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

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA, EVOLUÇÃO E BIODIVERSIDADE

Características morfo-funcionais digestivas como mediadoras da frugivoria por aves

Ana Cristina Vara Crestani

Rio Claro – SP
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Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Ecologia, Evolução e Biodiversidade.

Orientador: Prof. Dr. Marco Aurélio Pizo

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"*Tornar-se exige paciência e rigor em igual medida*"

Michelle Obama

RESUMO

Características morfológicas digestivas parecem explicar não só o quanto da dieta de aves é composta por frutos como também quais espécies de frutos são consumidas. Sabe-se que o processo de escolha dos frutos é determinado em parte pelo conjunto de característica dos frutos carnosos, como nutrientes e compostos químicos de defesa, que interagem e influenciam a escolha e consumo por animais, e por processos digestivos que determinam a eficiência do animal em extrair e assimilar a energia dos frutos. Este trabalho buscou entender: (1) como a variação de características morfológicas do trato digestivo de espécies de aves se relaciona com o consumo de frutos com diferentes quantidades de açúcares, lipídios e compostos fenólicos e (2) a capacidade digestiva de uma espécie de ave (*Euphonia violacea*) com alto grau de frugivoria que consome frutos pobres em nutrientes em relação à manutenção energética e requerimentos de nitrogênio. Para isso, (1) foram realizadas medidas morfométricas do trato digestivo de nove espécies de aves com diferentes porcentagens de frutos em suas dietas (grau de frugivoria). As porcentagens de açúcares solúveis, lipídios e fenóis totais foram quantificadas para sete espécies de frutos consumidos por estas aves. Essas características, combinada com o consumo médio dos frutos pelas aves, foram utilizadas para a seleção de modelos e análises filogenéticas. O grau de frugivoria foi explicado pela área da moela e peso do fígado. Além disso, o comprimento do intestino e o peso de fígado se relacionaram com o consumo de açúcares solúveis, lipídios e fenóis totais. Como segundo capítulo (2), foram realizados experimentos contrastando indivíduos de *E. violacea* e uma espécie de ave frugívora menos especializada (*Thraupis sayaca;* Thraupidae) com dietas artificiais com diferentes porcentagens de açúcares e nitrogênio. Ambas as espécies aumentaram a ingestão de alimento ofertado conforme a diminuição da concentração de açúcar, mantendo a energia corpórea, e não foram encontradas diferenças nos consumos de nitrogênio entre as espécies. Concluiu-se que os traços fisiológicos investigados não explicam o consumo de frutos com baixo valor nutricional. Assim, este trabalho investigou como características fisiológicas digestivas e morfológicas do trato digestivo das aves se relacionam com o consumo de frutos com determinada composição nutricional e química.

Palavras-chave: dieta especializada, balanço de energia, açúcar, lipídios, fenóis.

ABSTRACT

Digestive morphological characteristics seem to explain not only how much of the diet of birds is composed of fruits but also what species of fruit are consumed. The fruit selection process is determined in part by the characteristic set of nutrients and chemical defense compounds present in fleshy fruits, which interact and influence the choice and consumption by animals, and by digestive processes that determine the efficiency of the animal in extracting and assimilating the energy of the fruits. In this study, we explored (1) the relationship between digestive traits and the consumption of fruits with different nutrient and chemical compounds and, (2) the digestive capacity of a bird species that exploit low-energy fruits (*Euphonia violacea*) regarding to energy intake and nitrogen requirement. We performed (1) morphological characterization of the digestive structures in nine species of birds whit different percentage of fruit in their diet (frugivory degree). Using seven fruit species, which are consumed by sampled birds, we analyzed the percentage of soluble sugar, lipids and total phenols. Together with the rate of consumption, we combined all this traits in multiple regressions models and phylogenetic effect. We found that frugivory degree is explained by gizzard area and liver mass, while the intestine length and liver mass also have a relationship with consumption of sugar, lipids and phenols. As a second chapter (2), we conducted trials using captive individuals of *E. violacea* and a less-specialized bird (*Thraupis sayaca*; Thraupidae), and artificial diets with distinct amounts of sugar and nitrogen. Both species were able to compensate energy intake as sugar density decreased and they did not differ in mass-corrected maintenance nitrogen requirement. Thus, this study have investigated the relationship between birds digestive structures and process with the nutritional and chemical characteristic of fruit that the birds consume.

Keyword: diet specialization, energy balance, sugar, lipids, phenols.

SUMMARY

1. INTRODUCTION

Frugivory is a process by which animals obtain food resources and the outcome of these interactions determines the persistence, distribution and evolution of the animals involved (BARBOSA et al. 2012). A large proportion of plants rely on seed dispersal by animals to reproduce and colonize new sites (JORDANO, 2000). Thus, frugivory is not only fruit consumption by animals but an important ecological process, essential to the functionality and architecture of the communities it maintains. For the Neotropical region, the interaction between frugivorous birds and fruits needs to be better explored in relation to the traits that mediate it (DUGGER et al. 2018, KISSLING et al. 2009). This region is of extreme importance because it has the world's largest diversity of frugivorous birds and, consequently, of plants dispersed by them.

Fruit selection is determined in part by physiologic and digestive processes that determine the efficiency in extracting and assimilating energy, and by the nutrients and chemical defenses present in fruits, which influence the choice and the rate of fruit consumption by animals (BICUDO et al., 2010; HERRERA, 1987). Bird digestive structures (e.g. gizzard, liver, and intestine) are related to food mechanical processing, breakdown of nutrients, food transit time, absorption and detoxification of chemical compounds present in the pulp (JORDANO, 1987; HERRERA, 1984; STANLEY and LILL, 2002).

Another important characteristic is the animal nutritional balance. For example, frugivorous birds have different nitrogen requirements and many species supplement their diet by consuming arthropods and other invertebrates (HERRERA et al. 2009). For frugivorous birds, the minimum nitrogen requirements are lower compared to the omnivorous birds, which explains in part the consumption of fruits with low protein values (IZHAKI, 1998; JORDANO, 2000). In fact, fleshy fruits in general present a low amount of nitrogen when compared to other resources used by birds (JORDANO 2000). However, the genus *Euphonia*, which present a high degree of frugivory and physiological peculiarities, is able to obtain all the nitrogen requirement exclusively from fruits, which is not common (HERRERA et al., 2009).

Understanding why a diet distinction among birds occurs will bring a more refined understanding of the mutualistic interactions between birds and plants and their reflexes on the community. For example, recognizing which characteristic allows some birds to consume a high percentage of fruits while others consume a mix of fruits and arthropods.

In addition, fruit traits can help us to understand why some plant species differ from one another in terms of frugivore attraction and reward. This investigation also contributes to other wide interactions topics such as the chemical aspects of the seed dispersal syndromes (CIPOLLINI, 2000), the hierarchical choices made by foraging frugivores (SALLABANKS, 1993), and the hierarchical decision process related to optimal foraging theory (PULLIAM 1974) and diet choice that ultimately influence niche width (PIERSMAET al. 1993).

If what the animal eats and its digestive efficiency determine the flow of energy to other trophic levels within the ecosystem (BICUDO et al. 2010), it is important to understand the trait-matching between fruits and birds, and how their interactions lead to different rates of frugivory. Therefore, the overarching goal of this work was to understand how fruit chemical and nutritional characteristics of fruits match with the digestive capacity of birds and how it reflects on the frugivory gradient, from predominantly frugivorous birds to predominantly insectivorous birds that also occasionally eat fruits.

In the first chapter of this study, we analyzed how the digestive structures of birds and their frugivory degree are related to fruits' nutritional and chemical composition they eat. In the second chapter, we tested the energy intake and nitrogen requirement of two frugivorous birds to understand the specialized feeding of fruit.

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2. CHAPTER 1 Fruit and morphologic digestive traits as drivers of frugivory by birds

2.1 Introduction

Characteristics of fruits and frugivorous animals can determine plant reproductive success and be key to structure ecological communities. Frugivorous birds do not consume fruits in a random way, although it is still not entirely clear why they consume certain species of fruits and not others, or why there is a consumption gradient (i.e. variance in how much of an animal's diet is composed of fruit) because fruits are resources almost always available and easy to obtain (LEVEY; MARTÍNEZ DEL RIO, 2001). Among several factors, fruit selection is influenced by the nutrients and chemical defenses present in fleshy fruits, and by digestive processes that determine the efficiency of the animal in extracting and assimilating the energy available in fruits (HERRERA 1987, BICUDO et al., 2010). The association between morphological characteristic of birds and the habit of eating fruits was pointed out in the past, especially for Mediterranean species (HERRERA, 1984; JORDANO, 1987). For Neotropical birds, the interplay between the nutritional and chemical characteristics of fleshy fruits and the feeding processes of frugivorous birds received more attention in recent years (PIZO et al., 2021). Now, a deeper investigation on the matching between bird and fruit traits is needed to understand the critical factors that determine the outcome of their mutualistic interactions.

In relation to nutrient content, two major groups of fruits can be identified: lipid-poor and sugar-rich fruits, and fruits that are lipid-rich and sugar-poor (MOERMOND; DENSLOW 1985, JORDANO, 2014). The formation of these groups is explained by the fact that lipids are hydrophobic substances and, consequently, lipid concentrations are negatively correlated with water. Additionally, sugar is positively related with the amount of water resulting in a sugar-rich fruits that are, consequently, poor in lipids. These groups may also vary in their concentrations of secondary chemical compounds. In comparison to sugar-rich fruits, lipid-rich fruits have lower amounts of defensive secondary compounds, while fruits rich in sugars and water have higher amounts of such compounds (CAZETTA et al., 2008). This is explained because lipid-rich fruits are quickly consumed while sugar-rich fruits tend to stay longer in the plant and, because of this exposure, sugary fruits are more prone to be attacked by pathogens (CAZETTA et al., 2008). Thus, sugar-rich fruits have greater amounts of secondary compound for defense then lipid-rich fruits (CAZETTA et al., 2008). However, is not very clear which of both nutrient, sugar or water, influence the persistence of the fruits in the plant.

The secondary compounds present in fruits may not provide energy, and may alter the digestive efficiency and nutrient absorption of frugivores (BICUDO et al., 2010). For example, amides can reduce the gut retention time in frugivorous bats (BALDWIN; WHITEHEAD, 2015) while phenols can reduce the absorption of sugar by mice and chickens (KARASOV et al., 1992). If sugar-rich fruits contain high levels of phenols (CAZETTA et al., 2008), which decrease the nutrient absorption (KARASOV et al., 1992, SANTIDRIAN et al., 1981) thus birds need to have strategies to overcome the effects of such compound present in fleshy fruits.

Avian physiological characteristics, such as the nutritional assimilation capacity, determine how much of the nutrients contained in a resource is used while internal morphological characteristics can indicate how the animal handle the food and overcome the effects of secondary compounds. Digestion of lipid molecules, for example, requires a longer retention time because it is performed in different stages, such as emulsification and hydrolysis, whereas water molecules and sugars can be rapidly absorbed by passive transport (MARTINEZ DEL RIO; KARASOV, 1990; MARTÍNEZ DEL RIO; RESTREPO, 1993). Considering the internal morphological characteristics of the digestive tract of frugivorous birds, gizzard area for example can determine the ability of birds to separate pulp from seeds and process invertebrates (STANLEY; LILL, 2002). The detoxification of chemical elements, as secondary compounds, is related to liver functions (BICUDO et al., 2010, JORDANO, 2000), while digestion of carbohydrates is related to the intestinal surface area and intestine length (PRICE et al., 2015). In addition, the time of food transit in the digestive tract also depend on the size of gizzard and intestine (JORDANO, 1987, HERRERA, 1984).

These differences in bird digestive features are reflected in their choice of fruits as food items (LEVEY; MARTÍNEZ DEL RIO, 2001). When omnivorous and insectivorous birds eat fruits, they usually prefer those with high amounts of lipids, since they have an enzymatic limitation to digest certain classes of sugar (FUENTES 1994; MARTINEZ DEL RIO et al., 1988). Taking into account that omnivores and insectivorous birds need also to process hard food (e.g. arthropods) and that the digestion of lipids needs a longer retention time then we could expect a well-developed gizzard and longer intestine in such birds. Beside the intestine length is related to carbohydrates digestion, we can expected that the length could increase the transit time of food. In contrast, birds that often consumes sugar-rich fruits have a passive intestinal permeability that allow the digestion of sugars (LEVEY & MARTÍNEZ DEL RIO, 2001, MARTINEZ DEL RIO; KARASOV, 1991). As sugars are quickly absorbed we could expect a

shorter intestine and smaller surface area. In addition, since sugar-rich fruits have higher levels of secondary compounds, frugivorous birds relying on such fruits may present a larger liver for detoxification.

Associated with the type of fruits eaten and digestive capacity of birds, the percentage of fruits in their diets also varies forming a gradient, with bird species presenting different frugivory degrees based on the percentage of fruits in their diet. Usually, insectivorous birds have less the 30% of fruits in their diet, omnivores between 30 – 60%, and predominantly frugivorous birds have more than 60% of fruits in the diet (LOPES, 2016). Considering that insectivores and omnivores interact more strongly with lipid-rich fruits while frugivorous consume sugar-rich fruits more often (LOPES, 2016), and that these associations can be related to the digestive feature of birds, we hypothesized differences in the digestive tract according to a frugivory gradient. We predicted that birds with higher degree of frugivory that consume sugar-rich fruits have a smaller gizzard, intestine, surface area, but larger liver than birds that consume more often lipid-rich. For insectivorous and omnivorous birds, with a low frugivorous degree, we predicted a higher consumption of lipid-rich fruits and longer intestine and surface area, voluminous liver and biggest gizzard (Figure 1). We also hypothesized that the phylogeny can explain the relationship between bird morphological features and their frugivory degree. ~~We also tested the hypothesis that morphological features can predict the nutritional and chemical composition of fruit eaten by birds.~~

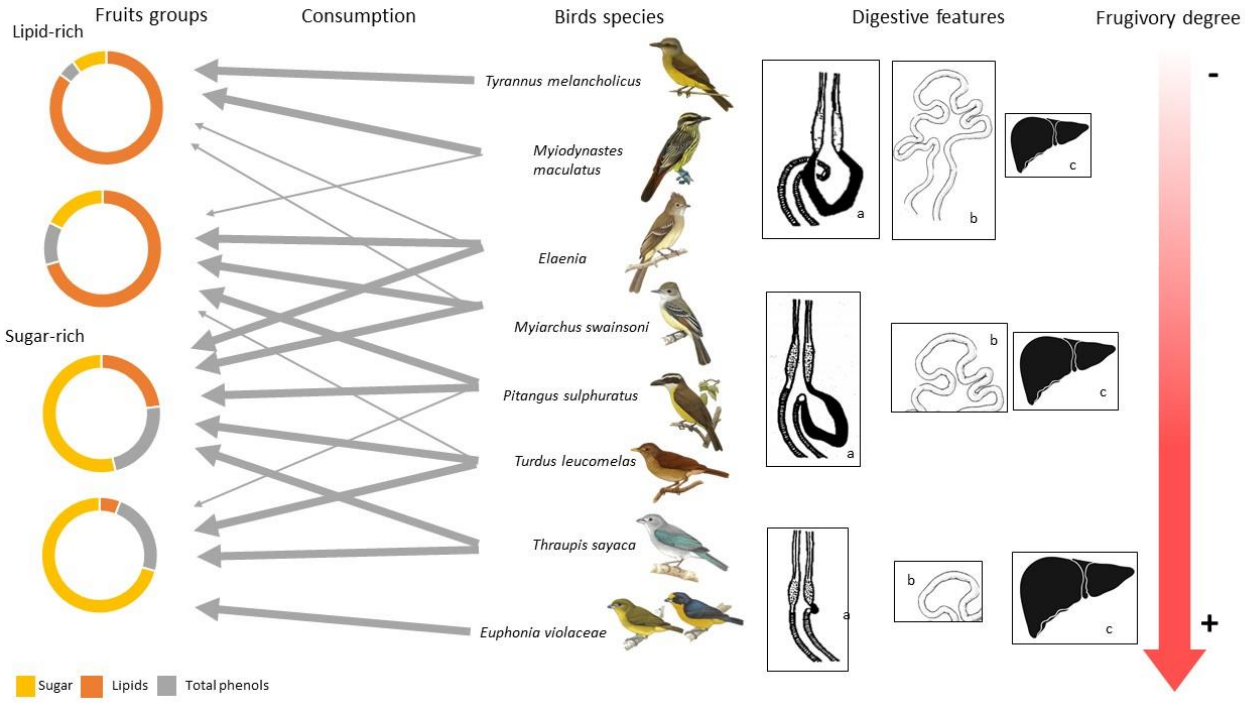

Figure 1. Predicted patterns of interactions between fruits with different percentages of secondary compounds, sugars, and lipids with birds with distinct digestive tracts (bird icons from RIDGELY 2015). In general, insectivorous birds that only occasionally feed on fruits (i.e. low frugivory degree; e.g. *Tyrannus melancholicus*), consume lipid-rich fruits, while predominantly frugivorous birds (higher frugivory degree; e.g. *Euphonia violacea*) consume mostly sugar-rich fruits. The thickness of the arrows is weighted by the frequency of fruit consumption. The black and white figures show, from top to bottom, a digestive tract with more developed structures (a – gizzard, b –intestine, c – liver), an intermediate and a simplified tract (with vestigial gizzard) (modified from JORDANO 2000). The vertical arrow depicts the frugivory gradient from birds with low percentage of fruits in the diet to birds with diet composed predominantly by fruits.

2.2 Methods

Bird traits

We selected common bird species that are easily to find the carcasses and they have different percentage of fruits in their diet. Eight bird species from four families were used to obtain the measurements of digestive tract and each species had from 1 to 6 individuals analyzed: *Tyrannus melancholicus* (n=5), *Myiodynastes maculatus* (n=3), *Elaenia mesoleuca* (n=2), *Elaenia spectabilis* (n=1), *Myiarchus swainsoni* (n=1), *Pitangus sulphuratus* (n=5) (Tyrannidae), *Turdus leucomelas* (n=5) (Turdidae), *Thraupis sayaca* (n=5) (Thraupidae), and *Euphonia violacea* (n=6) (Fringillidae). According to Gomes et al. (2022), these species have different frugivory degree: *T. melancholicus* and *M. maculatus* are predominantly insectivorous that eat fruits occasionally (< 30% of fruits in the diet). *Pitangus sulphuratus* is an omnivore with more than 50% of fruits in their diet. *Elaenia mesoleuca, E. spectabilis* and *M. swainsoni* are omnivores with intermediate frugivorous degree, having 50% of fruits in their diet. Although *Elaenia* are part of a family that is mostly insectivore (Tyrannidae), both species are considered highly frugivorous compared to most tyranid species (Marini & Cavalcanti 1998). *Turdus leucomelas* and *T. sayaca* are frugivores, with more than 60% of fruits in their diet, eating invertebrates occasionally. *Euphonia violacea* is considered primarily frugivorous, having 100% of its diet composed by fruits (GOMES et al.,2022, PARRINI, 2015, WILMAN et al., 2014).

The birds samples were obtained from the Institute of Biosciences of the Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Zoology Museum of the University of São Paulo, and the Center for the Recovery of Wild Animals of the Tietê Ecological Park in São Paulo (CRAS - PET). The carcasses were kept frozen until the mensuration of digestive tract. The carcasses come from birds killed by trampling or other unknown causes. We also used carcasses donated by the Zoology Museum of the University of São Paulo. The donations received by the Center for the Recovery of Wild Animals from the Tietê Ecological Park (CRAS - PET) come from animals that further died of unknown causes. No animal analyzed was killed for this work, and all carcasses analyzed have provenance and are stored in the Laboratory of Bird Ecology at the Institute of Biosciences of UNESP.

Morphologic analyses of digestive traits

For the morphological characterization of the digestive structures, we firstly weighted the carcass with a precision weighing scale to the nearest (0.01 g) to access the body mass (g). We then used a precision weighing scale and a digital caliper (0.01 mm) to obtain the liver mass (g) (wet weight), gizzard area (mm^2) (length × width), intestine length (mm) and intestinal surface area (mm^2) (adapted from BRUN et al., 2019 and JORDANO, 1987). The measured value of each structure was divided by the carcass' mass to obtain a proportional value. Details of the measurement of gizzard and intestine surface area can be found in the Supplementary Material (see fig. S1 and fig. S2).

Fruit traits

The fruit nutrient and total phenols amounts were used in the statistical analysis to identify how these compound have relationship with the bird morphological digestive structures. We selected seven wild tropical fruit species widely distributed across ecosystems and commonly eaten by frugivorous birds (PARRINI, 2015), *Acnistus arborescens*, *Cecropia pachystachya*, *Copaifera langsdorffii*, *Ficus guaranitica*, *Miconia albicans*, *Myrcia tomentosa*, and *Phoradendron rubrum*. Ripe fruits were collected from one to three plant individuals per species and immediately frozen. Samples were collected in and around the UNESP campus, including natural areas. Approximately 15 to 300g of fruits were oven-dried at 50°C for 48 hours. The seeds were manually speared from the pulp, which was ground to a fine powder in a coffee grinder. Depend of what extraction from 2 to 25 mg aliquots of pulp were weighed. All analyses were done in triplicate for each fruit species. We performed extraction to quantify total phenols, soluble sugar and lipids. Soluble sugar and lipids are presented in percentage of dry mass and total phenolics in percentage of dry mass weight in Gallic Acid equivalents. The detection principle for lipids is chloroform-methanol, for soluble sugar the principle was anthrone and methanol for total phenolics. Methodological details are provided in the Supplementary Material. Fruits with 0 to 10% of lipids in relation to dry weight were considered lipid-poor, sugar-rich fruits, while fruits with 20% to 80% lipids were considered lipid-rich, sugar-poor fruits.

Interactions

The interaction between birds and fruits were used in the statistical analysis to tested if the rate of fruit consumption is important for the relationship between fruits compound and the bird morphological digestive structures. To access fruit consumption by the bird species, we searched the literature (i.e., papers in peer-reviewed, indexed journals as well as dissertations

and theses) for studies with similar methodology that reported quantitatively the feeding visits of birds to focal fruiting plants. To be considered, the study should contain the selected species of bird and plant identification, study site details and sampling effort (i.e. total hours of focal observation). We found studies carried out from 1988 to 2018 in nature reserves, rural areas and urban parks in the Brazilian Atlantic Forest and Cerrado (Table S1). We also used unpublished data collected by the authors at a Cerrado spot in the UNESP campus. We calculated the rate of fruit consumption per bird species by dividing the total number of fruits consumed by a particular bird species per hours of observations.

Data analyses

We regressed the gizzard area, intestine length, intestine surface area, and liver mass against body mass to calculate the allometric relationship between structures (Ricklefs, 1996). As exploratory analysis, we calculated Pearson correlation coefficient between digestive structures and fruit nutrients and phenolic content. We did not removed from analysis the correlated structures.

To test the relationship between frugivory degree and bird traits, we ran linear multiple regression models using the frugivory degree as a continuous response variable and individual liver mass, gizzard area, intestine length and intestinal surface area as explanatory variables. Model selection was done by employing the stepwise forward procedure and the best model was determined by the lowest AIC (Table S2). We transformed the data when necessary using natural logarithm to achieve normality of model residuals, which was assessed by the Shapiro-Wilk test. Heteroscedasticity was checked graphically and all analysis were ran on R (R CORE TEAM, 2020).

To test the possible effect of phylogeny in the relationship between bird traits and frugivory degree, we first submitted our species list to birdtree.org database (JETZ et al., 2012) using the Hacket source tree. The phylogeny of birds containing up to 10 000 phylogenetic hypotheses, which resulted in a multiphylo file containing 5000 phylogenetic trees with resolution at the species level. Then we generate a consensus tree using the Tree Annotator, from BEAST (SUCHARD et al., 2018). We used this bird phylogenetic tree to calculate the phylogenetic signal of the traits: gizzard area, liver mass, intestine length and surface area. The phylogeny was used as co-variable in the phylogenetic generalized least square (GLS) models to test the relationship between the morphological traits of birds and frugivory degree using the PGLS function from the R package "caper" (R CORE TEAM, 2020).

We built separated models for each fruit nutrient component and phenolic content to address the relationship between the bird morphological traits and the chemical and nutrients composition of fruits in three approaches, considering each plant-bird interaction as a particular sample in all the models. The first approach was exclusively based on the percentage of fruit nutrient and phenolic contents, which we called “Fruit composition model”. The percentage of fruit nutrient component and phenolic content eaten were continuous response variables, while liver mass, gizzard area, intestine length, and intestine surface area (values summarized for each bird species as average of all individual values) were set as explanatory variables. In the second approach, called “Interaction frequency models”, we considered that the frequency of consumption might mediate the relationship between bird traits and fruit composition. Therefore, we rebuild the models above considering as a continuous response variable the percentage of fruits components multiplied by the rate of fruit consumption by each bird species to represent the intensity of fruit-bird interaction. The third approach, named “Fruit dependence model”, takes into account how much of the bird diet is composed of fruits, as expressed by frugivory degree, to address its role in affecting the link between fruit chemical composition and bird morphological traits. Thus, we took as a response variable the product of fruits components multiplied by fruit consumption rate and frugivory degree and the same explanatory variables described above (see Table 1). Due to the modest number of plant-bird interactions (n = 25), we conducted model selection similar as described above, by employing the stepwise forward procedure and the best model was determined by the lowest AIC.

2.3 Results

Among the bird morphological traits body mass and intestine length ($r = 0.83$, $p < 0.001$), gizzard and liver ($r = 0.79$, $p < 0.001$) and body mass and intestine surface are ($r = 0.76$, $p = 0.0001$) were correlated with each other (Figure S3). Bird gizzard, intestine length and intestine surface area had a significant allometric relationship with body mass ($p < 0.001$; Figure 2). Across bird species, we found a wide variation in morphological traits (Table 2). Body masses ranged from 11.1 to 66.6 g, gizzard area from 0 to 24.3 mm^2, intestine length from 3.7mm to 27.6 mm, intestine surface area from 21.5 to 121.8 mm^2, liver mass from 0.01 to 0.09 g and frugivory degree from 12 to 100% (Table S3). Fruit nutrients and total phenols were not correlated (Fig S4). Total phenols ranged from 0.4 to 5.2%, soluble sugar from 3.6 to 75.6% and lipids from 6.4 to 37.5% (Table 2). The rates of fruit consumption are expressed in Figure 3.

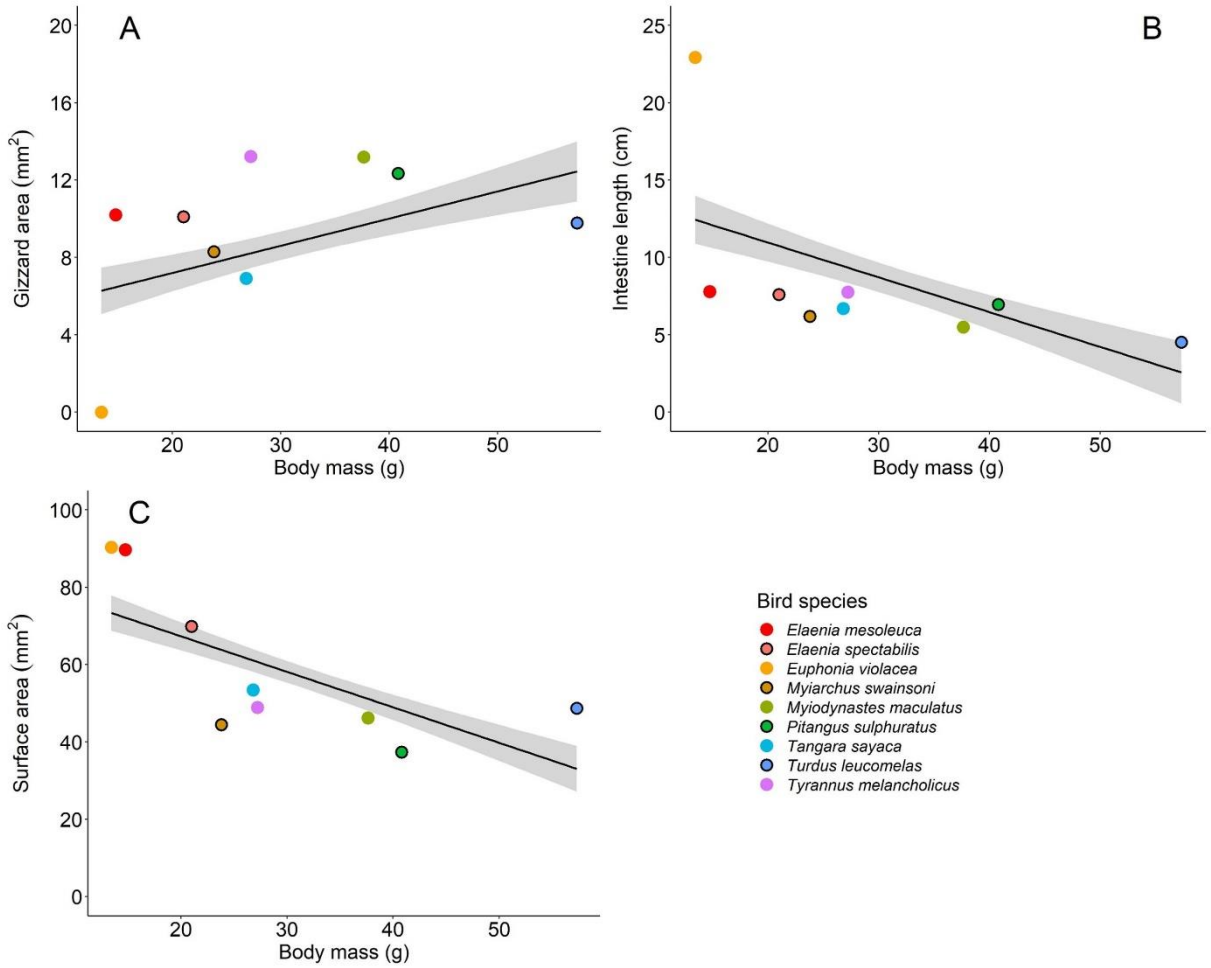

Figure 2. Significant allometric relationship between body mass and gizzard area (A), intestine length (B), intestine surface area (C).

Table 2. Morphometric traits of birds digestive tract used in this study. Data are presented as mean ± SD.

Birds species	Birds family	(n)	Body mass (g)	Gizzard area (mm^2)	Intestine length (mm)	Intestine surface area (mm^2)	Liver maas (g)	Frugivory degree[a] (%)
Myiodynastes maculatus	Tyrannidae	3	37.73± 12.68	12.64± 3.83	5.08±1.86	43.66±10.95	0.02± 0.007	29
Pitangus sulphuratus	Tyrannidae	5	40.94± 0.54	12.41± 2.34	6.95±1.03	37.27±13.04	0.03± 0.02	53
Tyrannus melancholicus	Tyrannidae	5	27.46± 6.12	13.04± 2.82	7.43±1.63	48.37±3.12	0.02± 0.004	12
Elaenia mesoleuca	Tyrannidae	2	14.75± 1.34	10.20± 2.96	7.80±0.42	89.70±2.82	0.02± 0.01	50
Elaenia spectabilis	Tyrannidae	1	21	10.10	7.60	69.90	0.02	50
Myiarchus swainsoni	Tyrannidae	1	23.80	8.30	6.20	44.50	0.02	50
Turdus leucomelas	Turdidae	5	57.10± 7.40	9.46±3.41	4.47±0.92	47.82±13.53	0.04± 0.006	64.9
Tangara sayaca	Thraupidae	5	27.22± 1.51	6.53±2.89	6.60±1.64	52.50±20.49	0.04± 0.01	66.4
Euphonia violacea	Fringillidae	6	13.80± 1.75	0.00±0.00	22.05±3.10	88.71±22.05	0.07± 0.01	100

[a] Frugivory degree from (GOMES et al., 2022; WILMAN et al., 2014).

Table 3. Percentage in dry mass of lipids, total phenols and soluble sugar from the fruit species analyzed.

Fruit specie	Fruit family	Lipids	Total phenolics	Soluble sugar
Acnistus arborescens	Solanaceae	17.44	0.64	56.88
Cecropia pachystachya	Urticaceae	10.43	1.55	52.53
Copaifera langsdorffii	Fabaceae	7.50	4.12	75.66
Ficus guaranitica	Moraceae	6.49	1.34	7.86
Miconia albicans	Melastomataceae	7.43	0.68	59.40
Myrcia tomentosa	Myrtaceae	6.61	05.25	7.16
Phoradendron rubrum	Santalaceae	10.34	1.16	31.93

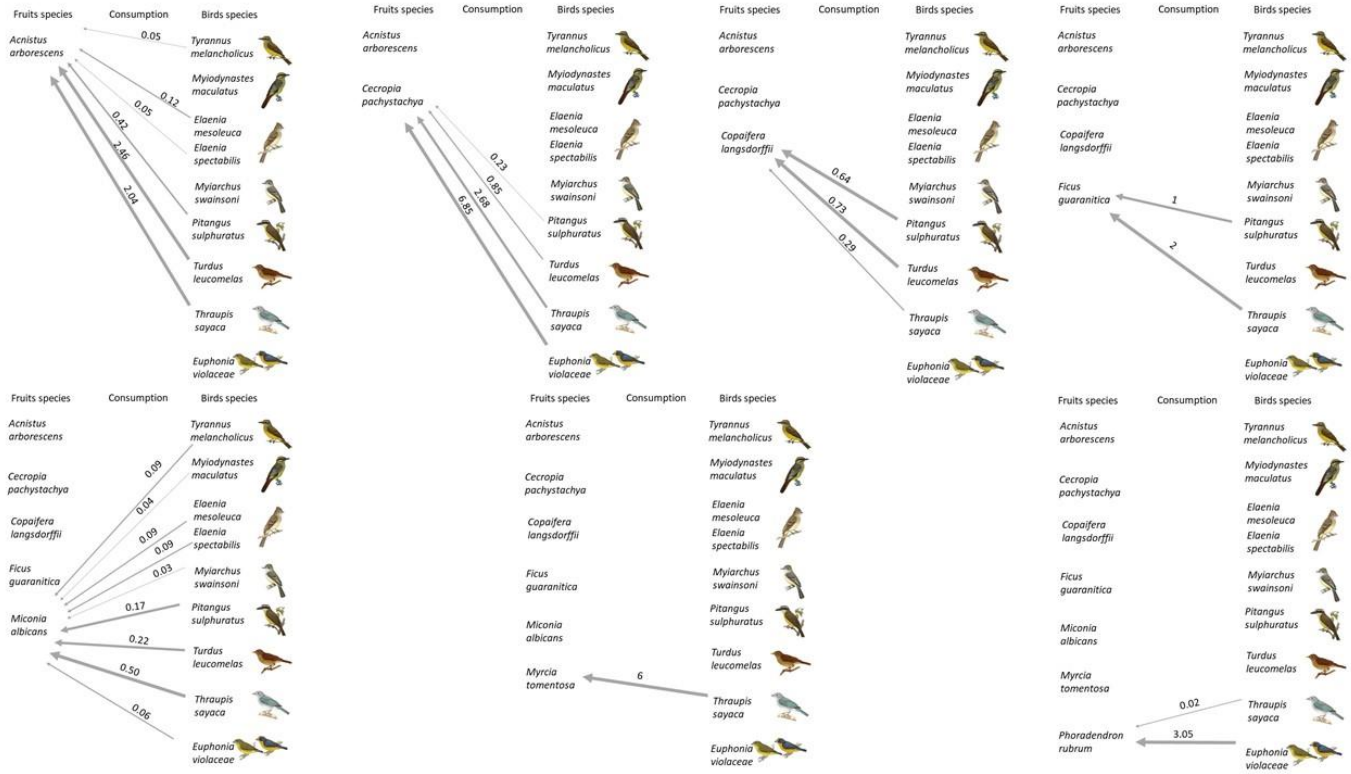

Figure 3. Rate of fruit consumption by bird species obtained by dividing the total number of fruits consumed by a particular bird species per the amount of time (in hours) spent by the authors observing that particular plant species (bird icons from Ridgely 2015). Data from the literature (see Table S1).

The frugivory degree had a negative relationship with gizzard area, while being positively related to the liver mass (Figure S5), whereas frugivory degree did not explain intestine length and surface area (Table S4). In the bird phylogenetic tree is possible to identify two distinct groups formed by *T. sayaca*, *E. violacea* and *T. leucomelas* and the other group formed by the tirannids *M. maculatus*, *P. sulphuratus*, *T. melancholicus*, *E. mesoleuca*, *E. spectabilis* and *M. swainsoni* (Figure 4). We found a phylogenetic signal for gizzard area, liver mass and intestine length (Table S5). The phylogenetic generalized least square showed a strong signal for gizzard area ($r^2 = 0.45$, $p = 0.02$) and liver mass ($r^2 = 0.41$, $p = 0.03$) (Figure 5, Table S6).

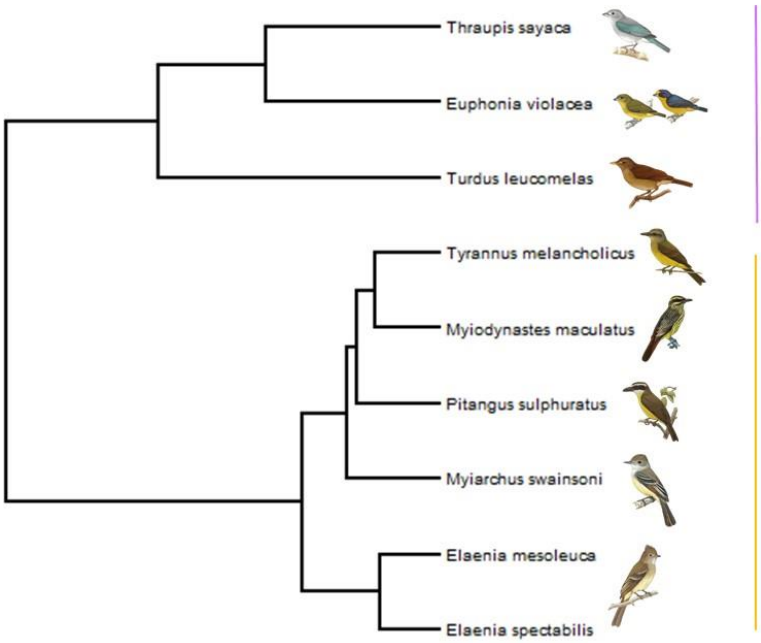

Figure 4. Phylogenetic tree of bird species considered in this study. The colored lines highlight two distinct groups (bird icons from Ridgely 2015).

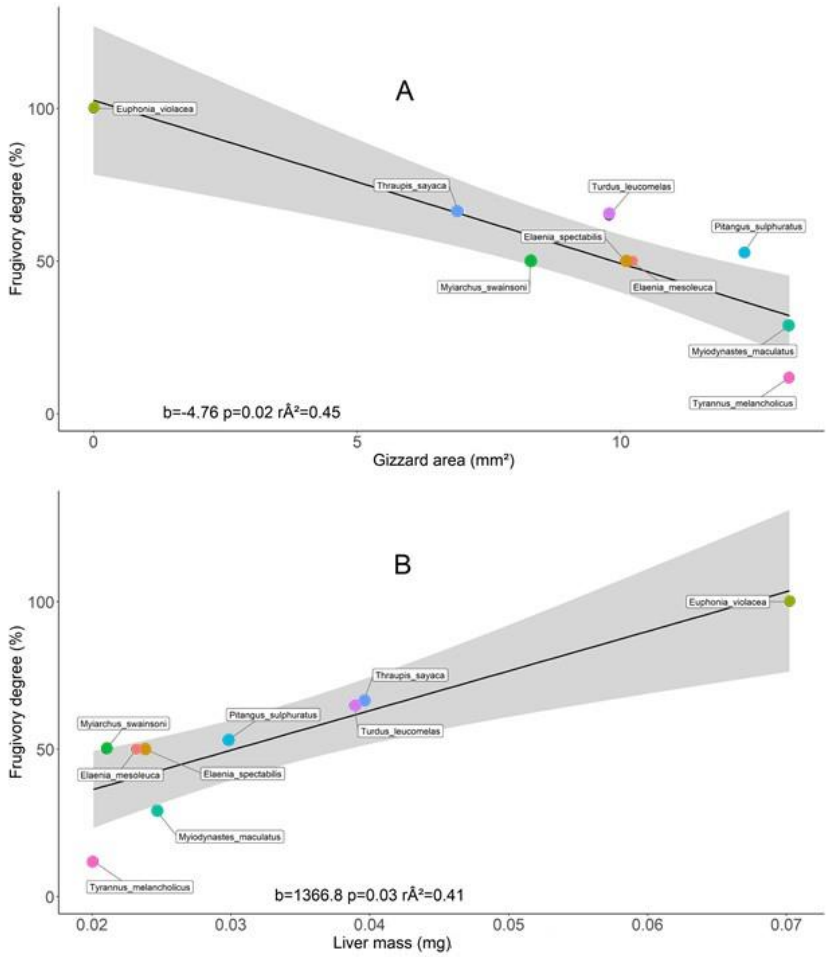

Figure 5. Phylogenetic linear regressions between bird digestive traits and frugivory degree: A: gizzard area and B: liver mass.

We found no relationship between bird morphological traits and the fruit nutrient composition in the Fruit composition model. In the Interaction frequency model, liver mass was positively related to total phenols, soluble sugar and lipids, whereas intestine length was negatively related to phenols (Figure 6). The Fruit dependence model showed a significant relationship of liver mass with total phenols, soluble sugar, and lipids, while intestine length was negatively related to total phenols, soluble sugar, and lipids (Figure 7). Further bird morphological traits were not related to the ingestion of any of the fruit components.

Table 2. Results from linear multiple regressions for Fruit composition, Interaction frequency and Fruit dependence models. The Interaction frequency model (that considers as a continuous response variable the percentage of fruit components multiplied by the rate of fruit consumption by each bird species) and the Fruit dependence model (that has as response variable the product of fruit components multiplied by the rate of fruit consumption and frugivorous degree) in relation to liver mass, intestine length, gizzard area and intestinal surface area. Std. error: Standard error, t: t value, and p: significant level

value at alpha = 0.05. Estimates and t values not shown for explanatory variables absent from the final models defined by the forward stepwise selection procedure.

	Fruit composition			Interaction frequency			Fruit dependence		
	Estimate (SE)	t	p	Estimate (SE)	t	p	Estimate (SE)	t	p
Total phenols									
Intercept	1.43 (0.25)	5.65	<0.001*	-4.04 (0.93)	-4.35	<0.001*	-5.65 (0.95)	-5.92	<0.001*
Liver			0.510	1.44 (0.38)	3.78	0.001*	1.82 (0.39)	4.75	<0.001*
Intestine			0.570	-0.25 (0.10)	-2.46	0.020*	-0.29 (0.10)	-2.83	0.009*
Gizzard			0.720			0.450			0.670
Surface			0.330			0.460			0.680
Soluble sugar									
Intercept	51.84 (3.58)	14.46	<0.001*	0.48 (0.70)	0.69	0.490	-1.12 (0.73)	-1.53	0.130
Liver			0.380	0.61 (0.17)	3.42	0.002*	1.35 (0.30)	4.50	<0.001*
Intestine			0.630			0.510	-0.19 (0.08)	-2.38	0.020*
Gizzard			0.320			0.900			0.520
Surface			0.990			0.980			0.940
Lipids									
Intercept	10.22 (0.79)	12.82	<0.001*	-1.22 (0.80)	-1.52	0.140	-2.83 (0.82)	-3.42	0.002*
Liver			0.530	1.06 (0.33)	3.21	0.004*	1.44 (0.34)	4.23	<0.001*
Intestine			0.820	-0.16 (0.08)	-1.86	0.070	-0.21 (0.09)	-2.30	0.030*
Gizzard			0.560			0.420			0.680
Surface			0.630			0.750			0.960

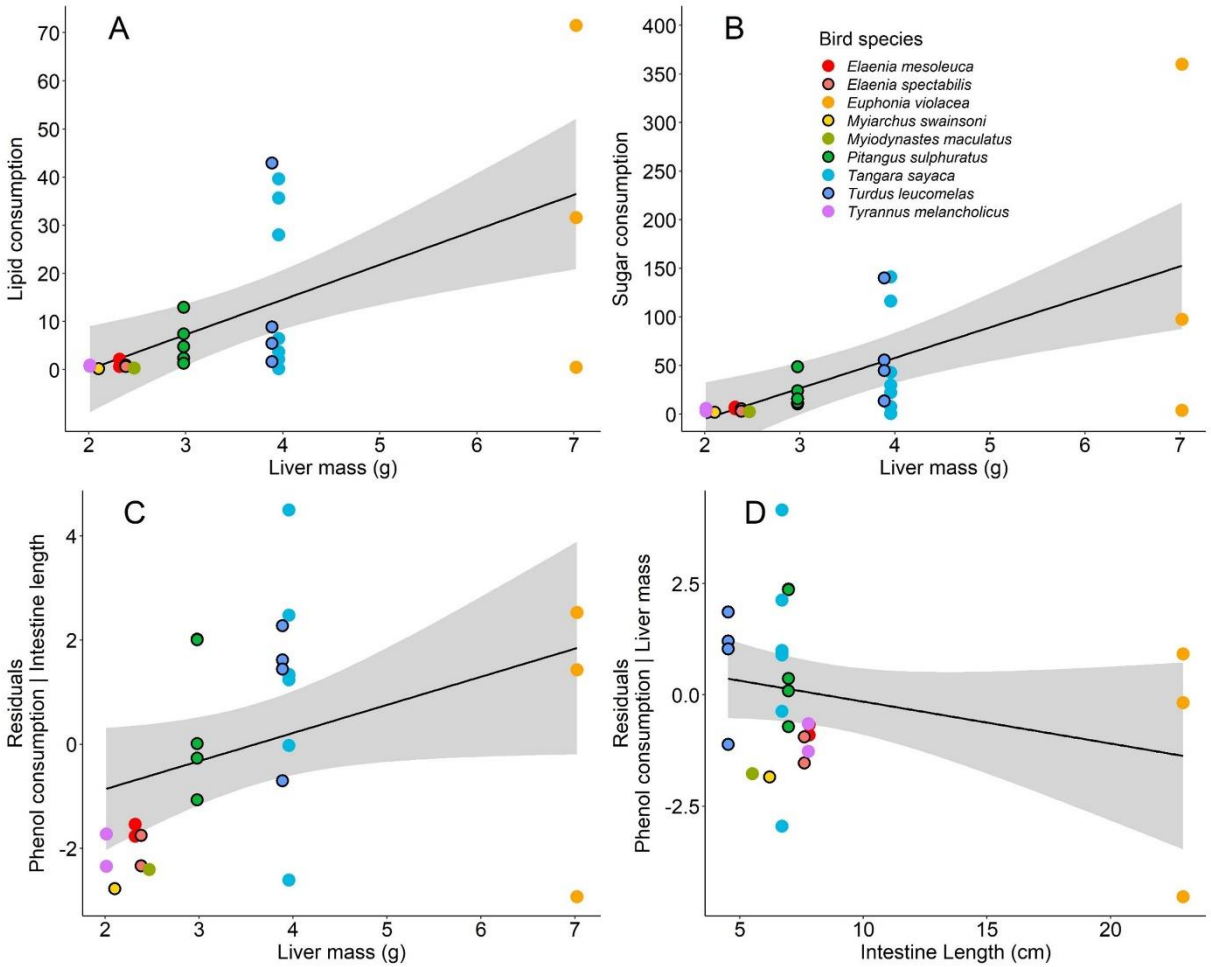

Figure 6. Partial regressions from the Interaction frequency models showing the independent relationship between: lipid consumption and liver mass (A), soluble sugar and liver mass (B), total phenols and liver mass (C), and total phenols and intestine length (D).

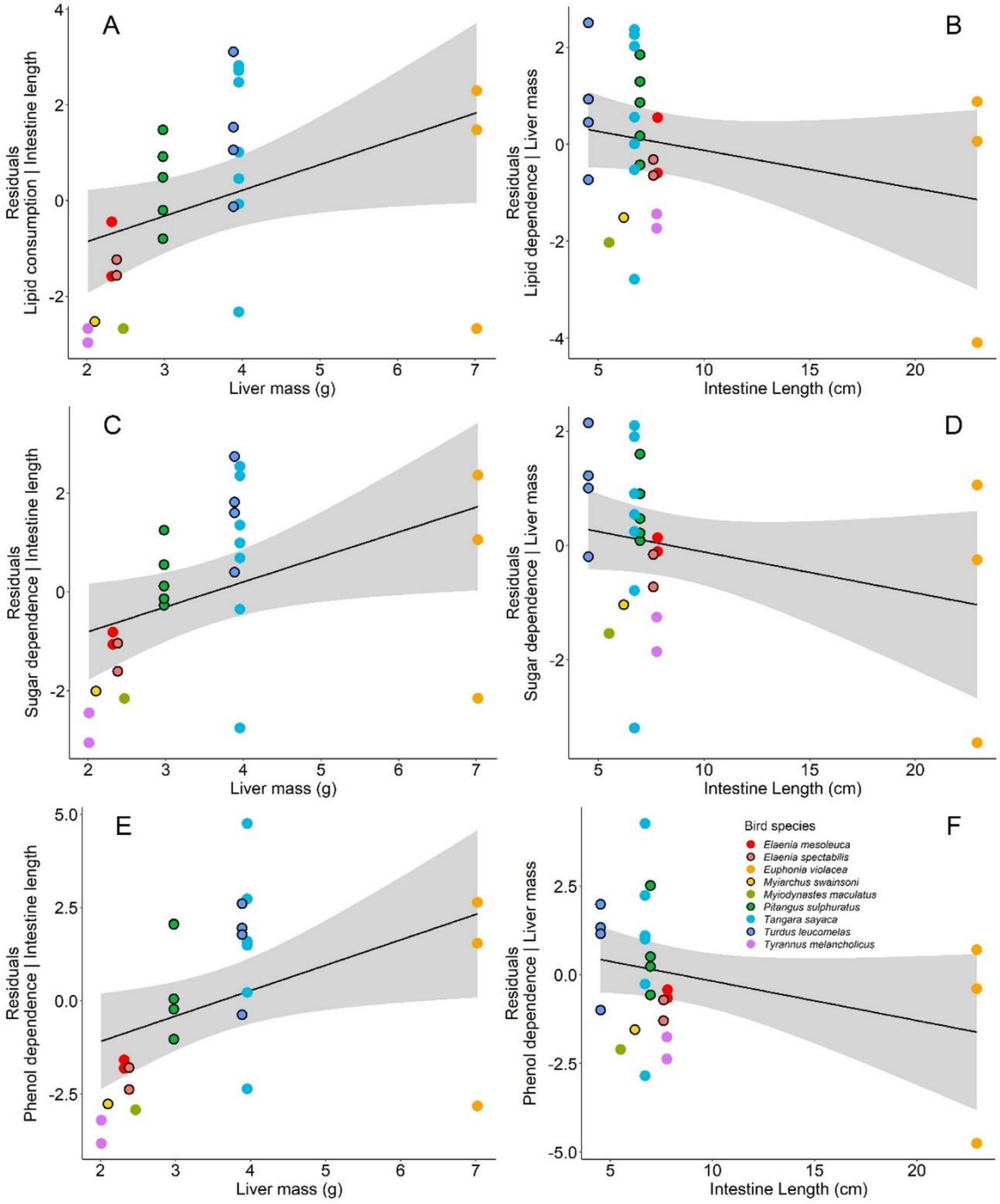

Figure 7. Partial regressions from the Fruit dependence models showing the independent relationship between: lipid and liver mass (A) and intestine length (B), soluble sugar and liver mass (C) and intestine length (D) and total phenols and liver mass (E) and intestine length (F).

2.4 Discussion

In this study, we provided a novel link between frugivory degree and morphological features for eight bird species while controlling for their phylogenetic relationships. In addition, incorporating the rate of fruit eating by birds and the percentage of fruits in their diet, our models revealed the importance of these aspects in predicting frugivory interactions. We could not explain the characteristics of the digestive tract just taking into account the fruit nutritional and chemical composition. Therefore, to find an explanation for digestive features is important to consider the frequency of bird-fruit interaction (Interaction frequency model) and the fruit proportion in a bird diet (Fruit dependence model).

The fruit nutrient analysis showed that the species sampled are mostly sugar-rich, lipid-poor if compared with other lipid-rich genera, for example *Cabralea* (Meliaceae) that produce fruits with more than 70% of lipids (PIZO et al., 2021), since the relative abundance of lipids in the studied fruits did not exceed 17.44%. Four of seven species of fruit analyzed presented more than 50% of soluble sugar, which could be expected since in the tropics sugar-rich are more abundant than lipid-rich fruit species (GALETTI et al., 2011). For total phenols, our results are close to other tropical fruits but this compound was not correlated with sugar as in Cazetta et al. (2008). Similar to Cazetta's results, we had few species that have high amounts of total phenols (*Myrcia tomentosa* with 5.25 % and *Copaifera langsdorffii* with 4.12 %) while the majority varied from 0.64 to 1.55%. Therefore, our fruits were sugar-rich, lipid-poor and presented low amount of total phenols.

Regarding fruit consumption, even though the insectivorous and omnivorous birds interacted with sugar-rich fruits sampled, they often feed on genera of fruits rich in lipids. For example, *Elaenia* and *Myiarchus* consume *Xylopia* (Annonaceae) and *Alchornea* (Euphorbiaceae), which around 44% and 67% of lipids, respectively (MOTTA-JÚNIOR, 1991; PARRINI; PACHECO, 2011; PIZO et al. 2021). Likewise, *Myiodynastes maculatus* consume genera of lipid-rich fruits as *Cabralea* (Meliaceae) with more than 71% of lipids (MOTTA-JÚNIOR, 1991; PIZO et al., 2021). As, we can consider that insectivores and omnivore birds presented, especially those from Tyrannidae, are the consumers of lipid-rich and sugar-rich fruits. Moreover, the rate of interaction is the factor that can play a difference between groups of insectivores/omnivore and frugivores. The Neotropical species of Tyrannidae present a heavy interaction with lipid-rich fruits in comparison with sugar-rich fruits (PIZO et al., 2021). However, since our fruits are mostly sugar-rich and the number of fruits consumed per hour for these species ranged from 0.03 to 2, we failed in find this pattern in our study. Even though insectivorous birds prefer

lipid-rich fruits due to their low capacity to handle with carbohydrate digestion (FUENTES, 1994; MARTINEZ DEL RIO et al., 1988), our data show that they occasionally eat sugary fruits but the low frequency of consume corroborates with this digestive limitation.

Phylogenetic analyses have supported the relationship between the bird feeding habits and their digestive structures (BARCELÓ et al., 2012; CRUZ-NETO et al., 2001). Our tree showed the phylogenetic distance of bird species separated into two groups, one composed of birds with more than 60% of frugivore degree and another with less than 50% of fruit in their diets. Adding a phylogenetic generalized least square model (GLS) we got an adaptive significance for our prediction about the relationship between birds' morphological features and their frugivory degree. Here, we focus on discussing the adaptive significance by linking the features with the characteristic of the fruits consumed.

We found an allometric relationship between morphological features with body mass (BARCELÓ; SALINAS; SABAT, 2012; BRUN et al., 2019; RICKLEFS, 1996). As expected, gizzard size increases with body mass. A muscular stomach is more evident in insectivorous birds compared to frugivores (RICKLEFS, 1996). Since those birds are insectivores or omnivorous, associated with this, they need a voluminous and muscular stomach to process invertebrates or fruits with big seeds (STANLEY and LILL, 2002). Both the linear multiple regression model and the phylogenetic generalized least square model showed that gizzard is related to frugivory degree. For birds whose diet is composed of a high percentage of fruit, the gizzard is not an important digestive structure. On the other hand, when different food items are incorporated and the diet has less than 50 % of fruits, the gizzard becomes larger and presumably more important. Besides gizzard performing mechanical processing of food, it is responsible for a preliminary proteolysis by secreting pepsin, then this organ is associate with a diet rich in protein as invertebrates (CAREY, 2012; DUKE, 1986; JORDANO, 2000). Our results are consistent with Jordano (2000), who showed the case of *Euphonia*, which has a high frugivory degree, low body mass and extrremely small or absent gizzard. Indeed, *Euphonia violacea* did not present gizzard and is the species with the higher frugivory degree. Regarding the processing of food protein , Herrera et al., (2009) have shown that some species of *Euphonia* are able to obtain the protein requirement feeding only on fruits. Combining the information that, *Euphonia* has a large intestine that allows the increase of fruit consumption and this genus can extract their requirement of protein than we can better understand the higher frugivory degree of this bird explained by their adaptations.

Based on our predictions, we expected a longer intestine with higher surface area for birds with lower frugivory degree, which are the largest birds sampled. We took this prediction considering the consumption of lipid-rich fruits by insectivorous birds and the retention time of lipid-rich fruits that demanded a multistep process to be digested (MARTINEZ DEL RIO; RESTREPO 1993, MARTÍNEZ DEL RIO et al., 1992). However, the proportionally longer intestine and its greater surface area found in smaller birds can be explained due to consumption, digestion, and absorption of carbohydrates. The consumption of low-quality food, such as fruits rich in water and sugar, can increase food ingestion to overcome the animal's energy balance (CRESTANI et al., 2021). In this case, a longer intestine allows a higher food intake while the flow of food through the gut still keeps fast due to sugar breakdown and absorption process and a higher intestinal permeability area (KARASOV et al., 2011; MARTINEZ; KARASOV, 1990). Moreover, Price et al., (2015) argue that high hydrolytic enzyme activities can provide compensation for reduced intestine length and surface area. Thus, insectivorous and omnivorous birds can present greater enzyme density to compensate for their smaller intestine and surface area.

Regarding liver mass, the allometric analysis revealed a negative relationship with body mass. To reinforce this finding, the linear multiple regression model and the phylogenetic generalized least square model also showed that birds with a high frugivory degree have a greater liver. First, regarding the capacity of sugar-rich fruit digestion, the liver is responsible for secreting bile that contains amylase, one of the enzymes involved in carbohydrate digestion, contributing with the levels of glucose to glycogenolysis (BRAUN; SWEAZEA, 2008, DUKE, 1986). This explains why birds with high frugivory degrees can consume sugary food. Secondly, these findings also corroborate our prediction that a diet with a high percentage of fruit can also mean a high exposure to secondary compounds. According to the Attraction/Defense fruit Hypothesis, the secondary metabolites present in fruits, as the phenolic group, can mediate the interaction with frugivorous animals, playing a role in fruit defense against pathogens (CAZETTA et al., 2008; CIPOLLINI, 2000; WHITEHEAD et al., 2015). Considering this, the presence of larger liver in birds that eat a high amount of chemical elements can be explained since the liver is the organ responsible for detoxification of such kinds of fruit components (BICUDO et al., 2010, JORDANO, 2000). For example, Herrera (1984) has shown that among Mediterranean birds that eat a combination of food items, those preadapted to eating fruits presented a larger liver. Consequently, animals that have a diet base on fruits, present a large liver to handle with secondary compound as phenols.

Additionally, both Fruit interaction and Fruit dependence models showed a positive relationship between the consumption of total phenols and liver mass, meaning that as the bird consumes a greater amount of total phenols larger is its liver. This also applies to consuming soluble sugar and lipids and can be explained due to the liver digestive function involving in the metabolism of fat and carbohydrates (DUKE, 1986). Our findings illustrate that even in low amounts, the consumption of lipids also demands a large liver responsible for its digestion through emulsification (DUKE, 1986). In fact, when the rate of fruit consumption and the diet dependence on fruits are incorporated into the analysis, the liver seems to be an important organ for nutrient digestion and detoxification of metabolites, did not directly investigated in this study. We highlight, however, that the studied fruits are mainly sugar-rich, and thus the liver mass may respond firstly to the need to deal with higher soluble sugar ingestion. In this sense, exploring a wider gradient of fruit lipid content may reinforce the direct relationship between liver and lipid ingestion, which deserves complementary studies. However, is important to point out that the birds sampled consume many other fruit species.

According to the Fruit interaction and Fruit dependence models, intestine length presented a negative relationship with total phenols. This means that the intestine gets shorter when the consumption of phenols increases, taking into account the rate of fruit consumption. When we combined the consumption with the percentage of fruit in the diet (Fruit dependence model), we found a negative relationship with total phenols, soluble sugar, and lipids. The shorter intestine is present in birds that do not eat much fruits. However, when they eat fruits, some strategies are present to compensate for their shorter intestines. One strategy is the greater villous amplification that enables higher paracellular absorption (CAVIEDES-VIDAL et al., 2007; PRICE et al., 2015). In this way, it could be important to consider the mechanisms by which macronutrients are absorbed in the intestine brush border. Another likely explanation for this pattern is that, as explained above, the digestive traits of our sampled birds, in particular intestine length, may be driven mainly by the soluble sugar gradient of fruit composition, which may constrain the adaptations of such organs to a better processing of further fruit compounds, such as phenols.

This study provides strong evidence of trait-matching between birds digestive and fruit nutritional and chemical characteristic. Future study could deeper investigate the digestive capacity of birds, by analyzing the enzymatic activities of nutrient digestion, rate of absorption, and chemical detoxification (BARCELÓ et al., 2012; BRUN et al., 2019; PRICE et al., 2015). In addition, experimental trials could be performed to better understand the effect of secondary

compounds in the fruits preference and gut transit of birds (BALDWIN & WHITEHEAD, 2015; WHITEHEAD et al., 2015). This approach can improve our knowledge about the outcome of frugivory and how it influences ecological communities.

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SUPLEMENTARY MATERIAL

Fruit traits and morphological digestive variation as mediators of frugivory by birds

Methods

Morphological digestive analyses

Description of measurements of gizzard and intestine surface area

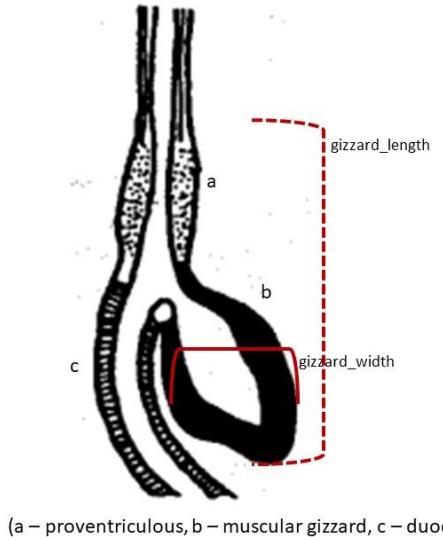

Fig S1. Measurement of gizzard area. Length from proventriculus to muscle gizzard base times muscular gizzard width (adapted from JORDANO, 1987).

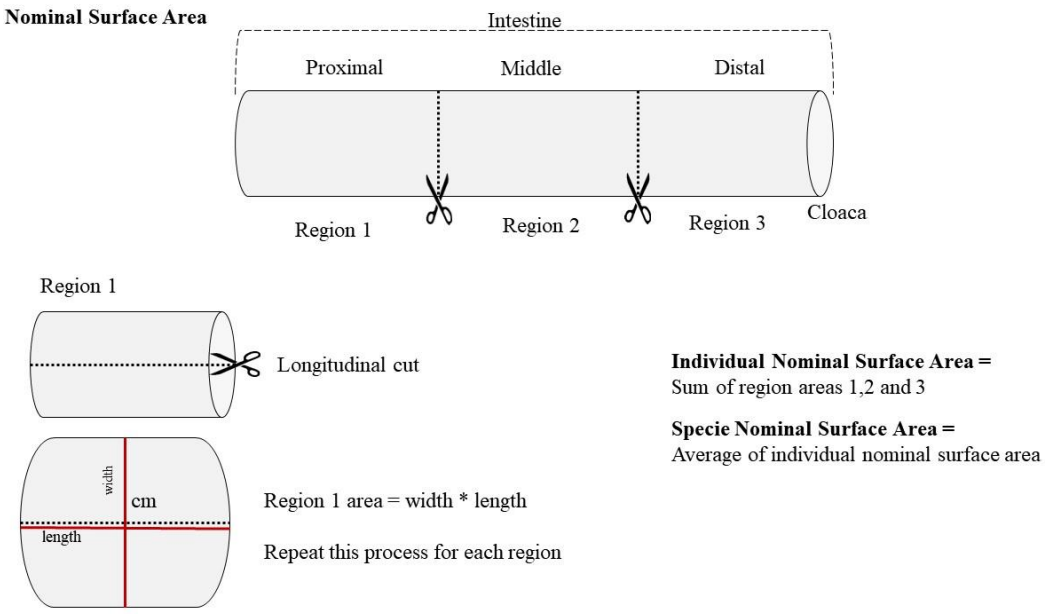

Fig S2. Nominal surface area. The intestine was divided in three regions, proximal, middle and distal (1, 2 and 3). Each region was carefully cut longitudinally with small surgical blunt scissors, opened, cleaned with destiled wather. Using a digital caliper, the length and width were took for each region. For each region we calculated length times width to access the region area. The sum of each region area represent the intestine surface area and the average of individual values of surface area represent the species nominal surface area value (adapted from BRUN et al., 2019).

Fruits' traits

Methods of chemical compound and nutrient extraction.

Total Phenol

1. Make 80% aqueous methanol.
    a. Using a graduated cylinder, add 800 ml methanol and 200 ml distilled water to a clean glass 1L bottle. Get the water from the large white dH20 container, not the distilled water tap in the sink.
2. Sample preparation
    a. Weigh out 25 mg aliquots of ground plant material into Eppendorfs.
    b. Label test tubes with sample number. Place a piece of clear tape over the label.
    c. Add 1 ml of 80% methanol to each tube using the repeater pipette.
    d. Cap tightly and sonicate for 20 min at room temperature.
    e. Centrifuge for 5min at 12000rpm
    f. Carefully remove tubes and transfer supernatant to a second clean 2mL test tube
    g. Add a second 1mL of 80% methanol to the plant material and repeat sonication and centrifuge
    h. Remove the supernatant and combine with the first extraction.
3. Make reagents

a. Folin reagent. Prepare a 10% vol/vol dilution of the concentrated stock (Folin-Ciocalteau reagent; Sigma cat. no F9252. For ~250 samples, make 50 ml of solution (5ml reagent and 45ml dH20)

b. Sodium carbonate (Na2CO3). Prepare a 74.2 mg/ml solution (=700 mM Na2CO3) in water. For ~250 samples, make 200 ml of solution (14.84 g in 200ml)

4. Make a serial dilution of gallic acid.
   a. Dissolve the 10mg of gallic acid in 10ml of 80% methanol to make a 1 mg/ml solution in a scintillation vial.
   b. Add 1mL of the 1mg/ml solution to a clean Eppendorf and add 500ul of 80% methanol to make a 0.6667 mg/ml solution
   c. From the 0.667mg/ml solution, remove 1ml to a clean Eppendorf and add 500uL of 80% methanol to make a 0.444 ml/ml solution
   d. Continue until you have 8 solutions at 1, 0.67, 0.44, 0.296, 0.198, 0.132, 0.088, and 0.059
5. Set up reaction.
   a. Label a fresh set of 2mL eppendorfs: one per sample, one for each gallic acid dilution, and two blanks (solvent only)
   b. To each centrifuge tubes, add (IN THIS ORDER—add each reagent to all tubes, then start next reagent):
      i. 80 μl distilled water
      ii. 20 μl sample (or 80% MeOH for blanks)
      iii. 200 μl dilute Folin reagent (10% vol/vol)
   c. Vortex each sample thoroughly
   d. To each sample, add 800 μl sodium carbonate solution (74.2 mg/ml)
   e. Incubate for 2 hours at room temperature
6. Set up 96-well plate.
   a. Make a diagram of where each sample will go in the 96-well plate. Add each sample to two duplicate wells
   b. Pipette 200 μl from each sample reaction tube into a well, making sure to keep track of which sample is in which well.
   c. Read the absorbance of each well on the plate using the plate reader set to read at 765nm
7. Copy data from plate reader into a spreadsheet. Subtract the average absorbance for the blanks from the values for the samples.
8. Calculate a standard curve from the blank-corrected gallic acid standards.
9. Calculate the concentrations of phenolics in your samples relative to the standards as 'gallic acid equivalents' using the regression equation between gallic acid standards and absorbance at 765nm.

Soluble Sugar. We followed the CheKine™ Plant Soluble Sugar Colorimetric Assay Kit. Catalog number MBS9719037. My BioSource.

Lipid extraction and quantification

Lipids were extracted from 2 mg of dried pulp using 2 mL of 2:1 chloroform-methanol. Samples were vortex, sonicated in a water bath for 20 min, and centrifuged for 5 min at

6000 g. The supernatant was collected, and a second extraction was performed on the pellet. Both extracts were combined and 1 mL of 0.6% NaCl was added. The mix was centrifuged for 10 min at 1500 g. The organic phase was collected in a clean vial, and evaporated under a stream of nitrogen. For the transesterification, 0.8 mL of hexane and 1.2 mL of 10% acetyl chloride in methanol were added. As an internal standard, 20 µL of octadecane 1 mg/mL were added. The samples were vortex and incubated at 100°C for 40 min. After the samples reached room temperature, 2 mL of 6% sodium carbonate and 0.4 mL hexane were added. The samples were vortex and centrifuged for 10 min at 1500 g. From the top layer, 200 uL were transferred to a clean vial, the solvent was evaporated under a stream of nitrogen and samples were resuspended in 100 uL of hexane. Samples quantification were performed injecting 5uL into an Agilent 7820 gas chromatograph coupled with a 5977 mass spectrometer equipped with an HP5-MS column (Agilent Technologies, Santa Clara, CA, USA). Lipids were separated using a temperature program of 70°C for 1 min, then ramped to 15°C per min to 150°C, then a second ramp 5 °C per min to 250°C, a final ramp of 10°C per min to 310°C, and then held for 5 min. Agilent files were converted to mzML using MSConvert, and data were processed using the R package metaMS (Wehrens et al., 2014). Total lipid concentration was calculated based on the area of the internal standard. We performed three analytical replicates per species.

Phenolics extraction and quantification. Phenolics were extracted from 25 mg of dried pulp using 1 mL of 80% methanol. Samples were vortex, sonicated in a water bath for 20 min, and centrifuged for 5 min at 10000 g. The supernatant was collected, and a second extraction was performed on the pellet. Both extracts were combined, and to 20 uL of samples, 80 uL of distilled water, 20 uL of samples, 200 uL of Folin reagent and 800 uL of sodium carbonate solution (74.2 mg/ml) were added. Then, 200 ul of the samples were transferred to a 96-well plate and absorbance was read at 765 nm. Phenolic concentrations were calculated as gallic acid equivalents. We performed three analytical replicates per species.

Interactions

Table S1. Sources of information of bird-fruit interactions used in this study.

Sources	Habitat
Allenspach et al. 2012	Cerrado
Alves, K. J. F. 2008	Mata Atlântica
Argel de Oliveira, M. M. 1999	Mata Atlântica
Athiê, S. 2009	Mata Atlântica
Camargo, M. G. G. 2014	Cerrado
Correia, J. M. S. 1997	Mata Atlântica
Gondim, M. J. C. 2002	Cerrado

Lopes, R. F. 2000	Mata Atlântica
Maruyama et al. 2013	Cerrado
Motta-Júnior and Lombardi. 1990	Cerrado
Motta-Júnior., J. C. 1991	Cerrado
Oniki et al. 1994	Mata Atlântica
Rabello et al. 2010	Mata Atlântica

Table S2. Model selection by employing the stepwise forward procedure. The best model is considered the one with the lowest AIC.

		Fruit composition models		Fruit interaction models		Fruit dependence model
		AIC		AIC		AIC
Total phenols	none	15.98	none	15.98	none	25.23
	Surface area	16.94	Surface area	16.94	Gizzard	27.03
	Liver	17.50	Liver	17.50	Surface area	27.22
	Intestine length	17.63	Intestine length	17.63	Intestine length	28.64
	Gizzard	17.85	Gizzard	17.85	Liver	38.29
Soluble sugar	none	158.93	none	158.93	none	16.60
	Gizzard	159.87	Gizzard	159.87	Gizzard	17.15
	Liver	160.08	Liver	160.08	Intestine length	18.32
	Intestine length	160.69	Intestine length	160.69	Surface area	18.47
	Surface area	160.93	Surface area	160.93	Liver	24.98
Lipids	none	77.72	none	23.67	none	23.67
	Liver	79.29	Gizzard	24.92	Gizzard	24.92
	Gizzard	79.37	Intestine length	25.32	Intestine length	25.32
	Surface area	79.47	Surface area	25.56	Surface area	25.56

Intestine length	79.66	Liver	31.32	Liver	31.32

Results

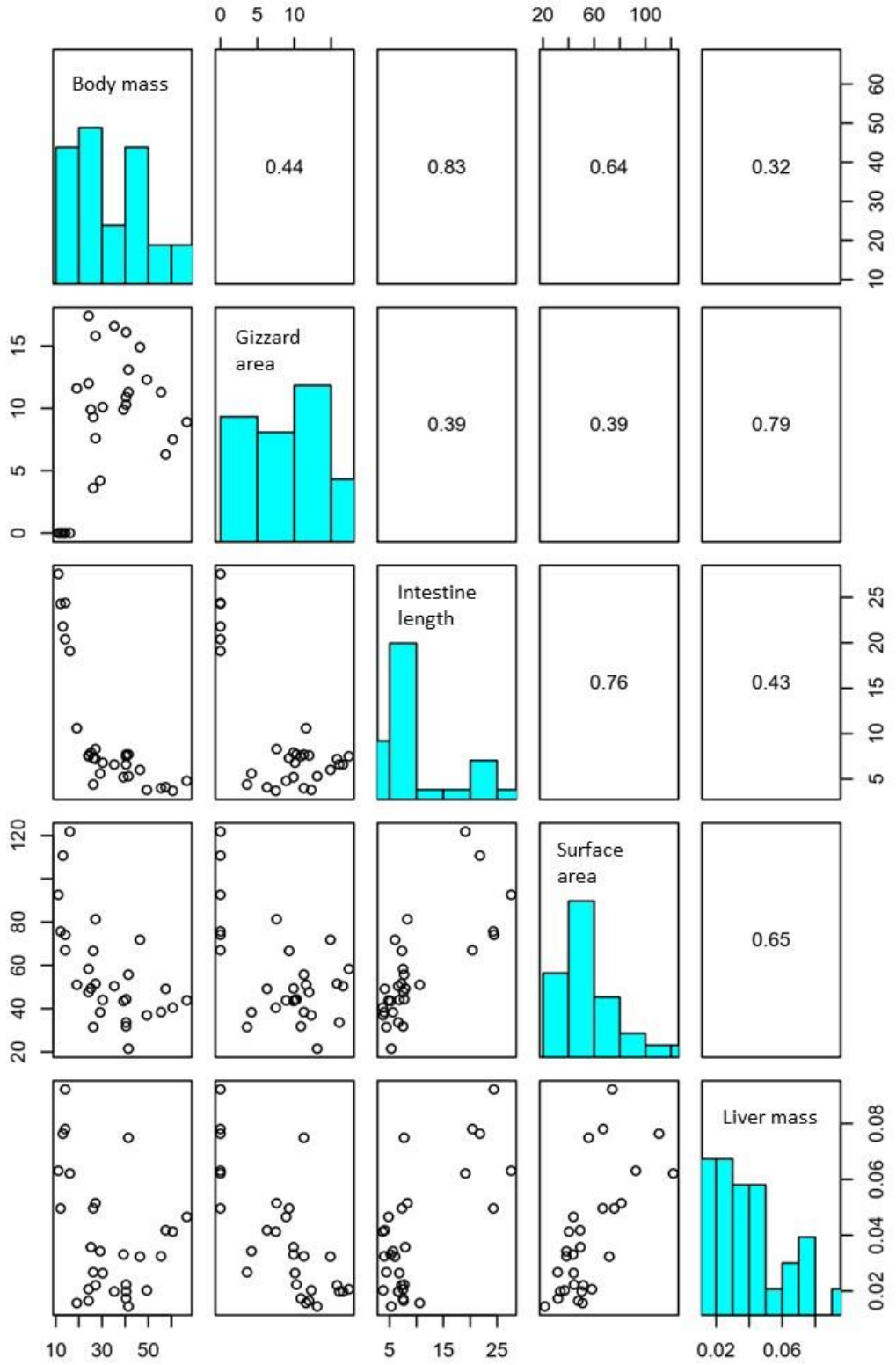

Figure S3. Pearson correlation matrix between morphological traits of birds.

Table S3. Measurement of body: body mass (g), gizz: gizzard diameter (cm), intest: intestine length (cm), surf: intestinal surface area (cm), and liv: liver mass (g) from 34 bird individuals from eight species and four families.

Birds species	Birds families	body	gizz	intest	surf	liv	frug
Euphonia violacea	Fringillidae	14,1	0	20,4	67	0,07801	100
Euphonia violacea	Fringillidae	13,1	0	21,8	110,7	0,07634	100
Euphonia violacea	Fringillidae	12,1	0	24,3	75,7	0,04959	100
Euphonia violacea	Fringillidae	11,1	0	27,6	92,6	0,06306	100
Euphonia violacea	Fringillidae	14,1	0	24,4	74,1	0,0922	100
Euphonia violacea	Fringillidae	16,1	0	19,1	121,8	0,06211	100
Tangara sayaca	Thraupidae	26,2	9,3	7,3	66,7	0,04962	64,9
Tangara sayaca	Thraupidae	26,2	3,6	4,4	31,5	0,02672	64,9
Tangara sayaca	Thraupidae	25,2	9,9	7,9	49,3	0,03571	64,9
Tangara sayaca	Thraupidae	27,2	7,6	8,3	81,3	0,05147	64,9
Tangara sayaca	Thraupidae	29,2	4,2	5,6	38,3	0,03425	64,9
Turdus leucomelas	Turdidae	57,5	6,3	4,1	49,1	0,04174	64,9
Turdus leucomelas	Turdidae	60,6	7,5	3,7	40,4	0,04125	64,9
Turdus leucomelas	Turdidae	66,6	8,9	4,8	43,8	0,04655	64,9
Turdus leucomelas	Turdidae	46,4	14,9	6	71,8	0,03233	64,9
Turdus leucomelas	Turdidae	55,5	11,3	4	38,4	0,03243	64,9
Elaenia mesoleuca	Tyrannidae	15,7	12,3	8,1	87,7	0,03185	50
Elaenia mesoleuca	Tyrannidae	13,8	8,1	7,5	91,7	0,01449	50
Elaenia spectabilis	Tyrannidae	21	10,1	7,6	69,9	0,02381	50
Myiarchus swainsoni	Tyrannidae	23,8	8,3	6,2	44,5	0,02101	50
Myiodynastes maculatus	Tyrannidae	39,3	9,9	5,2	43,5	0,03308	29
Myiodynastes maculatus	Tyrannidae	49,4	12,3	3,8	36,9	0,02024	29
Myiodynastes maculatus	Tyrannidae	24,2	17,4	7,5	58,3	0,02066	29
Pitangus sulphuratus	Tyrannidae	40,4	10,3	7,7	44,3	0,02228	53
Pitangus sulphuratus	Tyrannidae	41,4	13,1	5,3	21,5	0,01449	53
Pitangus sulphuratus	Tyrannidae	40,4	16,1	6,6	33,6	0,0198	53
Pitangus sulphuratus	Tyrannidae	40,4	10,9	7,5	31,8	0,01733	53
Pitangus sulphuratus	Tyrannidae	41,4	11,3	7,7	55,7	0,07488	53
Tyrannus melancholicus	Tyrannidae	30,3	10,1	6,8	44	0,0264	12
Tyrannus melancholicus	Tyrannidae	24,2	12	7,6	47,6	0,01653	12
Tyrannus melancholicus	Tyrannidae	19,1	11,6	10,6	51	0,01571	12
Tyrannus melancholicus	Tyrannidae	35,3	16,6	6,6	50,4	0,01983	12
Tyrannus melancholicus	Tyrannidae	27,2	15,8	7,2	51,5	0,02206	12

Figure S4. Pearson correlation between fruit nutrients and total phenols. Lipids: lipid, total phenols: phenols and soluble sugar: ssugar.

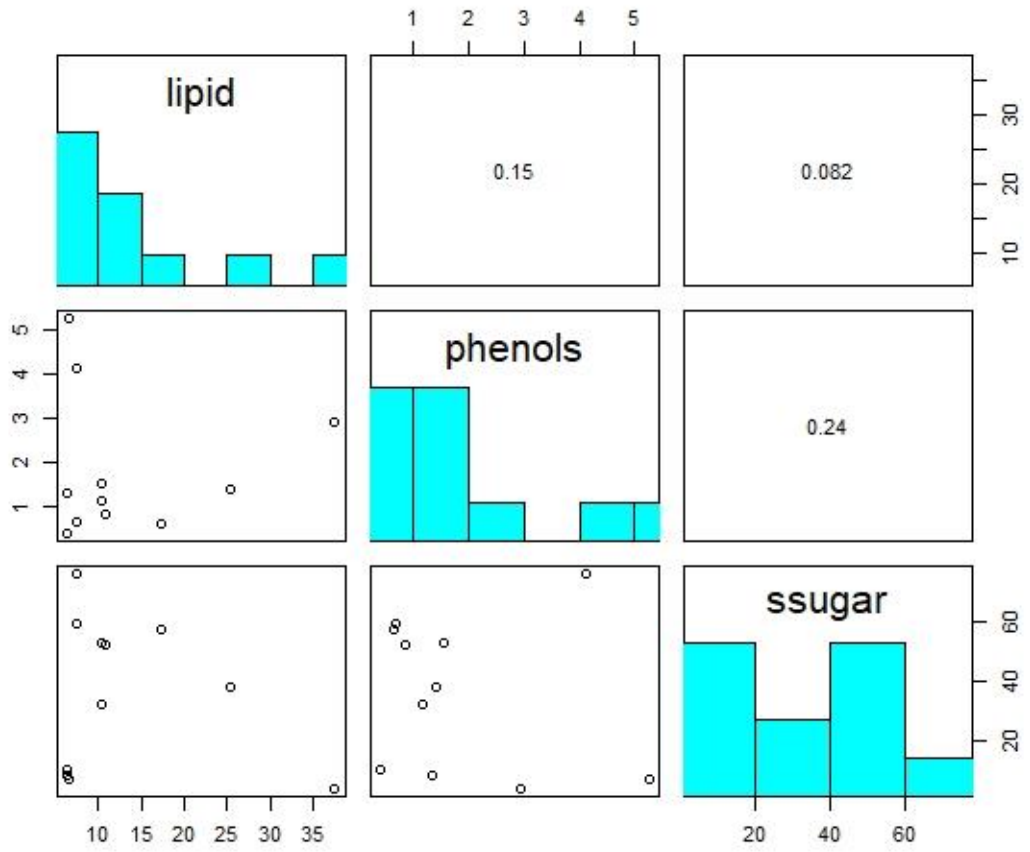

Figure S5. Partial regressions showing the independent relationship between frugivorous degree and gizzard area (A) and liver mass (B).

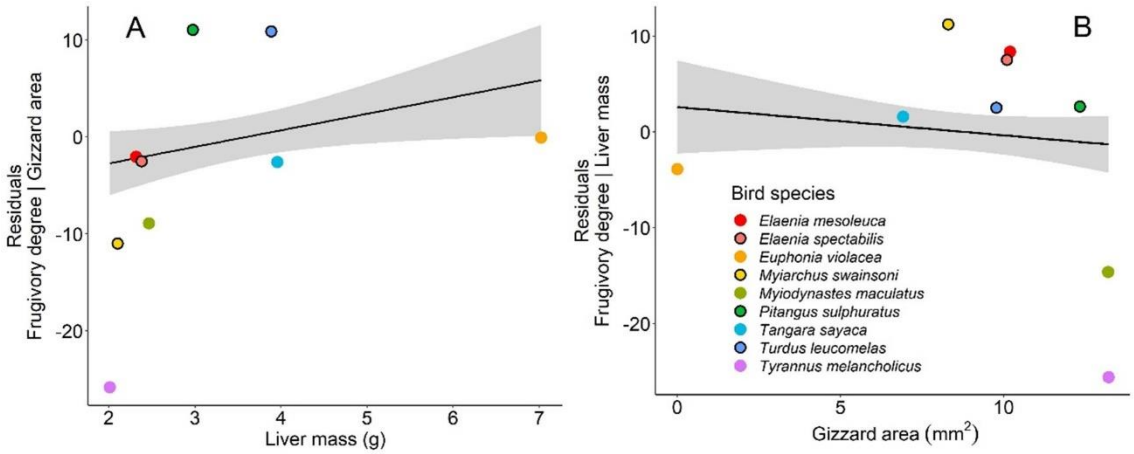

Table S4. Results from linear multiple regressions between Frugivory degree and liver mass, intestine length, gizzard area and intestinal surface area. Std. error: Standard error, t: t value, and

p: significant level value at alpha = 0.05. Estimates and t values not shown for explanatory variables absent from the final models defined by the forward stepwise selection procedure.

	Estimate (SE)	t	p
Intercept	64.23 (12.42)	5.17	< 0.001*
Liver	473.77 (180.83)	2.62	< 0.001*
Gizzard	-2.9 (0.72)	-3.99	< 0.001*
Intestine			0.21
Surface			0.98

Table S5. Significant phylogenetic signal for bird morphological features. $Phylo_{\lambda}$ represent phylogenetic signal for Pagel's lambda and p represent the significant level value at alpha = 0.05.

Morphological feature	$Phylo_{\lambda}$	p
Gizzard area	1.15	0.001
Liver mass	1.15	0.001
Intestine length	1.15	0.001

Table S6. Phylogenetic generalized least square models. Std. error: Standard error, t: t value, and p: significant level value at alpha = 0.05.

	Estimate (SE)	t	p
Gizzard	-4.76 (1.73)	-2.75	0.02
Liver	1366.83 (528.14)	2.58	0.03

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3. CHAPTER 2 Sugar and nitrogen digestive processing does not explain the specialized relationship between euphonias and low-quality fruits

3.1 APPENDIX

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Research

Sugar and nitrogen digestive processing does not explain the specialized relationship between euphonias and low-quality fruits

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In the Neotropical region, euphonias (*Euphonia* spp., Fringillidae) are the quintessential example of specialized bird frugivores, making the bulk of feeding visits to certain mistletoes (*Phoradendron* spp., Santalaceae) and epiphytes in the genus *Rhipsalis* (Cactaceae), whose fruits have high water and low sugar and protein concentrations. Surprisingly, a mechanistic explanation for such specialized, otherwise rare, relationships is lacking. Using captive birds and artificial diets, we contrasted euphonias with frugivorous tanagers in the genus *Thraupis* (Thraupidae), which rarely eats *Rhipsalis* fruits, to test the hypothesis that the digestive capacity of euphonias entails them to exploit such low-energy fruits. We expected that compensatory feeding in response to decreasing energy density would occur only in euphonias, whose higher reliance on fruits would entail a lower nitrogen requirement than the tanagers. Euphonias and tanagers were both able to compensate energy intake as sugar density decreased, and both species had the same mass-corrected energy intake at any given sugar concentration. Similarly, euphonias and tanagers did not differ in mass-corrected maintenance nitrogen requirement. Therefore, the physiological traits we investigated do not explain euphonias' specialization on *Rhipsalis* fruits. The fast rates of fruit passage typical of specialized avian frugivores as euphonias that entail the processing of a large volume of fruits and the putative better abilities of such birds to deal with secondary compounds likely present in *Rhipsalis* fruits are other possible mechanisms that should be considered in future studies to unveil the mechanisms underlying the intriguing specialized relationships between euphonias and certain fruits.

Keywords: compensatory feeding, diet specialization, energy balance, *Euphonia*, protein, sugar

Introduction

Unraveling the mechanisms that promote specialization (i.e. the restriction in the number of interacting partners) is a central issue for understanding the coevolution and maintenance of plant–animal interactions. Pairwise-specialized relationships between plants and frugivores are thought to be extremely rare in nature and less frequent than

in other plant–animal mutualisms or in interactions involving greater intimacy of partners (e.g. symbiotic interactions, parasitism; Thompson 1982, Jordano 1987, Blüthgen et al. 2007).

Mistletoes (i.e. aerial hemiparasitic plants) maintain with certain frugivores the best-studied cases of specialized seed dispersal systems. Across their worldwide distribution, mistletoes have independently developed close relationships with eight lineages of frugivorous birds (Reid 1991, Restrepo et al. 2002) and one marsupial species (Amico and Aizen 2000) that act as seed dispersers. In the Neotropical region, euphonias (*Euphonia* spp., Fringillidae) and the closely related genus *Chlorophonia* make the bulk of feeding visits to mistletoe species in the genus *Phoradendron* (Santalaceae; Restrepo 1987, Reid 1991, Restrepo et al. 2002). Euphonias form a group of 27 species of small (9–19 g) passerines that are the quintessential example of obligate avian frugivores for which fruits may represent up to 97% of their diets (Snow and Snow 1971, Hilty 2011). As an extreme adaptation to frugivory, euphonias, like other mistletoe specialists, have a simplified digestive tract with a vestigial gizzard, which promotes rapid transit of seeds through their guts and rapid processing of fruits (Wetmore 1914, Walsberg 1975). Euphonias are also able to meet nitrogen requirements exclusively from fruits, which is uncommon among frugivorous birds (Herrera et al. 2009). Evidence suggests that the close relationship of euphonias with mistletoes likely extends to other plants, especially epiphytes in the families Araceae, Cactaceae and Bromeliaceae. In Trinidad, 97% of 190 feeding records of *Euphonia violacea* were on fruits, 62% of which were on epiphytes (Snow and Snow 1971). In Costa Rica, euphonias fed so heavily on fruits of *Anthurium* (Araceae) to the point of forming a separate guild of frugivore (Loiselle and Blake 1990). In the Brazilian Atlantic forest, euphonias are responsible for 60% of feeding visits to the epiphytic cactus genus *Rhipsalis*, being the most effective seed dispersers (Guaraldo et al. 2013).

Despite the ubiquity of mistletoes and the historical interest in their specialized seed dispersal, the mechanisms promoting the specialization have surprisingly not been investigated in detail. Reid (1991) proposed a set of mechanisms related to fruit morphology, including fruit size and viscidity, and fruiting displays that would render foraging on mistletoe fruits inefficient for certain frugivores. Here, we explored the digestive traits of euphonias as a key to understand their close relationships with mistletoes and epiphytes. The emphasis on digestive traits is justified because (a) there is apparently no mechanical barrier preventing the consumption of mistletoes and epiphyte fruits by other potential consumers, (b) the low overall nutrient content of the epiphyte fruits eaten by euphonias makes them unusual in comparison to other fruits (below), (c) gut morphology of euphonias is also unusual, extremely simplified by the lack of a gizzard (Wetmore 1914) and (d) there is ample evidence that the digestive capabilities of avian frugivores may constraint the consumption of certain fruits (Levey and Martinez del Rio 2001).

We used *Rhipsalis* fruits as a model not only because their specialized interaction with euphonias is well documented but also because their nutrient content is better known than that of the fruits of *Phoradendron* mistletoes for which euphonias are the main or sole consumer (Guaraldo et al. 2013). *Rhipsalis* fruits are rich in water (89.0–93.7%) with very small amounts of proteins (1.9–5.5% dry weight) and lipids (0.7–2.0% dry weight), and low sugar concentration (3.0–7.8% wet weight), mainly represented by glucose with lower amounts of fructose and sucrose (Guaraldo et al. 2013). The few data available on *Phoradendron* fruits resemble *Rhipsalis* in regards to water, lipid and protein content (data on sugar unavailable; Supporting information). With high water and low protein contents, *Rhipsalis* fruits occupy the extremes of the water and protein distributions of Neotropical fleshy fruits (Supporting information), resembling in this regard the nectar of bird-pollinated flowers (McWhorter et al. 2003). Feeding on energy-diluted food is not trivial for frugivores because some species cannot compensate energy intake when sugar content is low (5–10%; Martínez del Rio et al. 2001). Likewise, the low protein content of *Rhipsalis* fruits may lead to a protein deficit in frugivorous birds (Izhaki and Safriel 1989, Izhaki 1992).

We measured the intake response to varying amounts of food energy of the violaceous euphonia *E. violacea* and compared it with that of the palm tanager *Thraupis palmarum* (Thraupidae). In another set of experiments, we compared the maintenance nitrogen requirements (MNR) of *E. violacea* with that of the sayaca tanager *Thraupis sayaca*. While the euphonia is a regular consumer of *Rhipsalis* fruits, both tanagers are occasional consumers (Guaraldo et al. 2013), which parallels their contrasting degrees of frugivory (i.e. reliance on fruit) that range from 50 to 60% in the tanagers to 100% in the euphonia (Wilman et al. 2014). We tested the hypothesis that the contrasting affinity to *Rhipsalis* fruits between euphonias and other birds is related to the digestive capacity to process and rely on watery fruits with low energy density and low protein content. Regarding the energy density issue, we predicted that compensatory feeding in response to decreasing energy density would occur only in *E. violacea*, leading to higher mass-corrected energy intakes. In relation to proteins, we expected that the higher frugivory degree of the euphonia would entail a lower MNR than the tanager. Since fleshy fruits typically have low protein contents, the higher the reliance of a bird on fruit, the lower should be its protein requirements, as observed in a comparison between frugivorous and omnivorous birds (Tsahar et al. 2006).

Material and methods

Animals: source and maintenance

Twelve individuals of *E. violacea*, six of *T. sayaca* and five of *T. palmarum* were obtained at Centro de Recuperação de Animais Silvestres do Parque Ecológico do Tietê (CRAS – PET) at São Paulo, Brazil and maintained separated in a large outdoor cage ($5 \times 3 \times 2$ m) within the facilities of the State University of São Paulo at Rio Claro, Brazil. Birds were held in these conditions for six months with water and a diet of papaya, banana, orange and avocado provided ad libitum and supplemented with commercial diets for frugivorous birds (NuTrópica, Brazil).

Before the experiments, birds were transferred to individual cages (1.0 × 0.5 × 0.5 m) and maintained in a climatic room (Fitotrom, Eletrolab, Brazil) at 25 ± 1°C, adjusted to 12 h light:12 h dark cycle. Birds received the same husbandry diet and had their body mass (m_b) measured daily during this five-day acclimation period (± 0.1 g – Ohaus Electronic Balance, USA). All birds adapted well to these conditions, as their m_b did not vary by more than 5% and showed no sign of feather loss, two conditions considered to be necessary to start the experimental trials. One day before the start of the trials, birds received only the experimental solutions to eliminate all residues of the husbandry diet.

General procedures

Experimental solutions (described below) were kept in a refrigerator before use and then removed 1–1.5 h before being offered to birds so that their temperatures would stabilize with the ambient temperature. All solutions had their osmotic concentrations determined before being offered. In cases where the osmotic concentrations differed by more than 5% from the values measured right after their preparation, solutions were discarded, and a new batch was prepared. We conducted two separate experiments to test compensatory feeding and to measure nitrogen requirements. Three trials were carried out for each experiment to measure the intake of solutions with different concentrations of sugar and protein. Each trial lasted for two days, and each individual was randomly assigned to a given solution in a given trial. Each trial was interspaced by a six-day period, when birds were returned to the outdoor cage and received the husbandry diet.

A trial started at dawn and ended at sunset, lasting for 12 h. After measuring the bird m_b, the experimental diet was offered by placing the solutions in a plastic feeder of known weight, which was again weighed after being filled with a precision of ± 0.001 g. The measured value represented the amount of food given. Three additional feeders, each with a solution of different concentrations, were placed in the climatic room, outside the cages, to control for evaporation. The feeders and the birds were again weighed 12 h later. We did not observe any spillover of the diet in the cages, so the difference between the weight of the feeder at dawn and sunset, after correcting for evaporation, was used as a measure of the volumetric intake of each bird. No food was given during the night, and the whole protocol was repeated the next day. Diets were offered to each individual for two consecutive days in a 12-h period, and each bird received all solutions in separate trials. A plastic film lined the cage bottom to retain the excreta that were collected every 33-h intervals using a syringe and immediately frozen at −20°C until analyses.

Compensatory feeding

For six *E. violacea* and five *T. palmarum* individuals, we offered sugar solutions whose compositions reflect the average sugar composition of different species of *Rhipsalis* consumed by euphonias: 77% of glucose, 10% of fructose and 13% of sucrose (Guaraldo et al. 2013). Based on this composition, three solutions containing 5, 10 and 20 g of these sugars diluted in 100 ml of water were prepared and used throughout the experiments. Each solution was complemented with 0.4 g of hydrolyzed casein (McWhorter et al. 2003). The energetic values of these solutions were calculated based on the caloric content of the different types of sugars and casein used to prepare the different solutions (Robbins 1994). Thus, each 100 ml of the solutions containing 5, 10 and 20% of sugar had an energetic content of 89.2, 171.6 and 336.5 kJ, respectively. In each trial, the energy content of the liquid diet offered to the birds was always two times higher than the estimated daily energetic requirements of the birds (field metabolic rate = $10.5 m_b^{0.681}$; Nagy 2005).

Nitrogen requirements

For the nitrogen requirement experiment, six individuals of each *E. violacea* and *T. sayaca* received liquid diets composed by 0, 0.2, 0.4 and 0.6 g of hydrolyzed casein (Merck KGaA)/100 ml H_2O and 10 g of sugar/100 ml H_2O (adapted from Tsahar et al. 2005). The hydrolysate casein had 7.0–8.5% of nitrogen. Nitrogen in excreta was analyzed using the Kjeldahl method (Mackereth et al. 1978).

Data handling and analysis

Compensatory feeding

A few individuals (one *E. violacea* and two *T. palmarum*) did not respond well when presented with a liquid diet during the first day of the first trial. Although all individuals were fully habituated to the liquid diet on both days of the second and third trials, only the data obtained during the second day of each trial was used in the subsequent analysis.

We used two approaches to test whether both species displayed compensatory feeding. The first approach follows the protocol outlined by Martinez del Rio et al. (2001): for each individual, we used regression analysis on log-transformed data to determine the slope (b) values of the relationship between volumetric intake and sugar concentration and an analysis of covariance (ANCOVA) with body mass as a covariate to test for the homogeneity of these slopes. To test whether both species displayed compensatory feeding, a power function relating volumetric intake and sugar concentration was fitted to the data (Martinez del Rio et al. 2001):

$$V = aC^{-b} \quad (1)$$

where V is the volumetric intake, C is the sugar concentration, and a and b are empirical constants. This function suggests that V decreases as C increases. Perfect compensatory feeding would occur when the values of b from Eq. 1 do not differ from −1 (Martínez del Rio et al. 2001). A one-sample t-test was used to verify whether the slope of this power function (obtained separately for each individual of each species) differed from the expected values of −1, and a two-sample t-test was used to compare the slopes between species.

In the second approach, we used a one-way repeated-measure ANOVA to test for within-species differences in volumetric (VI, in ml 12 h^{-1}) and energy intake (EI, in kJ 12 h^{-1}) as a function of sugar concentration (in g 100 ml^{-1}). A general linear model (GLM) was used to test whether VI and EI differed between species as a function of sugar concentration. In this model, we used species and sugar concentration as fixed categorical predictors, individuals as random factor, and m_b as a continuous (covariate) predictor. A post hoc Fisher test was then used, if necessary, to compare values of VI or EI between and within species.

The same procedure outlined above was used to test for within- and between-species differences in fractional changes in m_b (Δm_b, calculated as in Herrera et al. 2015) as a function of sugar concentration. In this case, however, we did not use m_b as a covariate in the GLM model to avoid collinearity. Thus, post hoc comparisons, if necessary, were carried out with untransformed values.

Nitrogen requirements

We determined apparent maintenance N requirements (MNR) for each individual bird using the x-intercept of a least square linear regression of the apparent N balance (N intake minus excreted N) on N intake (Smith and Green 1987). We compared mean MNR between *E. violacea* and *T. sayaca* using a general linear model (GLM), with species as fixed categorical predictor and m_b as a continuous (covariate) predictor.

In all analysis described above, values of VI, EI and Δm_b were log transformed (base10). Values are presented as mean $\pm$ 1 SEM and $\alpha = 0.05$ was assigned to all tests to determine statistically significant differences.

Results

Compensatory feeding

All individuals of *E. violacea* and *T. palmarum* increased their volumetric intake as sugar concentration decreased (Fig. 1). We found no difference in the exponent of the relationship between volumetric intake and sugar concentration among individuals of *E. violacea* ($F_{5,11} = 0.73$, $p = 0.62$) and *T. palmarum* ($F_{4,9} = 2.2$, $p = 0.21$). The mean exponent (b) of the power functions relating volumetric intake and sugar concentration for both species did not differ from −1 (*E. violacea*: $b = -1.01 \pm 0.05$, $t_5 = 0.13$, $p = 0.9$; *T. palmarum*: $b = -0.89 \pm 0.08$, $t_4 = 1.35$, $p = 0.25$) and also did not differ from each other ($t_9 = 1.23$, $p = 0.25$).

Volumetric intake varied as a function of sugar concentration for *E. violacea* ($F_{2,17} = 146.7$, $p < 0.001$; Fig. 2A) and *T. palmarum* ($F_{2,14} = 75.1$, $p < 0.001$; Fig. 2A), decreasing linearly with sugar concentration ($p < 0.001$ for all pairwise comparisons) for both species. Volumetric intake did not differ between the two species at any sugar concentration tested ($F_{2,26} = 1.81$; $p = 0.19$). Energy intake did not vary as a function of sugar concentration for *E. violacea* ($F_{2,17} = 1.86$, $p = 0.21$; Fig. 2B) and *T. palmarum* ($F_{2,14} = 0.96$; $p = 0.42$; Fig. 2B), being similar between species at all sugar concentration tested ($F_{2,26} = 1.83$; $p = 0.20$).

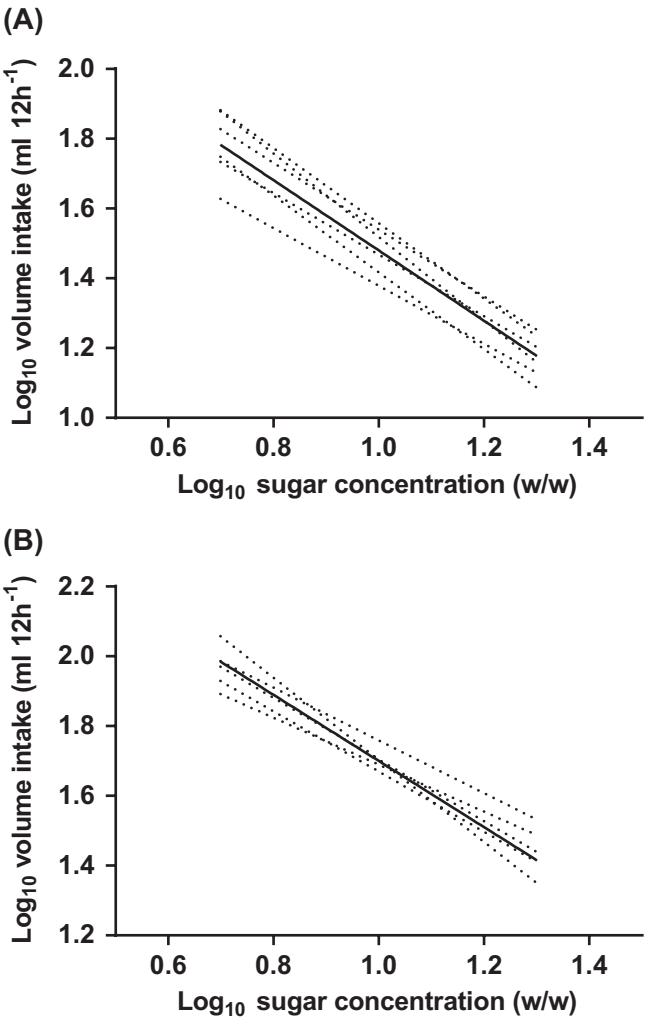

Figure 1. Volumetric intake of six *Euphonia violacea* (A) and five *Thraupis palmarum* (B) as a function of sugar concentration (w/w %). Dashed lines represent slopes for each individual regression (log_{10} transformed data) and solid lines is the common slope obtained from the average of each individual slope.

Relative change in body mass (Δm_b) was always positive and independent of sugar concentration for *E. violacea* ($F_{2,17} = 0.63$, $p = 0.56$) and *T. palmarum* ($F_{2,14} = 0.24$, $p = 0.79$; Table 1). The magnitude of Δm_b was similar for both species ($F_{2,26} = 0.72$, $p = 0.62$).

Nitrogen requirements

Nitrogen balance was significantly associated with total nitrogen intake for all individuals of *T. sayaca* and *E. violacea* ($Rs > 0.90$, $Ps < 0.0001$; Fig. 3). After controlling for the effects of body mass, mean MNR for *T. sayaca* (1.31 $\pm$ 0.17 mg N) was similar to that quantified for *E. violacea* (0.86 $\pm$ 0.08 mg N; $F_{1,11} = 2.04$, $p = 0.19$).

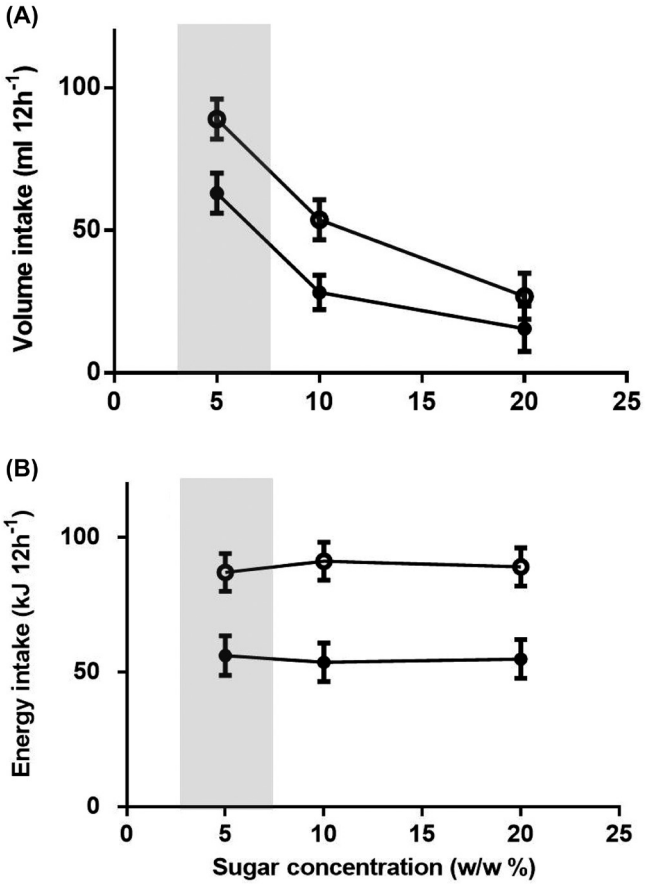

Figure 2. Relationship between volume (A) and energy (B) intake and sugar concentration (w/w %) for six *Euphonia violacea* (●) and five *Thraupis palmarum* (○). Shaded columns show the range of sugar concentration in *Rhipsalis* fruits. Variation expressed as mean (± 1 SEM).

Discussion

Compensatory feeding

Our hypothesis that the ability to compensate for food quality (low sugar concentration) would be limited to the specialist species was not supported by our findings since both *E. violacea* and *T. palmarum* compensated energy intake as sugar density decreased, and mass-corrected energy intake did not differ between the two species at any sugar concentration.

The intake rate of solutions with varying sugar densities has been widely examined in plant-eating birds, with contrasting responses when birds are exposed to low-sugar solutions. Some species are unable to compensate energy intake when fed dilute sugar solutions (5–10%), including specialized nectarivores (*Archilochus alexandrii*, *Chalcomitra amethystina*, *Coereba flaveola*, *Eugenes fulgens*, *Lampornis clemenciae*, *Nectarinia famosa*, *Selasphorus platycercus*; McWhorter and López-Calleja 2000, Fleming et al. 2004, Mata and Bosque 2004, Brown et al. 2010a, Köhler et al. 2010) and opportunistic nectarivores that feed heavily on fruits (*Colius striatus*, *Dicaeum hirundinaceum*, *Zosterops lateralis*; Brown et al. 2010b, Napier et al. 2013). In turn, some specialized nectarivores (*Cinnyris talatala*; Köhler et al. 2010) can compensate energy intake of 8.5% sugar solutions, and several opportunistic nectarivores compensate with 8.5% (*Lichenostomus virescens*; Napier et al. 2013) and 5% sugar solutions (*Cyanerpes cyaneus*, *Onychognathus morio*, *Ploceus cucullatus*, *Pycnonotus tricolor*; Brown et al. 2010c, 2012, Odendaal et al. 2010, Napier et al. 2013, Mancina and Herrera 2016). *Euphonia violacea* and *T. palmarum* compensated energy intake when fed 5% sugar solutions and are functionally more similar to opportunistic than to specialized avian nectarivores. Compensatory feeding may enable *E. violacea* and *T. palmarum* to process *Rhipsalis* fruits in the same way that it allows opportunistic avian nectarivores to feed on sugar-dilute nectar (Johnson and Nicolson 2008).

Compensating energy intake with the 5% sugar solution comes with the cost of having to process large volumes of watery food, particularly for *E. violacea* that drank a volume equivalent to 3–6 times its body mass. Ingestion of large volumes of pre-formed water is not trivial for birds and two strategies have been identified. While intestinal absorption of ingested water is nearly complete in hummingbirds (McWhorter and Martínez del Rio 1999), sunbirds and honeyeaters avoid the metabolic burden of processing excess water by limiting intestinal absorption and excreting most water directly in feces (McWhorter et al. 2004, Purchase et al. 2013). Excessive ingestion of water may represent an osmoregulatory challenge for the animal leading to electrolyte loss (Fleming and Nicolson 2003). Nectarivorous and frugivorous birds have kidneys with a reduced medullary area (Casotti et al. 1998, Barceló et al. 2012), and they can produce highly dilute cloacal fluids to conserve electrolytes (Fleming and Nicolson 2003). Renal morphology and physiology of *E. violacea* and *T. palmarum* have not being examined, but our findings suggest that their kidneys should be well adapted to process dilute food. Osmoregulatory constraints to processing solutions have been demonstrated in avian nectarivores and frugivores fed

Table 1. Initial (mb_i), final (mb_f) and relative change in body mass (Δm_b) of *Euphonia violacea* (n = 6) and *Thraupis palmarum* (n = 5) at different levels of sugar concentrations (SC, in w/w %). mb values in grams. Δm_b calculated as $[(mb_f - mb_i)/mb_i] \times 100$. Values are mean ± 1 SEM and number in parenthesis denote range of observations.

	Euphonia violacea			*Thraupis palmarum*		
SC	mb_i	mb_f	Δm_b	mb_i	mb_f	Δm_b
5	14.4 ± 0.2 (13.8–14.8)	14.8 ± 0.2 (14.1–15.3)	3.5 ± 0.6 (2.1–6.1)	41.2 ± 1.7 (36.8–47.6)	42.6 ± 1.6 (38.1–48.6)	3.3 ± 0.3 (2.1–4.1)
10	14.6 ± 0.2 (14.1–15.7)	15.2 ± 0.3 (14.6–16.2)	3.7 ± 0.4 (2.7–4.9)	41.8 ± 2.1 (37.1–49.5)	43.3 ± 2.1 (39.9–50.1)	3.6 ± 0.3 (2.6–4.3)
20	14.9 ± 0.3 (14.2–15.8)	15.5 ± 0.3 (14.6–16.4)	3.7 ± 0.5 (1.9–4.9)	40.2 ± 1.8 (36.1–47.3)	41.4 ± 1.7 (37.9–48.8)	3.2 ± 0.5 (1.4–4.9)

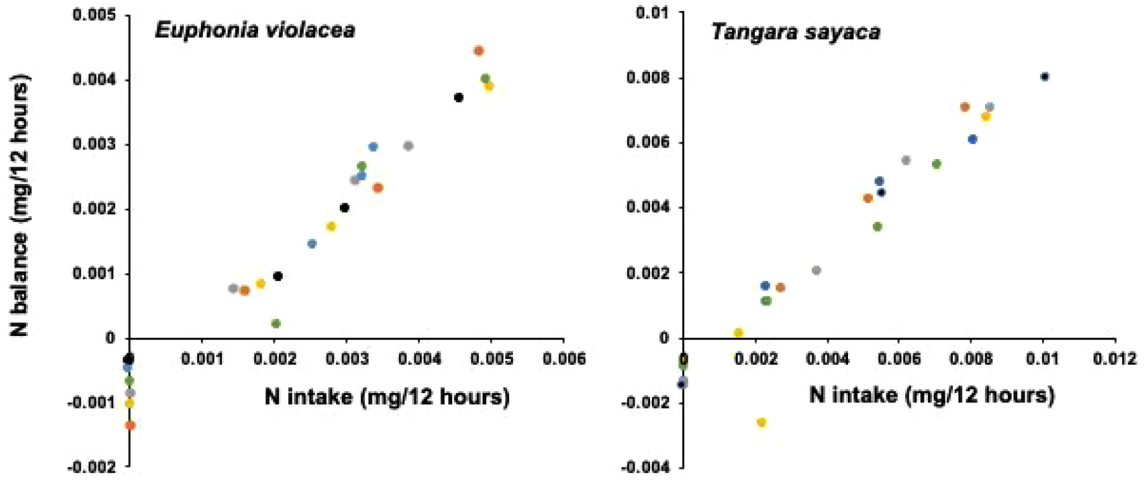

Figure 3. Nitrogen intake and nitrogen balance (N ingested – N excreted) in individuals of *Euphonia violacea* (n = 6) and *Thraupis sayaca* (n = 6) fed sugar solutions with different amounts of casein hydrolyzate as N source. We used the x intercept of the linear regressions (not shown) for each individual to estimate apparent maintenance N requirements. Different dot colors represent different experimental individuals.

solutions with a lower sugar concentration than in our study (2.5–3.5%; Purchase et al. 2010, Mancina and Herrera 2016). Therefore, it remains to be tested if the use of highly diluted *Rhipsalis* fruits (<5% sugar; Guaraldo et al. 2013) by our focal species is limited by osmoregulation. Finally, ingested water must be warmed to body temperature, and consumption of large volumes of dilute nectar increases metabolic rate due to food warming (Lotz and Nicolson 2002, Lotz et al. 2003). The cost of warming dilute sugar solutions by *E. violacea* and *T. palmarum* is unknown, but it can be significant for their energy budget given that it amounts to a 15% increase of metabolic rate in birds fed dilute food (~7% sugar; Lotz and Nicolson 2002).

Rhipsalis fruits are mainly consumed by several species of euphonias while other birds act as secondary consumers, including *T. palmarum* and *C. flaveola* (Guaraldo et al. 2013). In contrast to our focal species, *C. flaveola* is unable to compensate energy intake and loses body mass when feeding on solutions with low sugar content (9%-sugar solutions; Mata and Bosque 2004). Therefore, constraints in compensatory feeding explain (at least partly) the limited use of *Rhipsalis* fruits by *C. flaveola* but do not explain the contrasting feeding patterns of *E. violacea* and *T. palmarum*.

Nitrogen requirements

Euphonia violacea and *T. sayaca* did not differ in mass-corrected nitrogen requirements so we discarded lower MNR as a mechanism favoring the exploitation of the low-protein fruits of *Rhipsalis* by *E. violacea*. It has been shown that frugivorous and nectarivorous birds have lower MNR than omnivorous birds (Tsahar et al. 2006). Fruits were present in 66.4% of 107 fecal and stomach samples of *T. sayaca* retrieved from the literature (Arthur Gomes et al. unpubl.), revealing that both in terms of the overall contribution of fruits to the diet and MNR, it is closer to the end occupied by euphonias in the frugivory gradient.

Conclusions and future directions

Our experimental approach to separate specialist frugivorous birds from occasional consumers of *Rhipsalis* fruits failed to confirm that the ability to compensate for differences in food energy density or lower nitrogen requirement are the underlying mechanism permitting euphonias to cope with such low-energy and low-nitrogen fruits. The relatively small number of tested individuals, though most likely draw from different populations, might have precluded us from detecting interspecific differences. So, we cannot generalize from the current two-species study that such digestive mechanisms do not distinguish specialist versus generalist avian frugivores (Garland and Adolph 1994; but see Goymann and Schwabl 2021 for a plea on the value of 2-species comparisons). Anyway, our results call the attention to other possible mechanisms that should be considered in future studies. The fast rates of fruit passage typical of specialized avian frugivores as euphonias may be key to allow them to profit from the small amounts of proteins offered by *Rhipsalis* fruits. Euphonias were observed to eat more fruits per visit to *Rhipsalis* plants than other birds (7.3 ± 7.1 vs 5.5 ± 3.2 fruit visit, respectively; Guaraldo et al. 2013) and pass their seeds twice as fast through the guts than *Tangara seledon*, a 18.7-g tanager occasionally observed eating *Rhipsalis* fruits (14.2 ± 8.3 min vs 27.2 ± 7.6 min, respectively; Guaraldo et al. 2013). As noted by Izhaki (1992), low-protein diets may be tolerable if birds can consume and digest a large volume of food. The longer time taken by unspecialized frugivorous birds to process such nutritiously poor fruits may make them an unprofitable meal (Levey and Karasov 1992). Regarding fruits, our study could not perfectly simulate the complexity of fruit chemical profile that influences the choice of birds in the wild as, for instance, secondary compounds. *Rhipsalis* fruits may persist up to a year on the plant (Guaraldo et al. 2013), which likely entails the presence of secondary compounds (e.g. phenols) to defend

against the attack of insects and pathogens during this long-time span (the removal rate model of the defence-trade-off hypothesis sensu Schaefer et al. 2003). However, certain secondary compounds are known to interfere with protein assimilation, which may further lessen the nutritional value of *Rhipsalis* fruits (Izhaki and Safriel 1989, Izhaki 1992). As the detoxifying ability of birds is generally correlated with their degree of frugivory (Jordano 2000), one may hypothesize that secondary compounds may represent the mechanism favoring the specialization of euphonias on *Rhipsalis* fruits in detriment of less frugivorous birds. Likewise, the detailed nutritional profile of the fruits of other epiphytes and also mistletoes for which euphonias are the main consumers should be accessed to unveil the mechanisms underlying such hitherto intriguing specialized relationships.

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Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.bvq83bk96> (Crestani et al. 2021).

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4. CONCLUSION

The physiological and morphological traits of birds can have profound ecological and evolutionary consequences for their mutualism interactions. This study contributed to the understanding of the frugivory processes by showing how morphological digestive features is related to bird frugivory degree and the nutritional and chemical profile of the fruits they eat. We provided an integrative picture of mutualistic interactions of frugivores and plants by combining an experimental approach focus on physiology and trait matching between bird and fruit.

We used physiological traits to investigate the specialized relationships between *Euphonia* and low-quality fruits. After revealing that the intake response of varying amounts of food energy of *E. violacea* did not differ from a less-specialized bird (*Thraupis sayaca*; Thraupidae) does not explain the euphonia specialization on *Rhipsalis* fruits, we addressed other possible mechanisms that should be considered in future studies that investigated the specialization in frugivory interactions. For example, birds that consume fruits that are poor in nutrient, as *Rhipsalis*, may increase the consumption rate (GUARALDO, 2013). Another point to be considered is the fast rates of fruit passage through the gut presented by frugivores that consume sugary and aqueous fruits that are rapidly absorbed by passive transport (MARTINEZ; KARASOV, 1990; MARTÍNEZ DEL RIO; RESTREPO, 1993).

Regarding the relationship between digestive traits and the consumption of fruits with different nutrient and chemical compounds, our results show that the frugivory degree is related to gizzard area and liver mass and the consumption of sugar, lipids, and phenols has a relationship with intestine length and liver mass. In the same way, temperate fruit species should be incorporated into Interaction frequency and Fruit dependence model to compare the similarities and differences with tropical fruit results.

There are a huge spectra of trait-matching between birds and fruits that could be explored. For example, exploring the enzymatic activities for absorption in the avian intestine (PRICE et al., 2015), we could better understand the factors that can limit the consumption of some nutrients. This also contributes to explaining the fruit diet differences among insectivorous, omnivorous and frugivorous birds. The capacity of these groups to detoxify secondary compounds present in fruits also contributes to bird-fruit interaction. Future investigations adopting these approaches will improve the knowledge of ecological interactions and their outcomes for animal and plant communities.

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