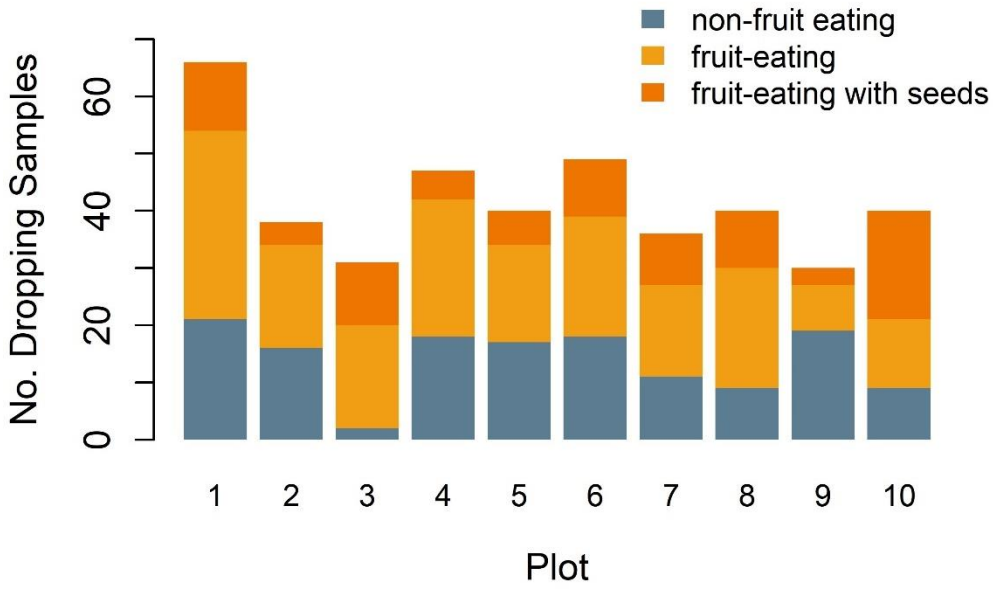

Fig. 7 Number of droppings collected from non-fruit eating, fruit-eating, and fruit-eating species with seeds of birds captured in plots of secondary Atlantic Forest in Corumbataí river basin, São Paulo.

4.3 SEED DISPERSAL

4.3.1 Quantification and identification of seeds

A total of 1,970 seeds were separated from the 65 dropping samples collected from 17 species (*Fig. 8*). The number of seeds collected per plot varied greatly, ranging from 11 to 696. We also observed substantial variation in the number of seeds found per bird species, ranging from 2 - 602, with between 1 - 195 seeds per dropping per species, and a median of 43 seeds per dropping per species. We classified the seeds as 42 unique morphospecies, with 28 morphospecies identified to family and 18 identified to genus (*Table 4*). Currently unidentified morphospecies will be identified using existing seed reference collections from W. Silva (Unicamp), M. Pizo (Unesp) and the group's own collections (collections are still being made by other students).

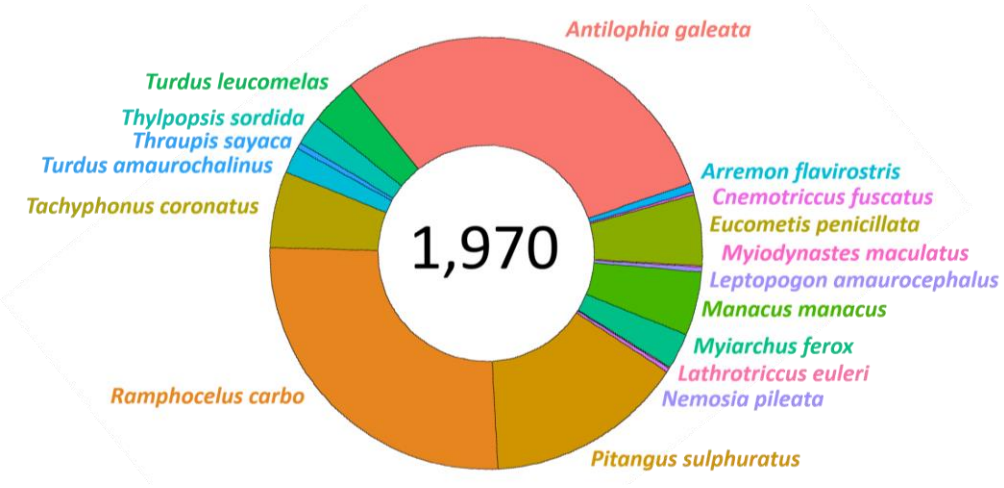

Fig 8. Proportion of seeds found in droppings per species from birds captured in ten plots of secondary Atlantic Forest in Corumbataí river basin, São Paulo. A total of 1970 seeds were recovered from 65 of 417 droppings. Colors are intended to be distinct and do not have significance.

4.3.2 Quantitative component of seed dispersal effectiveness

4.3.2.1 Habitat features

We observed differences in seed dispersal effectiveness (SDE) among plots due to variation in both sub-components, with plots 10 and 08 experiencing higher levels of seed dispersal, followed by plots 01 and 07 (*Fig. 9*). Conversely, seed dispersal in plots 02 and 09 was six-fold less effective (*Fig. 9*). We did not find any statistically significant model of plot SDE with respect to any of the four habitat features (*Supplemental Material 4*; *Fig. 10*). Therefore, variation in SDE among plots cannot be explained by forest cover, age, zoochoric tree species richness or abundance.

Table 4. Families and genera currently identified from the 45 morphospecies of seeds found in droppings. 28 morphospecies have been identified to family and 18 identified to genus.

Family	**Genus**	**No. Morphospecies**	**No. Seeds**
Amaranthaceae	unknown	1	7
Asteraceae	*Tilesia*	1	1
Euphorbiaceae	unknown	1	1
Fabaceae	unknown	1	2
Heliotropiaceae	*Myriopus*	1	8
Loranthaceae	*Struthanthus*	1	2
Melastomataceae	*Miconia*	2	1047
	unknown	2	134
Meliaceae	*Trichilia*	1	7
Moraceae	*Morus*	1	2
Myrtaceae	unknown	3	6
Peraceae	*Pera*	1	2
Piperaceae	*Piper*	2	186
Primulaceae	*Myrsine*	1	6
Rosaceae	*Rubus*	1	15
Rubiaceae	*Psychotria*	1	10
Salicaceae	*Casearia*	1	222
Sapindaceae	*Allophylus*	1	3
Siparunaceae	*Siparuna*	1	1
Solanaceae	*Cestrum*	1	8
	unknown	1	117
Urticaceae	*Cecropia*	1	49
Verbenaceae	unknown	1	1
unknown	unknown	17	133

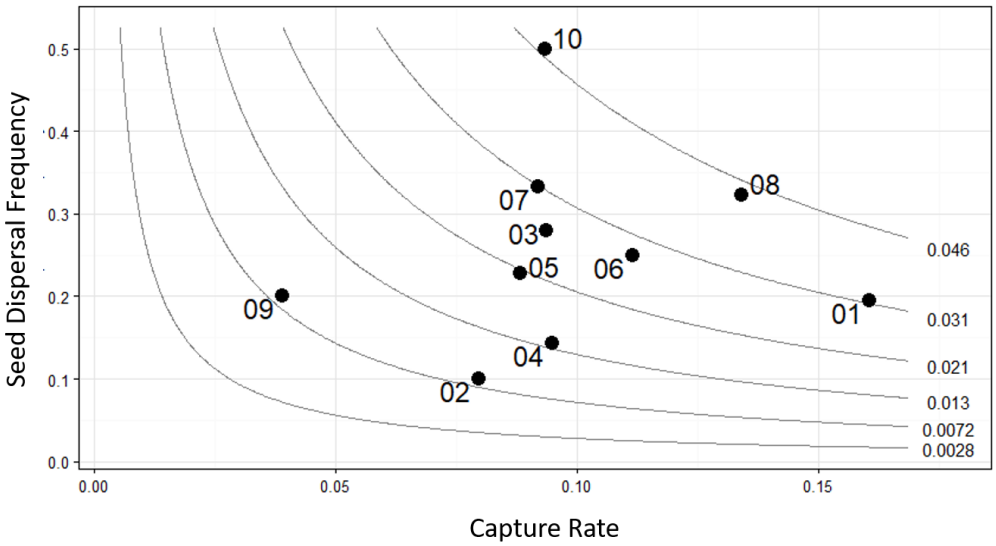

Fig 9. Seed dispersal effectiveness (SDE) landscape where each point represents one of ten plots of secondary Atlantic Forest where birds were captured in Corumbataí river basin, São Paulo. Isoclines connect all values of seed dispersal frequency and capture rate that yield the same SDE (Schupp, Jordano & Gómez 2010).

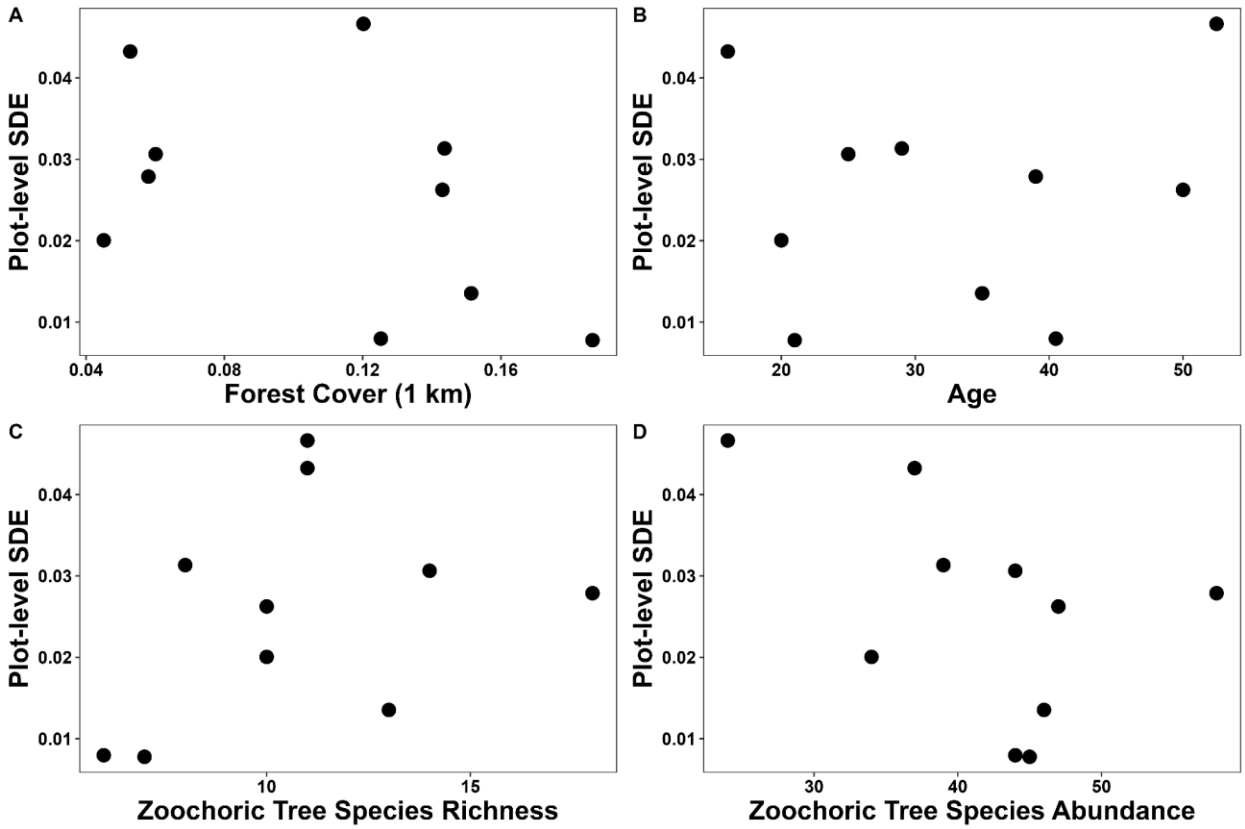

Fig 10. Relationship between habitat features and plot-level seed dispersal effectiveness (SDE) where each point represents a plot. A) Forest cover in a 1 km buffer around plot and SDE. B) Forest age estimated by historic satellite images and SDE. C) Zoochoric tree species richness within the plot and SDE. D) Zoochoric tree species abundance within the plot and SDE.

4.3.2.2 Fruit-eating birds

We observed differences in seed dispersal effectiveness among species, with *Antilophia galeata* being the most effective seed disperser in the region's secondary forests, followed by *Turdus leucomelas*, *Tachyphonus coronatus* and *Ramphocelus carbo* (*Fig. 11*). We tested for differences in SDE among bird species according to body mass and the proportion of diet that is composed of fruit. While no relationship was found between body mass and SDE (R^2 = 0.129, p-value = 0.157), a significant, positive relationship was found between the percentage of diet that includes fruit and SDE (*Fig 12*; R^2 = 0.19, p-value = 0.04). To evaluate whether the level of frugivory associated more strongly with a specific subcomponent of SDE, we modeled capture rate and seed dispersal frequency separately with the percentage of diet that includes fruit. No significant relationship was found with capture rate (R^2 = 0.145, p-value = 0.132) or seed dispersal frequency (R^2 = 0.0096, p-value = 0.708), indicating that diet is associated with the combination of seed dispersal frequency and capture rate, but not with individual sub-components (*Supplementary Material 4*).

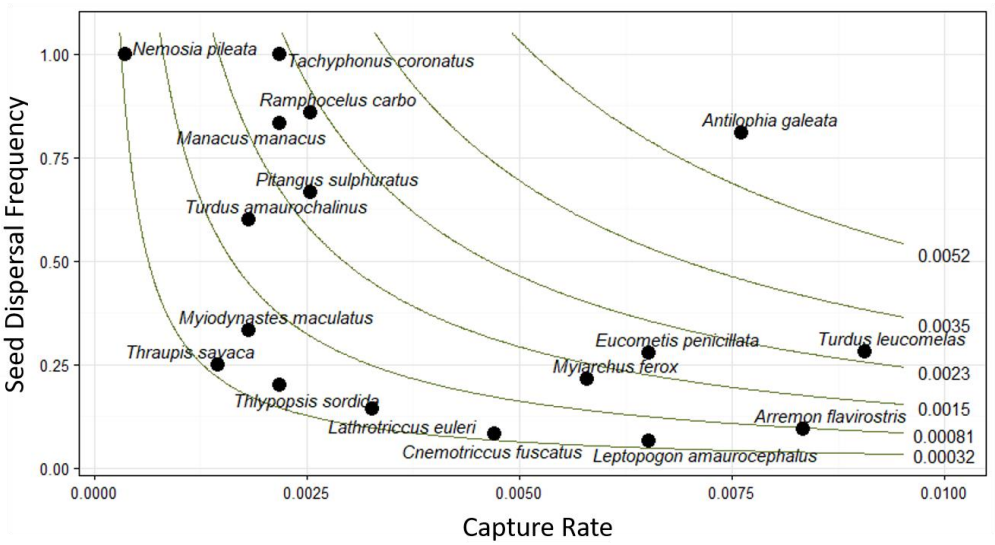

Fig 11. Seed dispersal effectiveness landscape where each point represents a species of bird captured across all ten plots in secondary Atlantic Forest in Corumbataí river basin, São Paulo. Isoclines connect all values of seed dispersal frequency and capture rate that yield the same SDE (Schupp, Jordano & Gómez 2010)

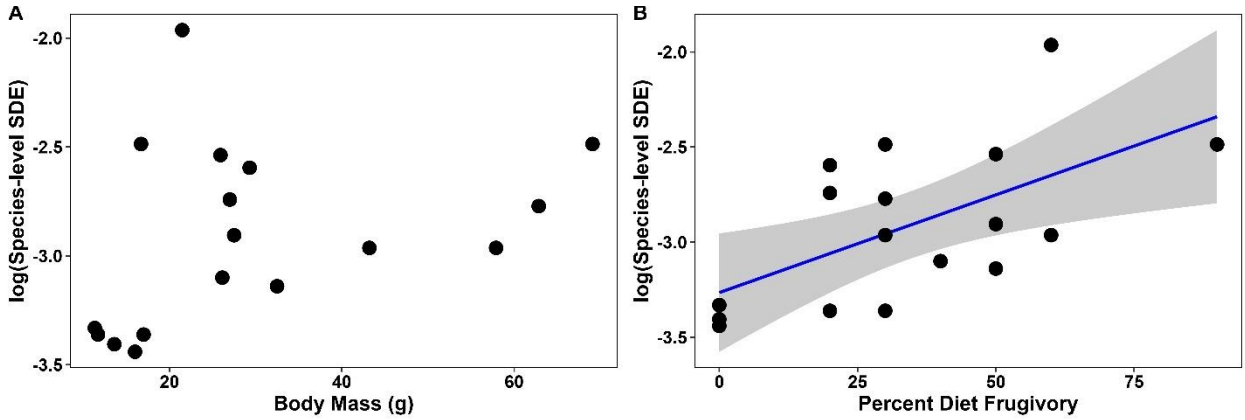

Fig 12. Relationship between bird species characteristics and log transformed species-level seed dispersal effectiveness (SDE) where each point represents a species. A) Body mass of each species. B) Degree of frugivory represented by the percent of diet composed of fruit for each species.

4.4 BIRD PLANT DIET

4.4.1 Metabarcoding of plant residue

Before quality filtering, the combined read count of the five pools resulted in a total of 161,170 paired reads, with a mean of 880.71 ± 4440.15 paired reads per OTU. All operational taxonomic units (OTUs) produced belonged to plant species, with a total of 183 OTUs produced. Following quality filtering, 50 OTUs were maintained while 111 were discarded due to having an insufficient number of reads (< 0.05% total paired reads from pool), including one sample of *Antilophia galeata* (sample number 360). An additional 23 OTUs were discarded due to an insufficient (< 98%) match with any organism. A total of 155,267 paired reads from 31 samples were maintained, with an average of 3168.71 ± 8204.28 per OTU. The mean number of reads retrieved per individual, as well as per species (adjusted for sampling effort), varied greatly, with an average of 5008.613 ± 10580.97 and 7554.07 ± 13215.44, respectively. We identified all 50 OTUs retained from 23 plant families. We assigned 48 to the genus level, with the remaining 2 OTUs classified as being one of two genera. The number of detections per OTU was relatively low (mean ± SD: 1.70 ± 1.81). For each bird individual sequenced, we retrieved on average 1.61 ± 1.08 OTUs, indicating that more sampling is necessary to register a majority of diet items (*Fig. 13*). At least an additional 30 samples will be sequenced to complement bird diet data in June of 2024.

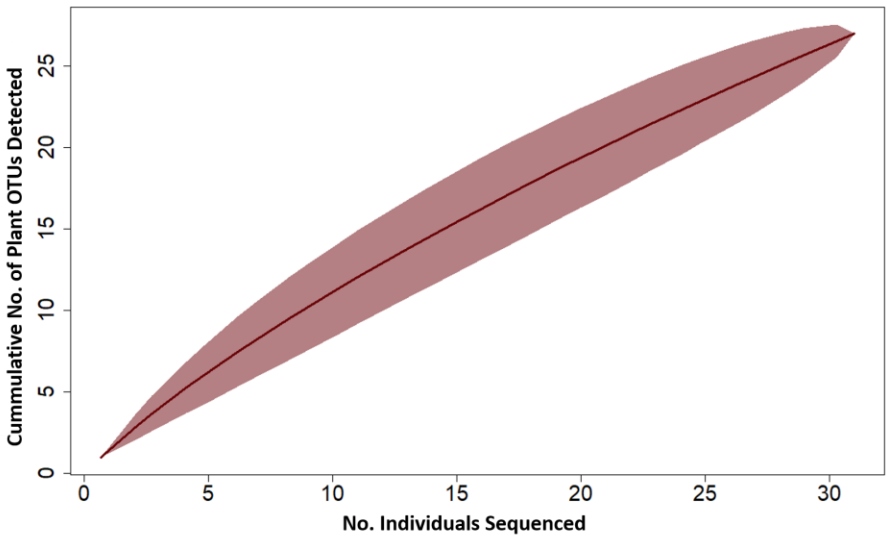

Fig 13. Accumulation curve of cumulative number of plant operational taxonomic units (OTU) detected per number of bird individuals (droppings) sequenced.

4.4.2 Plant diet profile

4.4.2.1 Fruit-eating birds

Consumption frequency ranged between 0.1 - 0.7, with *Miconia* spp. (Melastomataceae), *Casearia* spp. (Salicaceae), and *Serjania*/*Paullinia* (Sapindaceae) being most frequently consumed (*Fig. 14*). Richness of diet ranged between 1 - 3 genera with *Thlypopsis sordida*, *Tachyphonus coronatus*, *Manacus manacus*, *Turdus leucomelas*, and *Antilophia galeata* having an average of > 1 genera detected per individual (dropping) sequenced.

4.4.2.2 Plant features

Plants were classified as one of four life forms, with tree (n=14) being most common, followed by shrub (n=7), climbing (n=3), and herbaceous (n=2) life forms. We detected four genera of exotic plants: *Leucaena* (Fabaceae), *Morus* (Moraceae), *Oryza* (Poaceae), and *Tilesia* (Asteraceae) (*Fig. 15*).

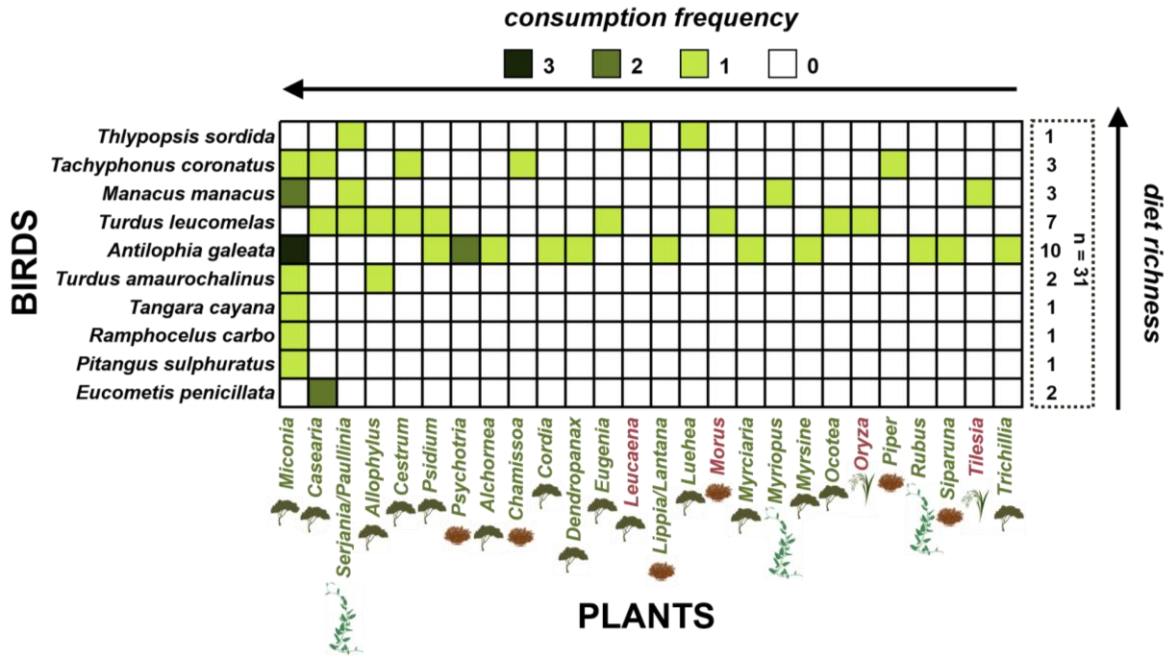

Fig. 14 Heatmap of plant genera present in bird diet. The color at each intersection between plant genera and bird species indicates the number of individuals that the plant genera was detected (0, 1, 2, or 3). Birds are ordered from top to bottom by decreasing diet richness and plants are ordered from left to right by decreasing consumption frequency. Icon next to plant genera indicates life form and corresponds to Fig. 15. Color of plant genera name indicates whether the genus is exotic or native, and corresponds to Fig. 15.

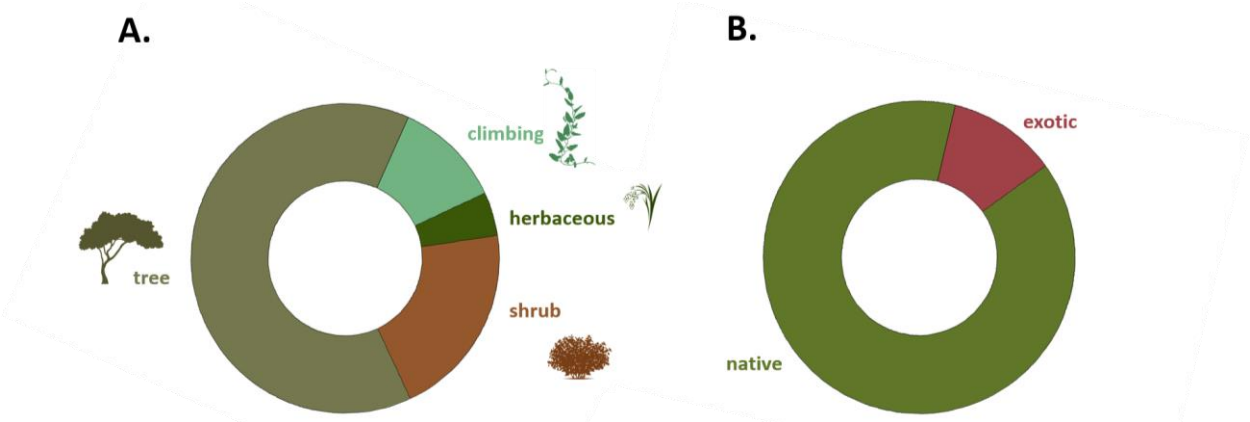

Fig. 15 Proportion of plant life forms and genera origin detected. A) Frequency with which trees, shrubs, climbing, and herbaceous plants were detected. Icon next to the plant life form corresponds to Fig. 14. B) Frequency with which exotic and native plant genera were detected. Color of plant origin corresponds to Fig. 14.

5 DISCUSSION

Our study evaluated the community of seed dispersers and plants being consumed in secondary forests by integrating bird capture data with the genetic analysis of bird droppings. Although SDE varied more than six times across plots, we did not observe an increase in plot-level quantitative SDE in relation to forest age, cover, or the zoochorous woody plant community. As expected, we found a significant, positive correlation between species-level SDE and the percent of bird diet composed of frugivory. Body mass, however, was not associated with species SDE. Our diet analysis indicated several native trees as important resources, while also revealed the presence of exotic species in bird diet as well as shade-intolerant plants most likely found outside forested patches, consistent with our predictions.

We observed a surprising amount of heterogeneity in plant-bird mutualisms among secondary forest communities, even among plots close in proximity and within the same forest fragment. Plot-level SDE values range from ~0.0072 to ~0.046, with the ten plots fairly evenly distributed across this range (*Fig. 9*). While forest age, cover, and the zoochorous woody plant community indicate habitat quality and serve as proxies for resource availability, the lack of relationship between these features and plot-level SDE may be due to features associated with secondary forest communities (SELWYN *et al.*, 2023; TABARELLI; PERES, 2001). These early successional communities are comprised of young plant individuals, which may have not reached reproductive age yet and, therefore, do not offer large amounts food resources to frugivores (GERARDO *et al.*, 2023). Additionally, previous land-use may have degraded the soil, resulting in low fruit productivity (JAKOVAC *et al.*, 2021; MANCINI *et al.*, 2020). Finally, our quantification of the plant community only accounted for woody, zoochorous individuals, and did not include other, potentially important resources from shrubs, vines, herbaceous plants, and even anthropogenic food sources, such as domestic animal feeders, planted orchards or discarded food (GUARIGUATA; OSTERTAG, 2001; SHUTT *et al.*, 2021). Differences in fruit availability from non-tree resources could explain why plots with similar habitat features had distinct SDE values. Plots 01 and 09 have a similar age, forest cover, and zoochorous woody plant community, yet plot 01 a SDE value four times that of plot 09 (*Table 1*; *Fig. 9*). Local variation in fruit availability would also offer an explanation as to why plots within the same forest fragment have radically different SDE values, such as plot 01 and plot 02 (*Fig. 9*). Simultaneously, local, temporary habitat features such as

resource availability would explain why plots very close in proximity, such as plot 06 and plot 07 have similar SDE values, while a third plot (plot 08) located in the same fragment, but further away, has a higher, distinct SDE value (HERRERA *et al.*, 2011). The absence of significant relationships between SDE and tested habitat features among plots may indicate that seed dispersal dynamics are being driven by other extrinsic factors (HOHMANN *et al.*, 2023; SCHUPP *et al.*, 2019). Alternatively, it is likely that these secondary forest communities are heavily driven by stochastic processes, causing SDE to be determined by the random arrival of species that is not reflected by tested covariates (BRADFER-LAWRENCE *et al.*, 2018; HIURA, 2001). Therefore, while seed dispersal dynamics may be defined by ephemeral, non-tree fruit resources, stochastic processes also likely play a role in determining plant-animal mutualisms in these secondary forest communities.

Analyses of quantitative SDE among bird species reaffirm knowledge of principal bird seed dispersers and show that SDE is determined by bird diet, but not by body mass. Bird species with the highest SDE values (i.e., *A. galeata*, *T. leucomelas*) are known as fruit-eaters and as being effective seed dispersers (e.g., Pizo 2004, Silva and De Melo 2011), while opportunistic frugivores that mainly consume insects (i.e., *L. euleri*, *C. fuscatus*) were found to have a lower SDE values (e.g., Silva and De Melo 2011, Gaiotti and Pinho 2013). Bird body mass is known to affect quantitative SDE by correlating with fruit ingestion rates (NEVO *et al.*, 2023; SCHUPP, 1993), however, our results did not show this relationship (*Fig. 12*). This may be explained by two non-exclusive reasons. First, body mass usually affects the number of fruits consumed per visit, but we only quantified the presence of seed in droppings, which seemingly does not correlate directly with disperser size. Second, frugivore avian assemblage in the area is composed of small to medium-bodied birds, lacking variation due to the absence of larger seed dispersers. Specifically, the largest captured fruit-eating bird in the area was *Leptotila verreauxi* (Columbidae; 146.88 g; de Lima et al. 2015). Larger, seed dispersing bird species are likely not yet present, or present in low numbers, due to the young age and dynamic nature of these forests, resulting in a bird community comprised of species with a lower body mass (MAHONEY *et al.*, 2018). The absence of larger avian frugivores is known to have negative impacts on long-distance seed dispersal and overall robustness of seed dispersal networks, which is consistent with the overall low seed dispersal we see in these areas (DONOSO *et al.*, 2020; WOTTON; KELLY, 2012). While

the percent frugivory of bird diet is significantly related to species-level quantitative SDE, when the individual subcomponents of seed dispersal frequency and capture rate were analyzed separately, we did not observe a significant relationship (*Supplementary Material 4).* Diet is therefore associated with the interaction between the subcomponents, indicating that diet determines the SDE of a bird species only when birds both are active in the area and frequently feeding on fruits. Changes in capture rate could be explained by the effect of fruit availability on fruit-eating bird activity, which can then explain changes in bird diet, affecting seed dispersal frequency (LOISELLE; LAKE, 1999; QUITIÁN *et al.*, 2019).

The diet composition of fruit-eating birds based on seeds and DNA indicates several native plant species as key resources, as well as confirmed the presence of exotic plants and potentially anthropogenic resources in bird diet. We see a high frequency of consumption of *Miconia* spp. (Melastomataceae), which is known to be a keystone resource in forest communities, with at 13% of Neotropical bird species depending on *Miconia* spp. As a food resource (MESSEDER *et al.*, 2020). While trees are most frequently consumed, we also detected the frequent consumption of climbing plants and shrubs, as expected in these early successional communities (MARQUES *et al.*, 2014). Half of the exotic species detected were present in the diet of *T. leucomelas*, a habitat generalist species found in both forested and urban areas (PIZO, 2004). The *Oryza* sp. Detected in *T. leucomelas* diet was likely cooked rice that was discarded by humans near the forest fragment, demonstrating the use of novel food sources (BATISTELI, 2020). Other exotic species detected include self-dispersed, dry fruit producing *Leucaena* sp. (Fabaceae), suggesting potential use of other plant parts (i.e. flowers, leaves) (MACHADO *et al.*, 2020). While not a known food source for fruit-eating birds, *Leucaena leucocephala* leaves are used as poultry and livestock feed (AYSSIWEDE *et al.*, 2010; D'MELLO; ACAMOVIC, 1989). We also see a strictly forest dwelling bird species, *M. manacus*, utilizing an exotic, fleshy-fruit producing Asteraceae (*Tilesia* sp.) that likely grows on the forest border (CESTARI; PIZO, 2013; FONSECA *et al.*, 2013). Overall, our diet analyses indicate that while birds depend on several native plant species, these fruit-eating birds are expanding the diet breadth to include novel food items and foraging outside of the forest, potentially connecting forest patches and introducing seeds from neighboring areas.

Diet analysis of fruit-eating bird species paired with knowledge of the most effective seed dispersers provides a new context to bird-plant mutualisms within these novel secondary forest communities. The presence of light-loving plants, such as climbing and herbaceous species, as well as human food items in fruit-eating bird diet suggests foraging in forest adjacent areas. The diet content of *T. leucomelas* combined with a high value of quantitative SDE of this species indicates that *T. leucomelas* is a particularly important species in connecting fragments and not only brings seeds to the forests, but also transports seeds to neighboring, open areas. However, *T. leucomelas* will therefore also be consuming and dispersing exotic species. Meanwhile, *A. galeata*, a forest species, principally consumes fruits from tree and shrub species that likely occur within the forest, suggesting that *A. galeata* as an important species for maintaining seed dispersal within the fragment (MELO *et al.*, 2003). While we were able to identify principal seed dispersers by quantifying SDE on a species level, our diet analysis provided additional context to the dispersal potential of the principal seed dispersers.

We applied traditional field methods in conjunction with molecular analyses to study plant-bird mutualisms in these novel, secondary forests. Although the lack of a positive correlation between SDE and habitat features known to affect SDE in other systems may be due to our limited sampling period of one year, it is possible that birds are moving across the region regardless of forest age or amount, resulting in spatially heterogeneous seed dispersal. Meanwhile, diet continues to determine quantitative SDE among bird species, although the lack of influence of bird size on SDE is likely due to a reduced number of larger bird species as a consequence of successional stage of these forests. Our genetic analysis of bird diet provided new insights that would otherwise have been undetected if relying solely on seed presence in droppings. Additionally, the detection of several diet items, such as *Leucaena* sp., raises further questions about bird diet, which may include other plant parts apart from fruit pulp and seeds. Our next steps include the expansion of our diet analysis to include all samples collected with seeds as well as further investigation into the use of border-dwelling plant species in bird diet. We also plan on using phenology data collected in these plots combined with camera trap data to evaluate the fruit resources available during our collection period. While bringing new knowledge of plants and their seed dispersers, our study highlights the need to further study plant-bird mutualisms in these novel, secondary forests.

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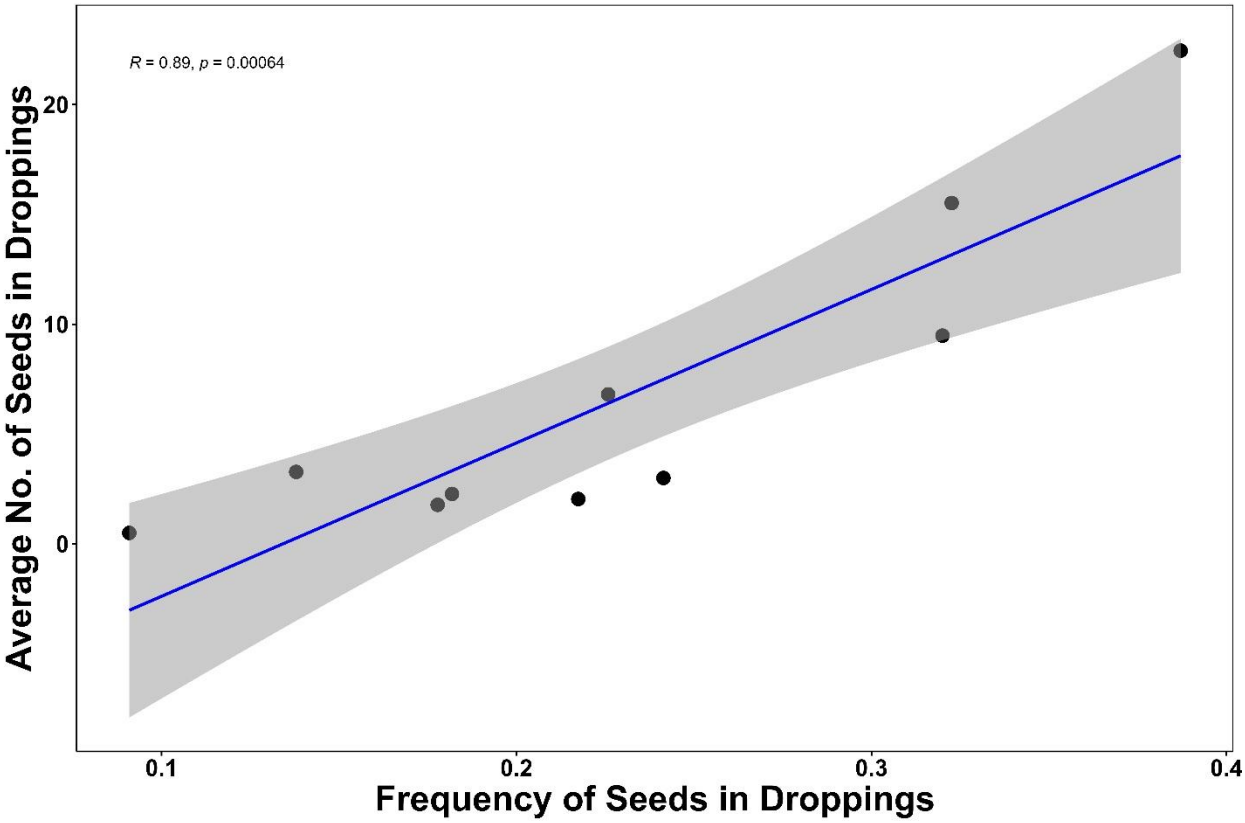
SUPPLEMENTARY MATERIAL

Supplementary Material 1 Zoochorous woody plant species present in study area of secondary forest plots in the Corumbataí River Basin in São Paulo, Brazil. Unpublished dataset from Brancalion et al.

Family	Species
Anacardiaceae	*Mangifera indica*
	Tapirira guianensis
Annonaceae	*Annona sylvatica*
Araliaceae	*Dendropanax cuneatus*
Arecaceae	*Syagrus romanzoffiana*
Boraginaceae	*Cordia ecalyculata*
Cannabaceae	*Celtis cf. iguanaea*
	Trema micrantha
Celastraceae	*Maytenus gonoclada*
Ebenaceae	*Diospyros inconstans*
Euphorbiaceae	*Alchornea glandulosa*
	Sapium glandulosum
Fabaceae	*Andira fraxinifolia*
	Copaifera langsdorffii
	Enterolobium contortisiliquum
	Inga vera
Lamiaceae	*Aegiphila integrifolia*
Lauraceae	*Nectandra lanceolata*
	Nectandra megapotamica
	Ocotea puberula
	Ocotea pulchella
Malvaceae	*Guazuma ulmifolia*
Melastomataceae	*Miconia albicans*
Meliaceae	*Cabralea canjerana*
	Melia azedarach
	Trichilia catigua
	Trichilia clausseni
	Trichilia elegans
	Trichilia pallida
Moraceae	*Ficus guaranitica*
	Maclura tinctoria
Myrtaceae	*Campomanesia xanthocarpa*
	Eugenia dodonaeifolia
	Eugenia florida
	Eugenia pyriformis
	Myrcia splendens
	Myrcia tomentosa
	Myrciaria floribunda
	Psidium guajava
	Psidium sartorianum
Nyctaginaceae	*Guapira opposita*

Peraceae	*Pera glabrata*
Primulaceae	*Myrsine coriacea*
	Myrsine umbellate
Rhamnaceae	*Rhamnidium elaeocarpum*
Rosaceae	*Eriobotrya japonica*
Rubiaceae	*Rudgea jasminoides*
Rutaceae	*Zanthoxylum rhoifolium*
	Zanthoxylum riedelianum
Salicaceae	*Casearia decandra*
	Casearia gossypiosperma
	Casearia sylvestris
Sapindaceae	*Allophylus edulis*
	Cupania vernalis
	Matayba elaeagnoides
Sapotaceae	*Chrysophyllum gonocarpum*
Siparunaceae	*Siparuna guianensis*
Solanaceae	*Acnistus arborescens*
Urticaceae	*Cecropia pachystachya*



Supplementary Material 2 Significant relationship between frequency of seed presence in droppings and average number of seeds in droppings (R = 0.89, p-value = 0.00064)

Supplementary Material 3 Summary of captures per species. Number of captures, recaptures, dropping samples collected, number of dropping samples with seeds, total number of seeds found in droppings, fruit-eating classification (y = yes, n = no) and body mass. Fruit-eating classification and body mass pulled from EltonTraits (WILMAN *et al.*, 2014).

Family	Species	Captures	Recaptures	Dropping Samples	Samples with Seeds	No. Seeds in Samples	Fruit-eating	Body Mass (g)
Bucconidae	*Malacoptila striata*	3	0	2	0	0	n	44.1
Caprimulgidae	*Nyctidromus albicollis*	1	0	0	0	0	n	57.84
Cardinalidae	*Habia rubica*	1	0	1	0	0	y	32.46
Coerebidae	*Coereba flaveola*	10	0	6	0	0	n	10.01
Columbidae	*Leptotila verreauxi*	11	0	9	0	0	y	146.88
Conopophagidae	*Conopophaga lineata*	8	1	7	0	0	y	25.21
Dendrocolaptidae	*Lepidocolaptes angustirostris*	4	1	4	0	0	n	29.59
Dendrocolaptidae	*Sittasomus griseicapillus*	10	0	4	0	0	n	13.12
Emberizidae	*Arremon flavirostris*	23	2	21	2	13	y	26.13
Emberizidae	*Asemospiza fuliginosa*	9	0	7	0	0	y	13.3
Emberizidae	*Coryphospingus cucullatus*	5	0	5	0	0	y	14.28
Emberizidae	*Sporophila sp.*	11	1	11	0	0	y	13.3
Emberizidae	*Volatinia jacarina*	2	0	2	0	0	y	9.94
Furnariidae	*Automolus leucophthalmus*	3	0	2	0	0	n	34.5
Furnariidae	*Clibanornis rectirostris*	3	0	3	0	0	n	48
Furnariidae	*Cranioleuca vulpina*	1	0	0	0	0	n	15.7
Furnariidae	*Synallaxis frontalis*	7	0	3	0	0	n	14
Furnariidae	*Synallaxis ruficapilla*	5	0	1	0	0	n	13.8
Furnariidae	*Xenops rutilans*	1	0	1	0	0	n	11.2
Galbulidae	*Galbula ruficauda*	2	0	1	0	0	n	26.5
Hirundinidae	*Stelgidopteryx ruficollis*	1	0	1	0	0	n	16.1
Momotidae	*Baryphthengus ruficapillus*	2	0	2	0	0	y	141.65
NA	*Myiothlypis flaveola x Basileuterus culicivorus*	4	2	4	0	0	n	13.19
Parulidae	*Basileuterus culicivorus*	28	1	27	0	0	n	10.5
Parulidae	*Geothlypis aequinoctialis*	1	0	1	0	0	y	13.1
Parulidae	*Myiothlypis flaveola*	93	20	85	0	0	n	13.19
Picidae	*Dryobates passerinus*	3	0	1	0	0	n	32.1
Pipridae	*Antilophia galeata*	21	2	21	17	602	y	21.48
Pipridae	*Manacus manacus*	6	0	6	5	94	y	16.7
Thamnophilidae	*Dysithamnus mentalis*	1	0	1	0	0	y	14.87

Thamnophilidae	*Taraba major*	4	0	4	0	0	y	59.2
Thamnophilidae	*Thamnophilus caerulescens*	8	0	8	0	0	y	21.1
Thamnophilidae	*Thamnophilus doliatus*	2	0	2	0	0	y	27.03
Thraupidae	*Conirostrum speciosum*	2	0	1	0	0	y	8.8
Thraupidae	*Eucometis penicillata*	18	1	18	5	105	y	27
Thraupidae	*Euphonia violacea*	3	0	3	0	0	y	15
Thraupidae	*Nemosia pileata*	1	0	1	1	6	y	16
Thraupidae	*Pipraeidea melanonota*	1	0	1	0	0	y	21
Thraupidae	*Ramphocelus carbo*	7	0	7	6	518	y	25.92
Thraupidae	*Stilpnia cayana*	2	0	2	0	0	y	18
Thraupidae	*Tachyphonus coronatus*	6	0	6	6	113	y	29.3
Thraupidae	*Thlypopsis sordida*	6	0	5	1	38	y	17
Thraupidae	*Thraupis sayaca*	4	0	4	1	9	y	32.49
Thraupidae	*Trichothraupis melanops*	1	0	1	0	0	y	22.58
Troglodytidae	*Troglodytes aedon*	2	0	2	0	0	n	10.85
Turdidae	*Turdus amaurochalinus*	5	0	5	3	43	y	57.9
Turdidae	*Turdus leucomelas*	25	0	25	7	68	y	69.1
Turdidae	*Turdus rufiventris*	1	0	1	0	0	y	69.44
Tyrannidae	*Cnemotriccus fuscatus*	13	0	12	1	4	y	13.6
Tyrannidae	*Lathrotriccus euleri*	9	1	7	1	2	y	11.33
Tyrannidae	*Leptopogon amaurocephalus*	18	2	15	1	7	y	11.7
Tyrannidae	*Myiarchus ferox*	16	0	14	3	53	y	27.5
Tyrannidae	*Myiodynastes maculatus*	5	0	3	1	2	y	43.2
Tyrannidae	*Myiornis auricularis*	1	0	1	0	0	n	5.3
Tyrannidae	*Pitangus sulphuratus*	7	0	6	4	293	y	62.85
Tyrannidae	*Platyrinchus mystaceus*	28	6	16	0	0	n	9.7
Tyrannidae	*Tolmomyias sulphurescens*	7	0	7	0	0	y	14.3
Tyrannidae	*Tyrannus melancholicus*	1	0	1	0	0	y	37.4

Supplementary Material 4 Linear regression model (LM) parameters, distribution, transformation, R^2 and p-value. Significant p-values are marked by an asterisk.

Model Parameters	Distribution	Transformation	R^2	p-value	Standard Error	t-value
Plot-level SDE ~ Plot age	Normal	None	0.017	0.718	-	-
Plot-level SDE ~ Plot forest cover	Normal	None	0.200	0.195	-	-
Plot-level SDE ~ Zoochoric woody plant richness	Normal	None	0.129	0.309	-	-
Plot-level SDE ~ Zoochoric woody plant abundance	Normal	None	0.233	0.158	-	-
Species-level SDE ~ Bird body mass	Non-normal	Log	0.129	0.157	-	-
Species-level SDE ~ Percent frugivory of bird diet	Non-normal	Log	0.246	0.041*	0.141	2.233
Capture rate ~ Percent frugivory of bird diet	Non-normal	Log	0.145	0.131	-	-
Seed dispersal frequency ~ Percent frugivory of bird diet	Non-normal	Log	0.009	0.708	-	-