

1 **Diet composition of reintroduced Red-and-Green Macaws (*Ara chloropterus*) reflects**
2 **gradual adaption to life in the wild**

3 Noelia L. Volpe^{1*}, Bettina Thalinger^{2,6}, Elisabet Vilacoba³, Thomas W.A. Braukmann^{2,4}, Adrián
4 S. Di Giacomo¹, Igor Berkunsky⁵, Darío A. Lijtmaer³, Dirk Steinke^{2,6} and Cecilia Kopuchian¹

5

6 ¹Laboratorio de Biología de la Conservación, Centro de Ecología Aplicada del Litoral
7 (CECOAL), CONICET, Corrientes, Argentina.

8 ²Centre for Biodiversity Genomics, University of Guelph, Guelph, Ontario, Canada.

9 ³División Ornitología, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” MACN
10 CONICET., Buenos Aires, Argentina.

11 ⁴Department of Pathology, Stanford University, Stanford, California, USA.

12 ⁵Instituto Multidisciplinario sobre Ecosistemas y Desarrollo Sustentable. Universidad Nacional
13 del Centro, Tandil, Buenos Aires, Argentina.

14 ⁶Department of Integrative Biology, College of Biological Science, University of Guelph,
15 Guelph, Ontario, Canada.

16 * Corresponding author: noelia.l.volpe@gmail.com

17 **ABSTRACT**

18 Over the last two centuries, the Red-and-green Macaw (*Ara chloropterus*) has become locally
19 extinct in Argentina. In an attempt to restore its key ecosystem functions as both disperser and
20 regulator of large-seeded plants, a reintroduction project was initiated at the Iberá National Park
21 in northeastern Argentina. The ability of released individuals to find food is crucial, in particular
22 when working with captive-bred animals, as long-term establishment of a self-sustaining
23 population depends on their short-term ability to exploit wild food sources. Monitoring of

24 feeding habits is usually conducted through behavioral observation, but in recent years DNA
25 metabarcoding has emerged as an alternative for obtaining highly resolved data on diet
26 composition. In this study we use a combination of both techniques to characterize the breadth
27 and composition of the reintroduced macaws' diet. In addition, we evaluate the efficiency of both
28 direct field observations and metabarcoding of feces as techniques to assess diet composition.
29 Individuals fed on a variety of plant species ($n = 49$) belonging to a broad phylogenetic spectrum
30 (28 families). Dietary richness estimated by direct observation and metabarcoding was similar,
31 though smaller than the combination of the two datasets as both techniques detected at least 15
32 species not recorded by the other method. While the total number of detected species was higher
33 for observational data, the rate of species-detection per sampling day was higher for
34 metabarcoding. These results suggest that a combination of both methods is required in order to
35 obtain the most accurate account of the total diversity of the diet of a species. The ability of the
36 reintroduced macaws to successfully exploit local food resources throughout the year indicates a
37 good level of adjustment to the release site, an important step towards the creation of a stable,
38 self-sustaining population of Red-and-green Macaws in Northern Argentina.

39 **Keywords:** *Ara chloropterus*, Conservation, Diet, Frugivory, Metabarcoding, Red-and-green
40 Macaw, Reintroduction, Trophic ecology

41

42 **RESUMEN**

43 En el transcurso de los últimos dos siglos, el Guacamayo Rojo (*Ara chloropterus*) se ha
44 extinguido en la Argentina. Buscando recuperar su rol ecológico tanto de dispensor como de
45 depredador de semillas de gran tamaño, se comenzó un proyecto de reintroducción de la especie
46 en el Parque Nacional Iberá, en la región noreste del país. La capacidad para encontrar alimento

47 por parte de los individuos liberados es crucial, particularmente cuando se trabaja con animales
48 provenientes de condiciones de cautiverio, ya que el establecimiento de una población
49 autosuficiente a largo plazo dependerá de la habilidad de éstos para explotar fuentes de alimento
50 silvestre a corto plazo. El monitoreo de hábitos alimenticios se realiza usualmente a través de
51 observaciones comportamentales. Sin embargo, en los últimos años la técnica del meta-código de
52 barras de ADN ha surgido como una alternativa para la obtención de datos de composición
53 dietaria con alto nivel de resolución. En este estudio, utilizamos una combinación de ambas
54 técnicas para caracterizar la amplitud y composición de la dieta de los guacamayos
55 reintroducidos. A su vez, evaluamos la eficiencia de la observación directa y el código de barras
56 genético de heces como técnicas para evaluar la composición de la dieta. Los individuos se
57 alimentaron de una amplia variedad de especies ($n = 49$), abarcando un amplio espectro
58 filogenético (28 familias). La riqueza dietaria estimada por observación directa y por meta-
59 código de barras genético fue similar, aunque menor a la resultante de la combinación de todos
60 los datos ya que ambas técnicas detectaron al menos 15 especies no registradas por el otro
61 método. Mientras que el número total de especies detectadas fue mayor para los métodos
62 observacionales, la tasa de detección de especies por día de muestreo fue mayor para el análisis
63 genético. Estos resultados sugieren que una combinación de ambos métodos es necesaria para
64 obtener la descripción más precisa posible de la diversidad dietaria total de una especie. La
65 capacidad de los guacamayos reintroducidos para explotar recursos alimenticios locales a lo
66 largo del año estaría indicando un buen nivel de adaptación al sitio de liberación, un paso muy
67 importante hacia el establecimiento de una población de Guacamayo Rojo estable y
68 autosuficiente en el norte de Argentina.

69 **Palabras clave:** *Ara chloropterus*, Conservación, Dieta, Frugivoría, Meta-código de barras,
70 Guacamayo Rojo, Reintroducción, Ecología trófica

71

72 **LAY SUMMARY**

- 73 • The Red-and-green Macaw reintroduction project aims to restore this species to
74 Argentina, where it is locally extinct. To assess if reintroduced macaws are
75 successfully adapting to life in the wild, we studied their foraging habits at the Iberá
76 National Park. Their food consumption was observed visually, and their feces were
77 analyzed with molecular methods for traces of the consumed plants.
 - 78 • Macaws fed from a large diversity of food items, exhibiting a flexible diet which
79 varied with fruit availability in different months. A combination of both methods was
80 required to obtain the most accurate account of the total diversity of the diet of a
81 species.
 - 82 • The reintroduced macaws were able to successfully locate and exploit food resources
83 throughout the year, indicating a good level of adjustment to the release site.
- 84

85 **INTRODUCTION**

86 Over the last two centuries, Northern Argentina has experienced substantial defaunation mainly
87 affecting large birds and mammals (Zamboni et al. 2017). One of the species that disappeared
88 was the Red-and-green Macaw (*Ara chloropterus*), one of the largest species of the order
89 Psittaciformes, last seen in the region almost 100 years ago and currently considered locally
90 extinct in Argentina (Collar et al. 2020). Psittacids have traditionally been considered plant
91 antagonists, acting as pre-dispersal predators by destroying seeds or removing them from the

92 parent plants before they become viable (Trivedi et al. 2004, de Faria 2007, Ragusa-Netto 2011).
93 Yet, an increasing body of literature highlights their importance as seed dispersers, being able to
94 transport fruits across longer distances than smaller frugivores (Tella et al. 2015, Blanco et al.
95 2018). Thus, with the loss of the Red-and-green Macaw from the north of Argentina, its key
96 roles as disperser and regulator of large-seeded plants in forests and savannas were removed
97 from the region. In an attempt to restore these ecosystem functions, in 2014 the NGO Rewilding
98 Argentina started the Red-and-green Macaw reintroduction project in the forests of the Iberá
99 Wetlands, located in the north of the province of Corrientes (Zamboni et al. 2017).

100 One key consideration for the establishment of a new population in a reintroduction
101 project is the ability of the animals to find food. Malnourished individuals will not only have a
102 reduced chance of survival but also low reproductive success (Williams et al. 2013, Yu et al.
103 2015, Renton et al. 2015). Long-term establishment of a self-sustaining population depends on
104 the short-term ability of released individuals to exploit wild food sources. For species that rely on
105 seasonal resources, such as frugivores, this will involve not only locating food sources in space
106 but also adapting to temporal changes in availability. Such dietary flexibility is a common
107 characteristic of psittacid feeding ecology and is demonstrated by their broad diets (Renton et al.
108 2015). For the Red-and-green Macaw, this can mean relying on as many as 54 different plant
109 species (Lee et al. 2014).

110 The Red-and-green Macaw reintroduction project relies entirely on captive-bred
111 individuals which are naïve to foraging in the wild and will thus face particular challenges
112 having to both find novel food sources and track their changes in availability along the year
113 (Peignot et al. 2008). In social or gregarious species such as parrots, uptake of new food items by
114 released individuals may be more successful if there are conspecifics already present in the area

115 (Jones and Duffy 1993, Ewen et al. 2012). Unfortunately, the Iberá National Park currently lacks
116 any native macaws or other large parrots from which reintroduced birds could learn to recognize
117 food items. To compensate for this, we developed a pre-release training program to encourage
118 captive-bred macaws to use wild fruits and seeds. Over the course of this program we were able
119 to identify over 30 local plant species the macaws were willing to consume (N.L.V., personal
120 observation). Although this indicates a potentially high diversity in the diet, it is likely that not
121 all food items consumed within the captive environment will be eaten in the wild (Plair et al.
122 2013, Amaya-Villarreal et al. 2015). The realized dietary breadth for the free roaming population
123 is determined by the ability of individuals to actually locate fruit bearing plants when they are
124 available. Hence, only monitoring of released populations can help to evaluate if released
125 macaws are succeeding at gathering food resources and to understand what key plant species are
126 needed for the persistence of the macaw population in the area.

127 Monitoring of feeding habits is usually conducted through behavioral observation.
128 Despite its common use, this technique comes with challenges and limitations because it is not
129 always feasible to adequately track individuals, in particular when working with animals as
130 mobile as macaws (Valentini et al. 2009a). The collected information is potentially incomplete,
131 as the observer might not have witnessed enough feeding events or not have been able to tell if
132 the foraged plants were actually ingested. DNA-based analysis of fecal matter can provide much
133 more detailed information on consumed food items while also removing the need to track
134 individuals for long periods of time (Valentini et al. 2009b, Oehm et al. 2011). In this context,
135 metabarcoding has emerged as a powerful tool for obtaining highly resolved data on diet
136 composition, which will be a reliable indicator of how well macaws are adapting to life in the
137 wild. Several studies have reported the use of metabarcoding to analyze the diet of different

138 animals, such as mammals (Lopes et al. 2020) and birds (Rytönen et al. 2019, McClenaghan et
139 al. 2019). However, despite the use of DNA barcoding to analyze seed dispersion (Lavabre et al.
140 2016, Galimberti et al. 2016), none of these studies applied metabarcoding to study the diet of
141 frugivorous bird species. We consider this approach a valuable method to shed light on this field.

142 The main objective of this study was to describe the diet of reintroduced macaws at the
143 Iberá National Park. In particular, we: 1) characterize dietary breadth and composition, and 2)
144 evaluate the efficiency of both direct field observations and metabarcoding as useful techniques
145 to assess diet composition.

146 **METHODS**

147 **Study Site**

148 The study was conducted at the Iberá National Park and the Iberá Provincial Reserve and
149 Provincial Park (27.8704°S, 56.8801°W, Figure 1) in the province of Corrientes, Argentina, a
150 wetland area consisting of flooded grasslands and savannas surrounding subtropical forest
151 patches of variable sizes (0.02 – 11 ha). The climate is subtropical humid, with mild winters and
152 no pronounced dry season. The average monthly temperature ranges from 15°C in June/July to
153 28°C in January/February with an average annual precipitation of 1800 mm (Neiff and Poi de
154 Neiff 2006).

155 **Project Description**

156 Macaws were donated to Rewilding Argentina by zoos, rescue centers and pet owners. They
157 spent a quarantine period at the Aguará Conservation Centre (Corrientes, Argentina), where they
158 were tested for a variety of diseases: mycoplasma, adenovirus, psittacine circovirus, Pacheco's
159 disease, paramyxovirus, influenza and chlamydia. In addition to this, individuals which showed
160 signs of significant physical or behavioral problems (e.g., inability to fly, human imprinting)

161 were removed from the candidate pool for release. Macaws deemed adequate were subsequently
162 moved to a pre-release aviary at the release site in Portal Camby Retá (Iberá National Park),
163 from where they were released after 11-16 months. For this study, we focused on two releases
164 which took place in June 2017 (7 macaws; 4 females, 3 males) and in February 2018 (2 female
165 macaws). However, visual feeding observations during pilot releases and DNA-based data
166 retrieved from a sample collected in 2019 were included in the respective datasets. Thus, the
167 time frame for observations and fecal sample collection was not identical. Food supplements
168 consisting of a mixture of commercial fruits, vegetables and seeds provided on tree-platforms
169 were available to the released macaws throughout the entire study period, applying a scheme of
170 decreasing food supplementation over time: Four daily food supplements were offered until
171 September 2017, three until December 2017, two until March 2018 and one from then onwards.
172 This reduction in food supplementation was needed in order to motivate the macaws to expand
173 their territory and forage from wild plants.

174 **Data Collection**

175 **Foraging observations.**

176 Between June 5 2017 and May 31 2018, we monitored the feeding activity of nine macaws, fitted
177 with VHF radio-collars (Holohil AI-2C). Each macaw was followed using Yagi antennas for at
178 least 4 consecutive hours each week for a total of 149 days. Every time we encountered macaws
179 feeding on wild plants, we recorded date, time, GPS location, part eaten and species being
180 consumed. We also included four additional foraging observations which took place during pilot
181 releases in September 2015 and March 2017.

182 **Fecal sampling and DNA barcode reference library.**

183 Feces were collected monthly between July 2017 and May 2018, with two additional samples
184 collected in June 2019 ($n = 10$ macaws). Collections were made opportunistically while tracking
185 the macaws with the aim to obtain at least one sample every two weeks, which led to a total of
186 96 samples collected on 61 different days (2 – 19 samples/month). Feces were collected between
187 1 and 40 minutes after defecation, except for two samples which were collected at a roost site
188 early in the morning. Samples were stored at room temperature in 15 ml falcon tubes filled with
189 ethanol (96 %) and after 1-3 months placed in a freezer at -20°C until further processing. Each
190 tube contained from one ($n = 86$) to multiple (2 to 4; $n = 10$) fecal samples.

191 Based on observations of feeding preferences during the pre-release stage we built a
192 DNA barcode reference library. We sampled 33 plant species that were expected to be used by
193 macaws as food sources. Leaf plant tissue was taken from 3 individuals for all but three species
194 for which we could only collect 1 or 2 samples. Samples were stored in silica gel until further
195 processing.

196 **Sample processing**

197 **DNA barcoding and reference library compilation.**

198 The DNA extraction from plant tissues and amplification of barcode markers were performed at
199 MACN following standard procedures; sequencing was done at the Centre for Biodiversity
200 Genomics (CBG) at the University of Guelph in Canada. For a detailed description of these
201 protocols see Supplementary Material 1A. In total, 65 of the 96 plant tissues produced ITS2
202 sequences corresponding to 24 different species; after filtering for contaminants and correcting
203 for base-call errors, these were uploaded to BOLD (DS-IBERAFLO) and Genbank (accession
204 numbers: MW845313-MW845377). Nine of the 33 sampled species expected to be eaten by the

205 macaws failed to amplify. Sequences for these species, together with those of 179 other plant
206 species occurring in the area (Arbo and Tressens 2002) were extracted from the ITS2 database
207 hosted by the University of Würzburg (accessed 16th June 2020; Ankenbrand et al., 2015) in
208 order to compile a custom reference database for maximum plausibility of the taxonomic
209 assignment. In case sequences of specific species were not available, entries of the respective
210 genus, family, tribe or order were used.

211 **Metabarcoding of fecal samples**

212 Metabarcoding of the fecal samples was carried out at the CBG and a detailed description of the
213 entire process is contained in Supplementary Material 1B. Feces were processed in a laboratory
214 dedicated to the handling of low-quality DNA samples with separate rooms for DNA extraction,
215 PCR preparation and post-PCR processing. All DNA extracts were subjected to a metabarcoding
216 approach using two consecutive PCRs and fusion primers (Elbrecht and Steinke 2019). PCR
217 conditions were optimized for maximum yield of target length fragments, while minimizing the
218 occurrence of non-target bands. The first round of PCR employed the primers ITS-u3 5'-
219 CAWCGATGAAGAACGYAGC-3' and ITS-u4 5'-RGTTTCTTTTCCTCCGCTTA-3' (Cheng
220 et al. 2016) and in the second PCR Illumina sequencing adapters were added using individually
221 tagged fusion (Elbrecht and Steinke, 2019; Supplementary Material Table S1). Sequencing was
222 carried out by the Advanced Analysis Centre at the University of Guelph using a 600 cycle
223 Illumina MiSeq Reagent Kit v3 and 5% PhiX spike in. Sequencing results were uploaded to the
224 Sequence Read Archive (SRA, Genbank, accession: PRJNA695029).

225 **Bioinformatic analyses**

226 Resulting sequence data were processed using the JAMP pipeline v0.67
227 (github.com/VascoElbrecht/JAMP). Sequences were demultiplexed, paired-end reads merged

228 using Usearch v11.0.667 with fastq_pctid=75 (Edgar 2010), reads outside a 100 bp to 430 bp
229 range were discarded and primer sequences trimmed by using Cutadapt v1.18 with default
230 settings (Martin 2011). Sequences with poor quality were removed using an expected error value
231 of 1.5 (Edgar and Flyvbjerg 2015) as implemented in Usearch. All sequences with less than five
232 reads were removed during the denoising process. The obtained haplotypes were mapped against
233 the custom sequence database; those without matches were subsequently blasted. Detailed
234 information on the mapping process and determination of the levels of taxonomic resolution can
235 be in Supplementary Material 1C.

236 The detected taxa were classified into five categories: Resource = wild local plants
237 known to be a food item or considered likely to be so based on its characteristics (fruit-producing
238 tree or vine); Provided = commercial fruits or vegetables included in the daily food supplements;
239 Provided/Resource = level of resolution did not allow to exclude either option; Contamination =
240 unlikely to have been eaten by the macaws (included algae and herbaceous or aquatic plants) and
241 Ambiguous = could be either of the previous categories. Only taxa classified as Resource were
242 included in the dietary analysis.

243 **Data Analysis**

244 Diet breadth was estimated as the number of wild species consumed by the macaws, including a)
245 species observed being eaten by the macaws, b) resource taxa detected in the feces resolved to
246 the species level and c) resource taxa resolved to the genus level but not detected at the species
247 level (e.g., *Tabebuia* sp. but not *Psidium* sp., as the latter is already represented by *Psidium*
248 *guajava*). Given that pine trees in forestry plantations are the only conifers present in the study
249 area, all conifer reads were treated as the same unit (*Pinus* sp.) regardless of their level of
250 resolution (order, family, genus or species). We ranked the relative importance of each consumed

251 species by estimating their proportion of occurrence in the diet, *i.e.* the number of days during
252 which a given food item was detected over the total number of sampling days (feces-collection
253 days and observation days; $n = 153$). To test whether macaws consumed each species according
254 to their availability in the area, we used the Spearman rank correlation to evaluate the
255 relationship between the number of feeding events and the proportion of fruiting trees of 14 of
256 the consumed species for which phenological information was available (see Supplementary
257 Material Figure S2). In order to compare results from both sampling methodologies, we
258 estimated the detection rate for each technique and used Pearson's correlations to assess if
259 foraging observations and fecal sampling data collected on the same dates led to similar
260 conclusions regarding changes in resource use patterns over time. All data were analyzed in R
261 3.6.2 (R Core Team 2020) using *tidyverse* and associated packages (Wickham et al. 2019).

262 Results are expressed as mean \pm standard error.

263 **RESULTS**

264 **Foraging Observations**

265 During the study period we recorded feeding bouts on 140 out of the 149 observation days,
266 adding up to 336 hours of records on feeding behavior ($n = 551$ feeding events). Macaws fed on
267 536 different individual trees, as well as five vines and one epiphyte. Macaws consumed mainly
268 fruits and seeds (98.5% of the events, 29 species), although they were occasionally observed
269 chewing on flowers (0.9% of the events, four species used) and leaves (0.6% of the events, one
270 species used).

271 **Fecal Analysis**

272 Illumina sequencing produced 23.2 million paired-end reads. Of the 96 samples, 13 did not
273 contain reads which passed the quality filtering process. Of the 8.1 million reads that passed all

274 quality filtering and denoising steps, 1.8 million (22%) could be assigned to sequences in the
275 custom reference database. The percentage of assignable reads varied between 0 and 99%
276 (average 24%) for the individual samples. After blasting previously unmapped sequences, 3.1
277 million (38%) did not result in a clear taxonomic assignment, 2.4 million (30%) were assigned to
278 fungi and 0.07 million (0.88%) matched to Viridiplantae. The majority of plant reads (custom
279 database plus Genbank) were assigned to species (57%); 17%, 21% and 5%, to genus, family,
280 and order respectively (Figure 2; see Supplementary Material Table S3 for information on
281 individual samples). Of all negative controls only 4 (2 PCR controls, the fume hood
282 (evaporation) control and the extraction control) contained reads which passed quality filtering.
283 However, only 10 reads (of one PCR control) could be mapped (to *Lens culinaris*). As the read
284 numbers in fecal samples assigned to *Lens culinaris* were always more than twice as high, we
285 refrained from correcting the read numbers and occurrence data.

286 On average, 4.63 ± 0.29 species were detected per sample ($n = 81$, range = 1 – 10 species,
287 Figure 3A). When taking into account just the resource taxa, the average dropped to 2.66 ± 0.16
288 species per sample ($n = 75$, range = 1 – 6 species, Figure 3B). The majority of reads
289 corresponded to confirmed food resources, but provided food was detected in 72% of the
290 samples (Figure 4A). Similarly, provided food was present in the feces throughout all sampling
291 months although its relative presence decreased towards the end of the study period, coinciding
292 with the reduction in food supplementation (Figure 4B).

293 **Diet Composition**

294 Macaws exhibited a diverse diet feeding on 49 plant species from 28 different families (Table 1).
295 Most of the feeding activity was concentrated on a small number of species, which were detected
296 on more than 15 sampling dates. Particularly important was *Psidium guajava*, which was

297 detected on 59% of the days. *Syagrus romanzoffiana* and *Inga edulis* were detected over 26%
298 and 20% of the days, respectively, while *Ficus luschnathiana*, *Enterolobium contortisiliquum*,
299 *Croton urucurana*, *Sapium haematospermum*, *Pinus* sp. and *Melia azedarach* appeared in the
300 diet on 10-14% of the days. The 40 remaining species were present in the diet on less than 10%
301 of the sampling days. The monthly recurrence of use of the different food items varied between
302 species. While most of them were detected as being consumed only during one month, others
303 were part of the diet for most of the year (e.g., *P. guajava*, *S. romanzoffia* and, *I. edulis*; Table 1).

304 The relationship between resource use and availability varied between species. Plants
305 with relatively short fruiting periods such as *Eugenia myrcianthes* and members of the family
306 Euphorbiaceae, or those that were eaten mainly at their ripe stage, such as *Ocotea diospyrifolia*,
307 *F. luschnathiana*, and *I. edulis* were consumed as they became available, with the number of
308 feeding events correlated to the monthly availability of each species (Spearman rho = 0.63 – 0.83,
309 $P < 0.05$). On the other hand, the intensity of use of species with longer fruiting periods was less
310 predictable and not associated to their availability (Spearman rho = - 0.02 – 0.56, $P > 0.05$). Some
311 species, like *P. guajava*, were used in high proportions even in months during which they had a
312 low relative abundance, while others were only used for a few months (*Enterolobium*
313 *contortisiliquum*) despite being available for most part of the year (Supplementary material Table
314 S2).

315 **Comparison Between Techniques**

316 Of the 49 plant species identified as being part of the macaw diet, 13 were detected by both
317 techniques, while 17 were detected only by metabarcoding (7 of them resolved to genus) and 19
318 only by direct observation (Table 1, Fig 4). Of these 19 species not detected by metabarcoding, 7
319 could actually not be detected because they were not present in the database used for species

320 identification. Both techniques identified *P. guajava* as the most important species for the
321 macaws, but the relative use of the remaining shared species was more variable. For example,
322 *Croton urucurana* was consumed in only 4% of the sampling days based on the observational
323 data, but on 25% of the days based on the metabarcoding results (Figure 5).

324 While the total number of detected species was higher for observational data, the rate of
325 species-detection per sampling day was higher for metabarcoding. When comparing data
326 collected on the same dates ($n = 39$ days) metabarcoding detected 28 species (0.72 species day-
327 1) while observational data detected 22 species (0.56 species day-1). The difference between
328 techniques was more pronounced when looking at detection rates each month, with an average of
329 1.81 ± 0.27 species detected per feces collection day ($n = 11$; range = 1 – 4 species), compared
330 to only 1.35 ± 0.11 species per observation day ($n = 11$, range = 0.8 – 2 species).

331 Results from both techniques exhibited a similar pattern regarding the rate of increase in
332 dietary richness since the date of release (Pearson correlation, $n = 39$, $r = 0.95$, $P < 0.05$),
333 showing a sharp initial growth during the first months and a flattening of the curve at around the
334 7-month mark, both leading to a similar final diet breadth estimate of 30-32 species (Figure 6).
335 The overall pattern of changes in dietary breadth along the year was also similar between
336 techniques (Pearson correlation, $n = 11$, $r = 0.6$, $P < 0.05$), with peaks in July, September and
337 December, followed by a decrease towards Autumn (Figure 7). The pattern of use of specific
338 food items along the year differed between techniques, with observational data underestimating
339 the use of many of them. At least ten species occurred in the diet of the macaws for much longer
340 than observed (Figure 8). Metabarcoding revealed that the group of macaws began to eat some of
341 the species several months before we first observed them doing so (e.g., *C. urucurana*, *Pinus* sp.)
342 or did it for 1-7 months longer than recorded (e.g., *Eugenia uniflora*, *Ficus luschnathiana*). In the

343 case of *Cecropia pachystachya*, its use was only observed during the pilot release of 2015, but
344 metabarcoding highlighted that the macaws were also eating it during the 2017-2018 releases,
345 though it was never visually detected.

346 **DISCUSSION**

347 **The Diet of Reintroduced Red-and-green Macaws**

348 Reintroduced Red-and-green Macaws showed a good level of adjustment to life in the wild in the
349 Iberá National Park and surrounding areas, being able to exploit a large variety of the food
350 resources available at the site. In the one-year period studied, released macaws fed from a variety
351 of plant species ($n = 49$) belonging to a broad phylogenetic spectrum (28 families). The observed
352 dietary richness lies within the expected range for the species. The most exhaustive diet studies
353 for wild Red-and-green Macaw populations to date (> 100 feeding bouts observed, > 24 months of
354 data), report a dietary breadth ranging from 10 species (Pantanal, Ferreira 2013) to 51-54 species
355 (Amazonian rainforest, Adamek 2012; Lee et al. 2014). The overall diet composition detected in
356 this study was similar to previous findings, with a high prevalence of detections concentrated on
357 species from the families Fabaceae, Arecaceae and Euphorbiaceae. Additionally, 32 of the plant
358 species eaten in the Iberá National Park belonged to the same genera as those eaten by red-and-
359 green macaws in other regions of South America (Desenne 1994, Nycander et al. 1995, Santos
360 2001, Antas et al. 2002, Ragusa-Netto and Fecchio 2006, Haugaasen 2008, Adamek 2011,
361 Scherer-Neto and Terto 2011, Ferreira 2013, Lee et al. 2014).

362 Macaws in this study were able to locate food sources after their release and throughout
363 the whole year, with a monthly dietary richness that ranged from 7 to 20 species. *P. guajava* and
364 *S. romanzoffiana* were the most frequently used plants, occurring in the diet for 12 and 11
365 months, respectively. The fruits and seeds of these species can be eaten by the macaws at both

366 ripe and unripe stages and are produced year-round, making them a reliable food source.
367 Selection for plant species with a relatively constant production of food has also been recorded
368 for other psittacids (Bonadie and Bacon 2000, Robinet et al. 2003), with palm trees in particular
369 being an important food source for macaws living in wetland and savanna areas (Yamashita and
370 Machado de Barros 1997, Brightsmith and Bravo 2006, Nunes and dos Santos 2011). The strong
371 beak of these large psittacids allows them to feed not only on the pulp but also the nuts of palm
372 fruits (Galetti 1997), granting them access to an interior rich in lipids and proteins (Litchfield
373 1970, Tella et al. 2020). The use of *Psidium guajava* by macaws has seldom been reported in the
374 literature (*Ara severa*, 2 events, Lee et al. 2014), although it is commonly used by other smaller
375 psittacids (Paranhos et al. 2009, Silva and Melo 2013). Studies on the nutritional content of seeds
376 of this species indicate it is a good source of proteins, as well as vitamins, and antioxidants
377 (Uchôa-thomaz et al. 2014), while the pulp of ripe fruits has a high moisture content which can
378 become important during the hot summer (Medina and Pagano 2003).

379 The composition of the diet varied along the year, responding at least in part to changes
380 in fruit availability. For example, macaws relied on multiple species from the Myrtaceae family
381 throughout spring (September to mid-December), switching to Euphorbiaceae during summer
382 (December-February). This shifting between species as they become available, also known as
383 diet switching, is the most common response to food resource fluctuation among psittacids
384 (Renton et al. 2015), allowing them to adapt to the spatial and temporal variability of fruits,
385 seeds and flowers. Metabarcoding results showed that some of the species were consumed
386 outside their fruiting stage, which would confirm the ingestion of non-reproductive plant
387 structures such as bark. The manipulation and chewing of bark was observed throughout the
388 study, but was not considered a feeding event as it was unclear whether ingestion had occurred.

389 The extent and function of the consumption of bark in Psittacids is yet to be determined, but it
390 has been hypothesized to be associated with detoxification, chemical or mechanical aid in
391 digestion, or absorption of nutrients (Warburton 2003, de Araujo and Marcondes-Machado 2011).

392 Two of the most frequently detected species, *Pinus elliottii* and *Melia azedarach*, were
393 not native to the area. *Melia azedarach* has frequently been detected in the diet of psittacids
394 living in modified landscapes (including the Red-and-green Macaw; Scherer-Neto and Terto,
395 2011)). Pine cones are a much less common food resource, having only been reported for one
396 other macaw species (Silva 2018). The use of exotic plants is not uncommon in psittacids, and
397 has allowed some species to survive and even thrive in human-dominated landscapes (Matuzak
398 et al. 2008). They might be a useful resource when faced with a scarcity of native plants (Hamm
399 et al. 2020), but relying on exotic species can be problematic as it can lead to conflicts with
400 farmers (Bucher 1992) or lead to sudden population drops when plantations are harvested (Dear
401 et al. 2010). Additionally, the presence of exotic plants is usually associated with human
402 settlements and hence, feeding on them can increase the risk of capture by poachers, the greatest
403 threat for psittacids (Berkunsky et al. 2017).

404 **Comparison Between Techniques**

405 Dietary richness as estimated by direct observation and metabarcoding were similar (32 and 30
406 species respectively) though smaller than the one obtained combining the two datasets (49
407 species). Based on the selected observation metrics and the availability of DNA reference
408 sequences in the study area, both techniques seem to be equally effective at describing changes
409 in dietary richness over time, generating a similar curve of increase since time of release and
410 similar patterns across the year.

411 The main difference between the results was the specific composition of the diet, with
412 both techniques detecting at least 15 species not recorded by the other method. In the case of
413 metabarcoding, several species, such as *S. romanzoffiana* and *I. edulis*, were expected to be
414 present in the feces, but could not be detected albeit visual observations showed they were being
415 consumed frequently and in large quantities by the macaws. Additionally, neither these two, nor
416 five more species observed to be part of the diet produced an ITS2 barcode sequence during the
417 construction of the DS-IBERAFLO database. Only ITS2 sequences for three of these species
418 could be found at the University of Würzburg's database. For the remaining four species, we
419 included sequences of the respective genus and/or family in the reference database, but despite
420 these efforts, none of the expected plants could be detected. Incomplete reference libraries
421 always pose a challenge for taxonomic assignments of metabarcoding data, especially in tropical
422 and subtropical habitats containing a lot of only superficially investigated biodiversity. In this
423 study, primer specificity and plant tissue traits additionally complicate the situation. For example,
424 ITS sequences of palm trees (Arecaceae) were not included in the original metabarcoding primer
425 evaluation (Cheng et al. 2016) and a superficial analysis showed at least one mismatch at the
426 forward and reverse priming site for *A. aculeata* and *S. romanzoffiana*. Furthermore, the failure
427 to produce ITS sequences from palm trees for the custom reference database indicates a tissue-
428 specific problem such as the presence of inhibitory substances, low DNA content, or strong cell
429 membranes warranting additional homogenization steps during lysis. The digestive process or
430 the potentially lower DNA quantities in the consumed palm tree nuts could further decrease the
431 detection probability in fecal samples (King et al. 2015, Thalinger et al. 2017). All in all, the
432 failure to detect some of the consumed species is likely a combination of these different factors.
433 In the future, the availability of better reference databases and the development of optimized

434 sample processing protocols will undoubtedly improve detection probabilities and the level of
435 resolution of the results, facilitating large-scale DNA metabarcoding studies.

436 On the other hand, the failure to detect species by direct observation of feeding events
437 was likely a product of observation bias. The results of an observational study can be influenced
438 by the experience levels of observers and constrained by their ability to follow the individuals
439 across the terrain (Ford et al. 1990, White and Garrott 2012). As a consequence, isolated food
440 sources located in inaccessible locations will never be recorded by traditional methods, and those
441 that are consumed only sporadically are likely to be missed.

442 When taking into account the sampling effort, metabarcoding showed advantages to
443 direct observation: It had a higher detection rate than observational data, as each fecal sample
444 was a summary of multiple feeding events. The detection rate of food items in fecal samples is
445 influenced by the gut transition time (*i.e.* the time between its ingestion and defecation). For
446 frugivorous birds, gut transit time has been estimated to range from a few minutes up to several
447 hours, with longer duration in larger birds (Oehm et al. 2011, Wotton and Kelly 2012). There is
448 currently no clear information on how long plant DNA can be detected in the feces of
449 frugivorous birds, but studies on piscivorous birds show that prey items can be detected for up to
450 4 days after ingestion (Deagle et al. 2010) and that detectability is affected by meal size. When
451 portions are small, detection rate can plummet within 24-32 hours after ingestion (Thalinger et al.
452 2017). Out of 48 Red-and-green Macaw fecal samples containing fruit and vegetable DNA and
453 for which the timeframe of food availability was known, 98% contained DNA of species that had
454 been eaten within the last 24 hours. Based on this pattern we expect each fecal sample of Red-
455 and-green Macaws to predominantly contain remains of food ingested on either the same or the
456 previous day, encompassing multiple feeding events. As a consequence of this high detection

457 rate, fewer field days will be required to obtain information on dietary breadth than when
458 working with observational data. For example, two fecal samples collected on separate days in
459 November yielded the same number of resource species ($n = 8$) as 10 days of conducting
460 foraging observations.

461 Metabarcoding also provided more detailed information on the role of particular species
462 in the diet of the macaws, by highlighting the importance of species that were not considered
463 significant due to an apparent low frequency of use (e.g., *Eugenia uniflora*). It also showed that
464 the macaws were exploiting a number of wild species significantly earlier than estimated by
465 observation (e.g., *Croton urucurana*). This information not only showed that the macaws were
466 expanding their diet quicker than expected, but in some cases it also indicated that they were
467 moving farther distances than detected by tracking. For example, pine trees are only located
468 outside the boundaries of the Iberá National Park. The presence of *Pinus* sp. in fecal samples
469 collected in July indicates that some of the macaws were feeding at forestry plantations outside
470 of the protected area months before we were able to observe them. The downside of the
471 technique is its inability to quantitatively compare between plant species in a fecal sample
472 without additional tests and comparisons between food sources. Thus, estimating the relative
473 importance of each species at any given time from DNA-based metabarcoding data is not
474 advisable (Deagle et al. 2019, McClenaghan et al. 2019). Even if we can determine the
475 prevalence of a species in the diet by looking at the proportion of samples in which it appears,
476 we miss the fine-level detail that can be achieved through an observational study in which one
477 can measure the number of items consumed, the time spent feeding on a given species, etc.

478 **Final Remarks**

479 There is still no consensus on what is the optimal method to study the diet of a species, as
480 shortcomings have been reported for all of them. Observational data can be subject to observer
481 bias or inability to record feeding signs correctly (Shrestha and Wegge 2006, Matthews et al.
482 2020). Macroscopic fecal inspection is limited by the fact that mastication and digestion by the
483 consumer can render dietary elements unidentifiable (Tutin and Fernandez 1993, Hayward 2013)
484 while DNA-based studies are susceptible to inadequate reference databases and amplification
485 biases (Piñol et al. 2015, Mallott et al. 2018, McClenaghan et al. 2019, Scasta et al. 2019). In the
486 present study, we corroborate that single techniques are more limited than a combined approach
487 in which the observation and metabarcoding of feces complement each other. By considering the
488 results of both methods we were able to increase the number of detected food species by over
489 50% and better estimate the timeline of addition of novel plants in the diet after the release of the
490 macaws. We recommend the use of a combination of observational and genetic tools in diet
491 studies, implementing a question-oriented approach to determine the primary method of data
492 collection. When the main focus of a study is to describe dietary composition, effort should focus
493 on fecal sampling as metabarcoding can yield more detailed results with reduced logistical effort
494 (assuming an adequate reference library is available). When the diet study has a behavioral
495 component which requires information other than the identity of the consumed species (e.g., part
496 of the plant ingested, intensity of use, etc.), then an observational approach will be needed, with
497 fecal sampling filling the gap regarding species which are rarely eaten.

498 The combination of methodological approaches allowed us to establish that the Red-and-
499 green Macaws are eating at least 49 different plant species, indicating that they have adapted
500 well to their environment after their release and the gradual decrease in their food

501 supplementation. This is a necessary step towards the establishment of a new population in the
502 site. In turn, this suggests that the macaws are slowly re-gaining their dual ecological role as both
503 regulators and disperser of seeds (Blanco et al. 2018), exhibiting a potential to affect a large
504 diversity of local plants. On one hand, Red-and-green Macaws fed on seeds and flowers from 24
505 species of plants; by destructing reproductive structures macaws would be actively reducing
506 these species reproductive output. On the other hand, macaws were seen transporting fruits for
507 distances of up to 900m (N.L.V., personal observation), evidencing their potential role as long-
508 distance seed dispersers. Although germination experiments would need to be conducted in
509 order to confirm that the transported seeds are actually viable, we can expect that at least a
510 portion of them will contribute to plant recruitment (Tella et al. 2020). In a fragmented landscape
511 like the wetlands, where ground connectivity between forest patches is restricted by flooded
512 terrain, seed dispersal by terrestrial vertebrates is likely limited (Nield et al. 2020). In this
513 scenario, the presence of large bodied frugivorous birds such as the macaws can become vital to
514 maintain gene flow between forest fragments, in particular of plant species with large seeds
515 which cannot be transported by smaller birds (Baños-Villalba et al. 2017).

516 The ability of the reintroduced macaws to successfully locate and exploit food resources
517 throughout the year, despite their captive-bred origin, can be considered as a good indicator of
518 their acclimatization to the release site. This is an important step towards the creation of a stable,
519 self-sustaining population of Red-and-green Macaws in the North of Argentina which can, in
520 turn, serve as a source of individuals for the colonization of other areas from which the species
521 has disappeared.

522

523 **LITERATURE CITED**

- 524 Adamek, K. A. (2011). Temporal Variation in Space and Resource Use of Macaws in the
525 Southeastern Peruvian Amazon.
- 526 Amaya-Villarreal, Á. M., A. Estrada, and N. Vargas-Ramírez (2015). Use of Wild Foods During
527 the Rainy Season by a Reintroduced Population of Scarlet Macaws (*Ara macao*
528 *cyanoptera*) in Palenque, Mexico. *Tropical Conservation Science* 8:455–478.
- 529 Ankenbrand, M. J., A. Keller, M. Wolf, J. Schultz, and F. Förster (2015). ITS2 Database V:
530 Twice as Much. *Molecular Biology and Evolution* 32:3030–3032.
- 531 Antas, P. T. Z., R. De Souza Yabe, L. A. Carrara, and E. R. Vasques (2002). *Ecologia e Biologia*
532 *Básica das Espécies de Araras da RPPN SESC Pantanal, Brasil*. SESC, Departamento
533 Nacional 1.
- 534 de Araujo, C. B., and L. O. Marcondes-Machado (2011). Diet and feeding behavior of the
535 yellow-faced parrot (*Alipiopsitta xanthops*) in Brasilia, Brazil. *Ornitología Neotropical*
536 22:79–88.
- 537 Arbo, M. M., and S. G. Tressens (2002). *Flora del Iberá*. EUDENE, Universidad Nacional del
538 Nordeste, Corrientes, Argentina.
- 539 Baños-Villalba, A., G. Blanco, J. A. Díaz-Luque, F. V. Dénes, F. Hiraldo, and J. L. Tella (2017).
540 Seed dispersal by macaws shapes the landscape of an Amazonian ecosystem. *Scientific*
541 *Reports* 7:7373.
- 542 Berkunsky, I., P. Quillfeldt, D. J. Brightsmith, M. C. Abbud, J. M. R. E. Aguilar, U. Alemán-
543 Zelaya, R. M. Aramburú, A. Arce Arias, R. Balas McNab, T. J. S. Balsby, J. M. Barredo
544 Barberena, et al. (2017). Current threats faced by Neotropical parrot populations.
545 *Biological Conservation* 214:278–287.
- 546 Blanco, G., F. Hiraldo, and J. L. Tella (2018). Ecological functions of parrots: an integrative
547 perspective from plant life cycle to ecosystem functioning. *Emu - Austral Ornithology*
548 118:36–49.
- 549 Bonadie, W. A., and P. R. Bacon (2000). Year-round utilisation of fragmented palm swamp
550 forest by Red-bellied macaws (*Ara manilata*) and Orange-winged parrots (*Amazona*
551 *amazonica*) in the Nariva Swamp (Trinidad). *Biological Conservation* 95:1–5.
- 552 Brightsmith, D., and A. Bravo (2006). Ecology and Management of Nesting Blue-and-Yellow
553 Macaws (*Ara ararauna*) in Mauritia Palm Swamps. *Biodiversity and Conservation*
554 15:4271–4287.
- 555 Bucher, E. H. (1992). Neotropical parrots as agricultural pests. In *New world parrots in crisis:*
556 *solutions from conservation biology.* (S. R. Beissinger and N. F. R. Snyder, Editors).
557 Smithsonian Institution Press, New York and London, pp. 201–219.

- 558 Cheng, T., C. Xu, L. Lei, C. Li, Y. Zhang, and S. Zhou (2016). Barcoding the kingdom Plantae:
559 new PCR primers for ITS regions of plants with improved universality and specificity.
560 *Molecular Ecology Resources* 16:138–149.
- 561 Collar, N., P. F. D. Boesman, and C. J. Sharpe (2020). Red-and-Green Macaw (*Ara*
562 *chloropterus*), version 1.0. In *Birds of the World* (J. del Hoyo, A. Elliot, J. Sargatal, D. A.
563 Christie and E. de Juana, Editors). Cornell Lab of Ornithology, Ithaca, NY, USA.
- 564 Deagle, B. E., A. Chiaradia, J. McInnes, and S. N. Jarman (2010). Pyrosequencing faecal DNA
565 to determine diet of little penguins: is what goes in what comes out? *Conservation*
566 *Genetics* 11:2039–2048.
- 567 Deagle, B. E., A. C. Thomas, J. C. McInnes, L. J. Clarke, E. J. Vesterinen, E. L. Clare, T. R.
568 Kartzinel, and J. P. Eveson (2019). Counting with DNA in metabarcoding studies: How
569 should we convert sequence reads to dietary data? *Molecular Ecology* 28:391–406.
- 570 Dear, F., C. Vaughan, and A. M. Polanco (2010). Current Status and Conservation of the Scarlet
571 Macaw (*Ara macao*) in the Osa Conservation Area (ACOSA), Costa Rica. *UNED*
572 *Research Journal* 2:7–21.
- 573 Desenne, P. (1994). Estudio preliminar de la dieta de 15 especies de psitácidos en un bosque
574 siempreverde, Cuenca del Río Tawadu, Reserva Forestal El Caura, Edo. Bolívar,
575 Venezuela. In *Biología y conservación de los psitácidos de Venezuela* (G. Morales, I.
576 Novo, D. Bigio, A. Luy and F. Rojas-Suarez, Editors). Gráficas Giavimar, Caracas, pp.
577 25–42.
- 578 Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST.
579 *Bioinformatics* 26:2460–2461.
- 580 Edgar, R. C., and H. Flyvbjerg (2015). Error filtering, pair assembly and error correction for
581 next-generation sequencing reads. *Bioinformatics* 31:3476–3482.
- 582 Elbrecht, V., and D. Steinke (2019). Scaling up DNA metabarcoding for freshwater
583 macrozoobenthos monitoring. *Freshwater Biology* 64:380–387.
- 584 Ewen, J., D. Armstrong, K. Parker, and P. Seddon (Editors) (2012). *Reintroduction Biology:*
585 *Integrating Science and Management*. John Wiley & Sons.
- 586 de Faria, I. P. (2007). Peach-fronted parakeet (*Aratinga aurea*) feeding on arboreal termites in
587 the Brazilian Cerrado. *Revista Brasileira de Ornitologia* 15:457–458.
- 588 Ferreira, L. P. (2013). Dieta e uso do hábitat da arara-vermelha *Ara chloropterus* no pantanal de
589 mato grosso campo grande –.
- 590 Ford, H. A., L. Bridges, and S. Noske (1990). Interobserver Differences in Recording Foraging
591 Behavior of Fuscous Honeyeaters. *Studies in Avian Biology* 13:199–201.

- 592 Galetti, M. (1997). Seasonal abundance and feeding ecology of parrots and parakeets in a
593 lowland Atlantic forest of Brazil. *Ararajuba* 5:115–126.
- 594 Galimberti, A., S. Spinelli, A. Bruno, V. Mezzasalma, F. De Mattia, P. Cortis, and M. Labra
595 (2016). Evaluating the efficacy of restoration plantings through DNA barcoding of
596 frugivorous bird diets. *Conservation Biology: The Journal of the Society for*
597 *Conservation Biology* 30:763–773.
- 598 Hamm, J. O. E., G. M. Bond, L. C. Exley, and E. A. Korein (2020). Reduced diet breadth in the
599 Scarlet Macaw *Ara macao* of the Área de Conservación Osa (ACOSA), Costa Rica:
600 Implications for conservation and ecotourism. *Bird Conservation International*:1–11.
- 601 Haugaasen, T. (2008). Seed predation of *Couratari guianensis* (Lecythidaceae) by macaws in
602 central Amazonia, Brazil. *Ornitología Neotropical* 19:321–328.
- 603 Hayward, C. E. (2013). DNA barcoding expands dietary identification and reveals dietary
604 similarity in Jamaican frugivorous bats.
- 605 Jones, G. C., and K. Duffy (1993). Conservation management of the echo parakeet *Psittacula*
606 *eques echo*. *Dodo J.Wildl.Preserv.Trusts* 29:126–148.
- 607 King, R. A., W. O. C. Symondson, and R. J. Thomas (2015). Molecular analysis of faecal
608 samples from birds to identify potential crop pests and useful biocontrol agents in natural
609 areas. *Bulletin of Entomological Research* 105:261–272.
- 610 Lavabre, J. E., L. J. Gilarranz, M. A. Fortuna, and J. Bascompte (2016). How does the functional
611 diversity of frugivorous birds shape the spatial pattern of seed dispersal? A case study in
612 a relict plant species. *Philosophical Transactions of the Royal Society B: Biological*
613 *Sciences* 371:20150280.
- 614 Lee, A. T. K., D. J. Brightsmith, M. P. Vargas, K. Q. Leon, A. J. Mejia, and S. J. Marsden (2014).
615 Diet and geophagy across a western Amazonian parrot assemblage. *Biotropica* 46:322–
616 330.
- 617 Litchfield, C. (1970). Taxonomic patterns in the fat content, fatty acid composition, and
618 triglyceride composition of Palmae seeds. *Chemistry and Physics of Lipids* 4:96–103.
- 619 Lopes, C. M., M. De Barba, F. Boyer, C. Mercier, D. Galiano, B. B. Kubiak, R. Maestri, P. J. S.
620 da Silva Filho, L. Gielly, E. Coissac, T. R. O. de Freitas, and P. Taberlet (2020).
621 Ecological specialization and niche overlap of subterranean rodents inferred from DNA
622 metabarcoding diet analysis. *Molecular Ecology*:mec.15549.
- 623 Mallott, E. K., P. A. Garber, and R. S. Malhi (2018). trnL outperforms rbcL as a DNA
624 metabarcoding marker when compared with the observed plant component of the diet of
625 wild white-faced capuchins (*Cebus capucinus*, Primates). *PLOS ONE* 13:e0199556.
- 626 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads.
627 *EMBnet.journal* 17:10–12.

- 628 Matthews, J. K., A. Ridley, B. A. Kaplin, and C. C. Grueter (2020). A comparison of fecal
629 sampling and direct feeding observations for quantifying the diet of a frugivorous primate.
630 *Current Zoology* 66:333–343.
- 631 Matuzak, G. D., M. B. Bezy, and D. J. Brightsmith (2008). Foraging ecology of parrots in a
632 modified landscape: seasonal trends and introduced species. *The Wilson Journal of*
633 *Ornithology* 120:353–365.
- 634 McClenaghan, B., E. Nol, and K. C. R. Kerr (2019). DNA metabarcoding reveals the broad and
635 flexible diet of a declining aerial insectivore. *The Auk* 136.
- 636 Medina, B. M. L., and G. F. Pagano (2003). Caracterización de la pulpa de guayaba (*Psidium*
637 *guajava* L.) tipo “Criolla Roja.” *Revista de la Facultad de Agronomía* 20:72–86.
- 638 Neiff, J., and A. Poi de Neiff (2006). Situación ambiental en la ecorregión Iberá. In *La Situación*
639 *Ambiental Argentina 2005* (A. Brown, U. Martinez Ortiz, M. Acerbi and J. Corcuera,
640 Editors). Fundación Vida Silvestre Argentina, Buenos Aires, Argentina, pp. 177–184.
- 641 Nield, A. P., R. Nathan, N. J. Enright, P. G. Ladd, and G. L. W. Perry (2020). The spatial
642 complexity of seed movement: Animal-generated seed dispersal patterns in fragmented
643 landscapes revealed by animal movement models. *Journal of Ecology* 108:687–701.
- 644 Nunes, A. P., and A. dos Santos Jr. (2011). Itens alimentares consumidos por psitacídeos no
645 Pantanal e planaltos do entorno , Mato Grosso do Sul. *Atualidades Ornitológicas On-line*
646 162:42–50.
- 647 Nycander, E., D. H. Blanco, K. M. Holle, A. del Campo, C. A. Munn, J. I. Moscoso, and D. G.
648 Ricalde (1995). Manu and Tambopata: nesting success and techniques for increasing
649 reproduction in wild macaws in southeastern Peru. In *The large macaws: their care,*
650 *breeding and conservation* (B. L. S. J. Abramson and J. B. Thomsen, Editors). Raintree
651 Publications, Fort Bragg, California, pp. 423–443.
- 652 Oehm, J., A. Juen, K. Nagiller, S. Neuhauser, and M. Traugott (2011). Molecular scatology: how
653 to improve prey DNA detection success in avian faeces? *Molecular Ecology Resources*
654 11:620–628.
- 655 Paranhos, S. J., C. B. de Araujo, and L. O. Marcondes-Machado (2009). Feeding behavior of
656 *Aratinga aurea* (Psittacidae) in Southwestern Minas Gerais State, Brazil. *Revista*
657 *Brasileira de Ornitologia* 17:187–193.
- 658 Peignot, P., M. J. E. Charpentier, N. Bout, O. Bourry, U. Massima, O. Dosimont, R. Terramorsi,
659 and E. J. Wickings (2008). Learning from the first release project of captive-bred
660 mandrills *Mandrillus sphinx* in Gabon. *Oryx* 42.
- 661 Piñol, J., G. Mir, P. Gomez-Polo, and N. Agustí (2015). Universal and blocking primer
662 mismatches limit the use of high-throughput DNA sequencing for the quantitative
663 metabarcoding of arthropods. *Molecular Ecology Resources* 15:819–830.

- 664 Plair, B. L., M. Lal, A. Ramadhar, and S. Ramsubage (2013). Status of Blue-and-yellow Macaws
665 *Ara ararauna* Reintroduced to the Nariva Swamp , Trinidad and Tobago. Living World,
666 Journal of Trinidad and Tobago Field Naturalists' Club:19–28.
- 667 R Core Team (2020). R: A language and environment for statistical computing. R Foundation for
668 Statistical Computing, Vienna, Austria.
- 669 Ragusa-Netto, J. (2011). Pre-dispersal seed predation by Blue-and-Yellow macaw (*Ara ararauna*,
670 Psittacidae), on fruit crops of the Pequi (*Caryocar brasiliense*, Caryocaraceae), in the
671 Brazilian Cerrado. *Ornitología Neotropical* 22:329–338.
- 672 Ragusa-Netto, J., and A. Fecchio (2006). Plant food resources and the diet of a parrot community
673 in a gallery forest of the southern Pantanal (Brazil). *Brazilian Journal of Biology*
674 66:1021–1032.
- 675 Renton, K., A. Salinas-Melgoza, M. Á. D. Labra-Hernández, and S. M. de la Parra-Martínez
676 (2015). Resource requirements of parrots: nest site selectivity and dietary plasticity of
677 Psittaciformes. *Journal of Ornithology* 156:73–90.
- 678 Robinet, O., V. Bretagnolle, and M. N. Clout (2003). Activity patterns, habitat use, foraging
679 behaviour and food selection of the Ouvea Parakeet (*Eunymphicus cornutus uvaeensis*).
680 *Emu* 103:71–80.
- 681 Rytönen, S., E. J. Vesterinen, C. Westerduin, T. Leviäkangas, E. Votka, M. Mutanen, P.
682 Välimäki, M. Hukkanen, M. Suokas, and M. Orell (2019). From feces to data: A
683 metabarcoding method for analyzing consumed and available prey in a bird-insect food
684 web. *Ecology and Evolution* 9:631–639.
- 685 Santos, M. P. D. (2001). Dieta da arara-vermelha-grande (*Ara chloroptera*) na Chapada das
686 Mangabeiras, Sul do Piauí, Brasil. *Tangara* 1:131–134.
- 687 Scasta, J. D., T. Jorns, J. D. Derner, S. Lake, D. J. Augustine, J. L. Windh, and T. L. Smith
688 (2019). Validation of DNA metabarcoding of fecal samples using cattle fed known
689 rations. *Animal Feed Science and Technology* 255:114219.
- 690 Scherer-Neto, P., and A. C. Terto (2011). Registros e documentação fotográfica da alimentação
691 da arara-vermelha-grande (*Ara chloropterus*) na região noroeste do Paraná
692 (Psittaciformes: Psittacidae). *Atualidades Ornitológicas* 159:37–42.
- 693 Shrestha, R., and P. Wegge (2006). Determining the Composition of Herbivore Diets in the
694 Trans-Himalayan Rangelands: A Comparison of Field Methods. *Rangeland Ecology &*
695 *Management* 59:512–518.
- 696 Silva, P. A. (2018). Massive consumption of unripe slash pine (*Pinus elliottii*) seeds by Blue-
697 and- Yellow Macaws (*Ara ararauna*). *Ornitología Neotropical*:9.
- 698 Silva, P. A., and C. Melo (2013). Foraging of the Golden-capped Parakeet (*Aratinga*
699 *auricapillus*) in an Anthropogenic Landscape in Brazil. *Ornitología Neotropical* 24:55–66.

- 700 Tella, J. L., A. Baños-Villalba, D. Hernández-Brito, A. Rojas, E. Pacífico, J. A. Díaz-Luque, M.
701 Carrete, G. Blanco, and F. Hiraldo (2015). Parrots as overlooked seed dispersers.
702 *Frontiers in Ecology and the Environment* 13:338–339.
- 703 Tella, J. L., F. Hiraldo, E. Pacífico, J. A. Díaz-Luque, F. V. Dénes, F. M. Fontoura, N. Guedes,
704 and G. Blanco (2020). Conserving the Diversity of Ecological Interactions: The Role of
705 Two Threatened Macaw Species as Legitimate Dispersers of “Megafaunal” Fruits.
706 *Diversity* 12:45.
- 707 Thalinger, B., J. Oehm, A. Obwexer, and M. Traugott (2017). The influence of meal size on prey
708 DNA detectability in piscivorous birds. *Molecular Ecology Resources* 17:e174–e186.
- 709 Trivedi, M. R., F. H. Cornejo, and A. R. Watkinson (2004). Seed predation on Brazil nuts
710 (*Bertholletia excelsa*) by macaws (Psittacidae) in Madre de Dios, Peru. *Biotropica*
711 36:118–122.
- 712 Tutin, C. E., and M. Fernandez (1993). Faecal Analysis as a Method of Describing Diets of
713 Apes: Examples from Sympatric Gorillas and Chimpanzees at Lope, Gabon. *Tropics*
714 2:189–197.
- 715 Uchôa-thomaz, A. M. A., E. C. Sousa, J. O. B. Carioca, S. M. D. Morais, A. D. Lima, C. G.
716 Martins, C. D. Alexandrino, P. A. T. Ferreira, A. L. M. Rodrigues, S. P. Rodrigues, J. C.
717 D. A. Thomaz, et al. (2014). Chemical composition, fatty acid profile and bioactive
718 compounds of guava seeds (*Psidium guajava* L.). *Food Science and Technology* 34:485–
719 492.
- 720 Valentini, A., C. Miquel, M. A. Nawaz, E. Bellemain, E. Coissac, F. Pompanon, L. Gielly, C.
721 Cruaud, G. Nascetti, P. Wincker, J. E. Swenson, and P. Taberlet (2009a). New
722 perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the
723 trnL approach. *Molecular Ecology Resources* 9:51–60.
- 724 Valentini, A., F. Pompanon, and P. Taberlet (2009b). DNA barcoding for ecologists. *Trends in*
725 *Ecology & Evolution* 24:110–117.
- 726 Warburton, L. S. (2003). The ecology and conservation biology of the Black-cheeked Lovebird
727 *Agapornis nigrigenis* in Zambia.
- 728 White, G. C., and R. A. Garrott (2012). *Analysis of Wildlife Radio-Tracking Data*. Academic
729 Press Inc, San Diego, California.
- 730 Wickham, H., M. Averick, J. Bryan, W. Chang, L. D. McGowan, R. François, G. Grolemond, A.
731 Hayes, L. Henry, J. Hester, M. Kuhn, et al. (2019). Welcome to the Tidyverse. *Journal of*
732 *Open Source Software* 4:1686.
- 733 Williams, D. R., R. G. Pople, D. A. Showler, and M. F. Child (2013). *Bird Conservation: Global*
734 *Evidence for the Effects of Interventions*. Pelagic Publishing Limited.

- 735 Wotton, D. M., and D. Kelly (2012). Do larger frugivores move seeds further? Body size, seed
736 dispersal distance, and a case study of a large, sedentary pigeon. *Journal of Biogeography*
737 39:1973–1983.
- 738 Yamashita, C., and Y. Machado de Barros (1997). The Blue-throated Macaw *Ara glaucogularis*:
739 characterization of its distinctive habitats in savannahs of the Beni, Bolivia. *Ararajuba*
740 5:141–150.
- 741 Yu, X., X. Li, and Z. Huo (2015). Breeding ecology and success of a reintroduced population of
742 the endangered Crested Ibis *Nipponia nippon*. *Bird Conservation International* 25:207–
743 219.
- 744 Zamboni, T., S. Di Martino, and I. Jiménez-Pérez (2017). A review of a multispecies
745 reintroduction to restore a large ecosystem: The Iberá Rewilding Program (Argentina).
746 *Perspectives in Ecology and Conservation* 15:248–256.
- 747
- 748

749 **TABLES**

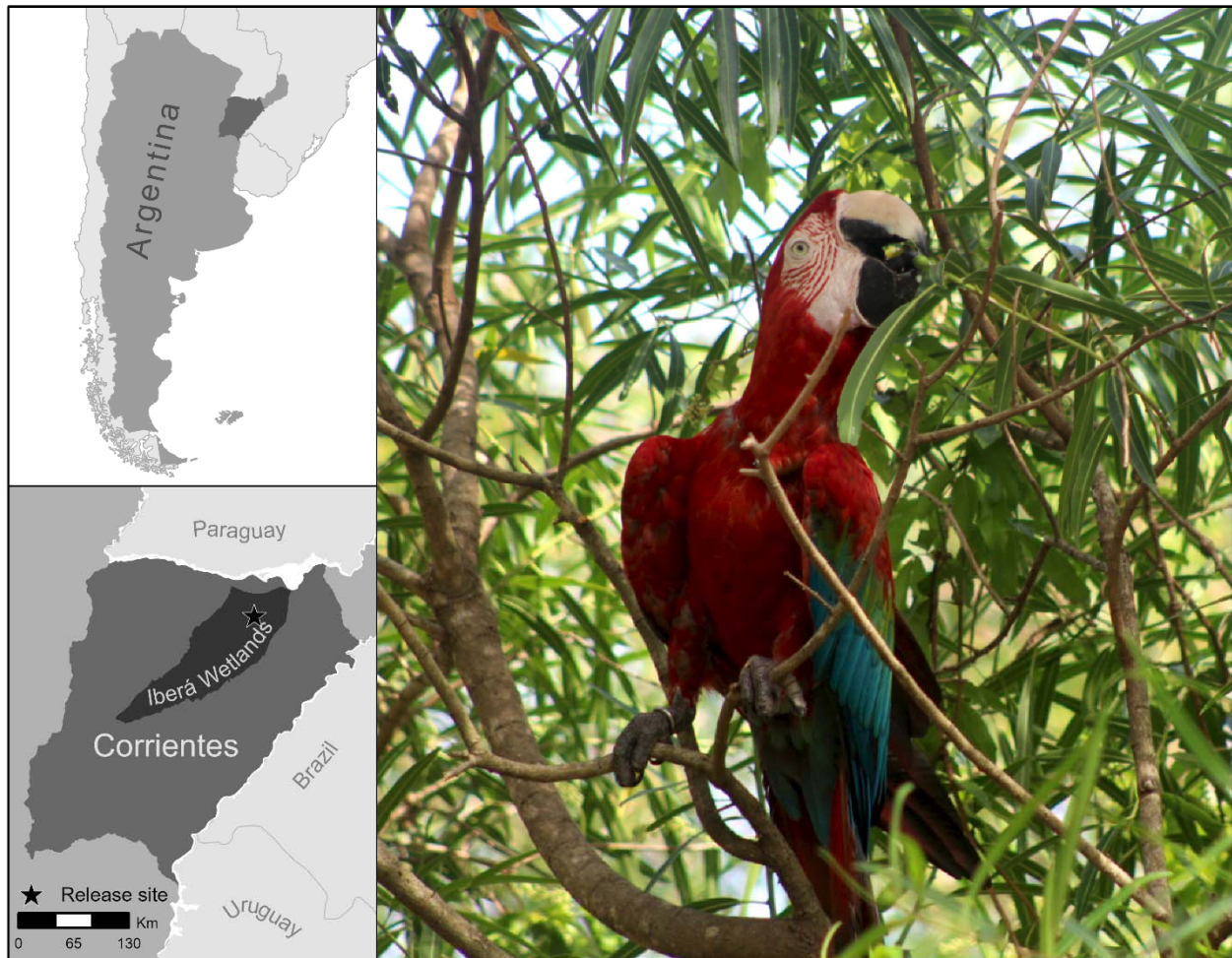
750 **Table 1.** Species used by Red-and-green Macaws released at the Iberá National Park; **Part:** part
751 of the plant consumed (only for direct observations); **Method:** technique used to determine
752 presence in the diet (O = Observation, M = Metabarcoding); **PO:** proportion of occurrence
753 (percentage of sampling days on which the species was detected in the diet); **Month:** month
754 during which feeding was detected by direct observation (black), metabarcoding (light grey) or
755 both methods (dark grey); **Total:** number of days sampled in a month.

Family	Scientific name	Part*	Method	PO	2017						2018					
					J	J	A	S	O	N	D	J	F	M	A	M
Myrtaceae	<i>Psidium guajava</i>	up,rp,us,rs	O,M	59												
	<i>Eugenia myrcianthes</i>	up,rp,us,rs	O,M	6												
	<i>Eugenia uniflora</i>	rp	O,M	5												
	<i>Blepharocalyx salicifolius</i>	-	M	1												
	<i>Eugenia pitanga</i>	-	M	1												
Fabaceae	<i>Inga edulis</i>	us,rs,a	O	20												
	<i>Enterolobium contortisiliquum</i>	rp,rs	O	13												
	<i>Delonix regia</i>	rs,f	O	3												
	<i>Chloroleucon tenuiflorum</i>	us	O	1												
	<i>Senna sp.</i>	-	M	1												
Euphorbiaceae	<i>Croton urucurana</i>	us	O,M	14												
	<i>Sapium haematospermum</i>	us,rs	O,M	12												
	<i>Alchornea triplinervia</i>	us	O,M	7												
	<i>Sebastiania commersoniana</i>	us	O,M	2												
Arecaeae	<i>Syagrus romanzoffiana</i>	up,rp,us,rs	O	26												
	<i>Acrocomia aculeata</i>	us,rs	O	1												
Moraceae	<i>Ficus luschnathiana</i>	rp,rs	O,M	14												
Pinaceae	<i>Pinus sp.</i>	us	O,M	11												
Meliaceae	<i>Melia azedarach</i>	us,rs	O,M	10												
Lauraceae	<i>Ocotea diospyrifolia</i>	rs	O	8												
Bignoniaceae	<i>Jacaranda mimosifolia</i>	us	O	3												
	<i>Handroanthus impetiginosus</i>	f	O	1												
	<i>Tabebuia sp.</i>	-	M	1												
	<i>Dolichandra unguis-cati</i>	f	O	1												
Solanaceae	<i>Cestrum nocturnum</i>	-	M	3												
	<i>Cestrum parqui</i>	u	O	2												
Tiliaceae	<i>Luehea divaricata</i>	us	O	3												
Sapotaceae	<i>Chrysophyllum gonocarpum</i>	u,r	O	3												
	<i>Pouteria gardneriana</i>	-	M	1												
Rhamnaceae	<i>Sageretia elegans</i>	r	O,M	3												
	<i>Gouania polygama</i>	us,rs	O	1												
Asteraceae	<i>Mikania cordifolia</i>	-	M	3												
Viscaceae	<i>Phoradendron bathyoryctum</i>	r	O	2												
Urticaceae	<i>Cecropia pachystachya</i>	l	O,M	1												
	<i>Urera sp.</i>	-	M	1												
Sapindaceae	<i>Allophylus edulis</i>	-	M	1												
	<i>Mangifera indica</i>	-	M	1												
	<i>Paullinia elegans</i>	rs	O	1												
Apocynaceae	<i>Tabernaemontana catharinensis</i>	-	M	1												
Annonaceae	<i>Annona emarginata</i>	rp	O	1												
Verbenaceae	<i>Vitex sp.</i>	-	M	1												
Salicaceae	<i>Xylosma venosa</i>	r	O	1												
Rubiaceae	<i>Psychotria carthagenensis</i>	-	M	1												
Primulaceae	<i>Myrsine sp.</i>	-	M	1												
Phytolaccaceae	<i>Phytolacca dioica</i>	-	M	1												
Passifloraceae	<i>Passiflora caerulea</i>	rp,rs,f	O	1												
Melastomataceae	<i>Miconia pusilliflora</i>	r	O,M	1												
Malvaceae	<i>Theobroma sp.</i>	-	M	1												
Cucurbitaceae	<i>Lagenaria sp.</i>	-	M	1												
Total					9	20	9	16	14	13	19	12	10	11	7	9

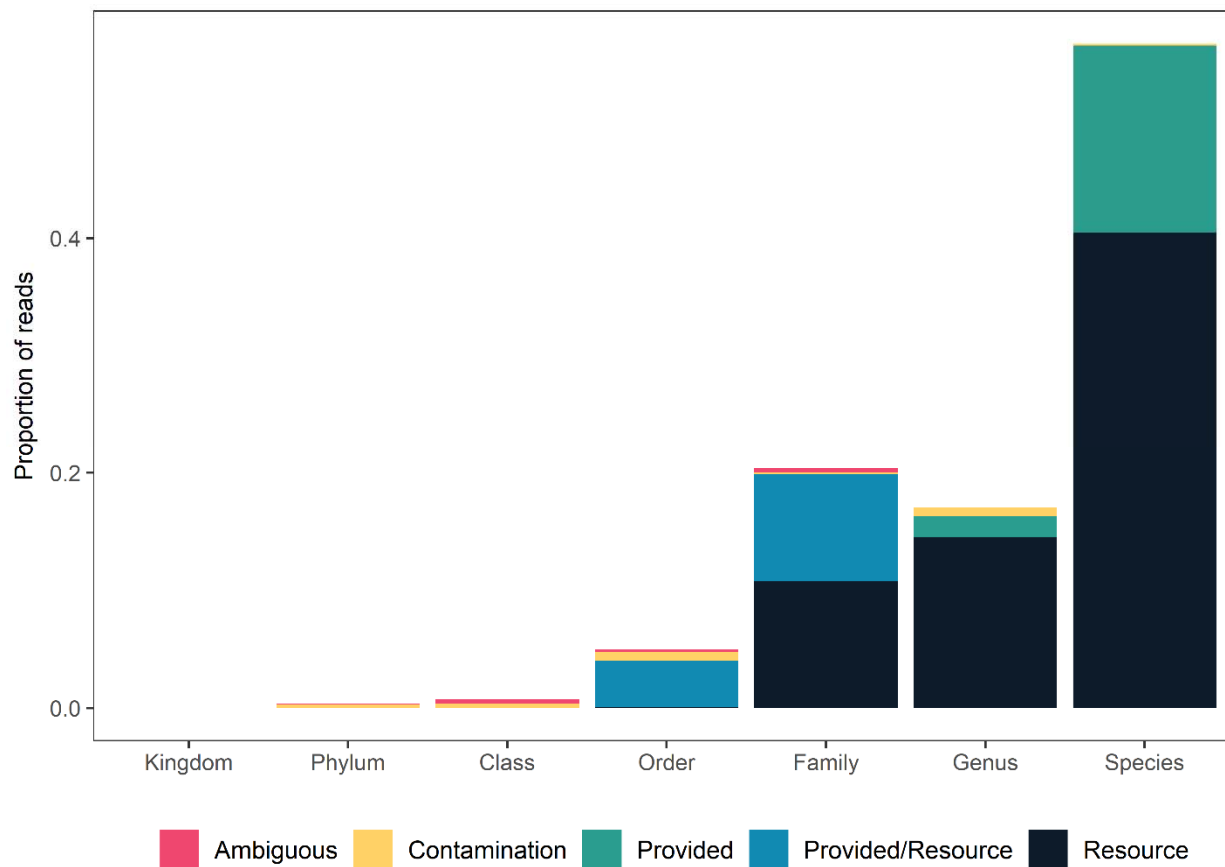
757 * **Part:** rs = ripe seed, us = unripe seed, rp = ripe pulp, up = unripe pulp r = ripe fruit (unclear if
758 seed or pulp), u = unripe fruit (unclear if seed or pulp), f = flower, l = leaf, a = aril.

759

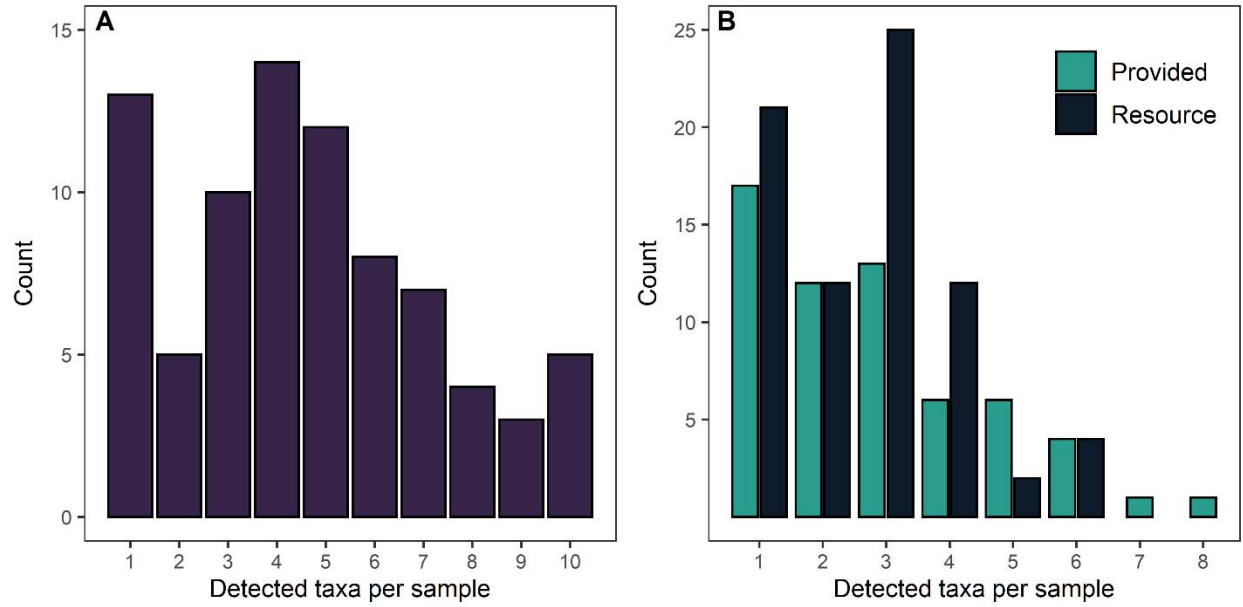
760 **FIGURES**



761 **FIGURE 1. Right:** Location of the study site, Portal Camby Retá (Iberá National Park), in the
762 Iberá Wetlands region located in the province of Corrientes, northeastern Argentina. **Left:**
763 reintroduced Red-and-green Macaw feeding on a wild fruit (*Sapium haematospermum*).
764



765
766 **FIGURE 2.** Distribution of reads among taxonomic levels and categories of use, based on
767 1,840,811 reads from 83 samples (**Resource** = wild local plants; **Provided** = commercial fruits
768 or vegetables; **Provided/Resource** = level of resolution does not allow to exclude either option;
769 **Contamination** = Accidental presence, byproduct of fecal sample collection; **Ambiguous** =
770 either of the previous categories).
771



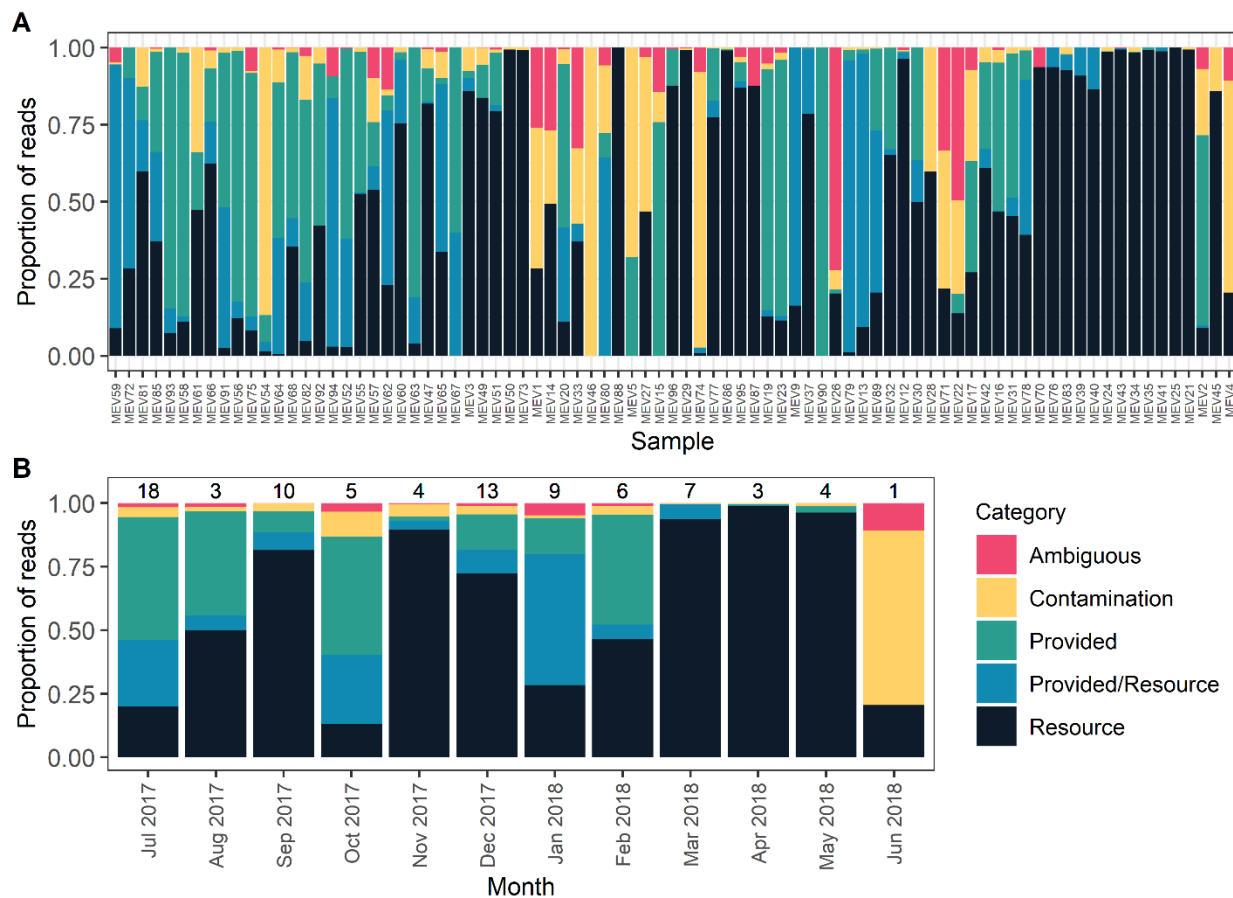
772

773 **FIGURE 3.** Plant diversity in Red-and-green Macaw feces collected at the Iberá Wetlands. (A)

774 Distribution of the total number of taxa detected at the species or genus level per sample; (B)

775 Distribution of the number of provided taxa and resource taxa detected at the species or genus

776 level per sample ($n = 83$ samples).



777

778

779 **FIGURE 4.** Distribution of plant reads proportions between samples (**A**) and months (**B**).

780 Samples in the upper panel (**A**) are sorted based on their collection date (Jul-15-2017 to Jun-2-

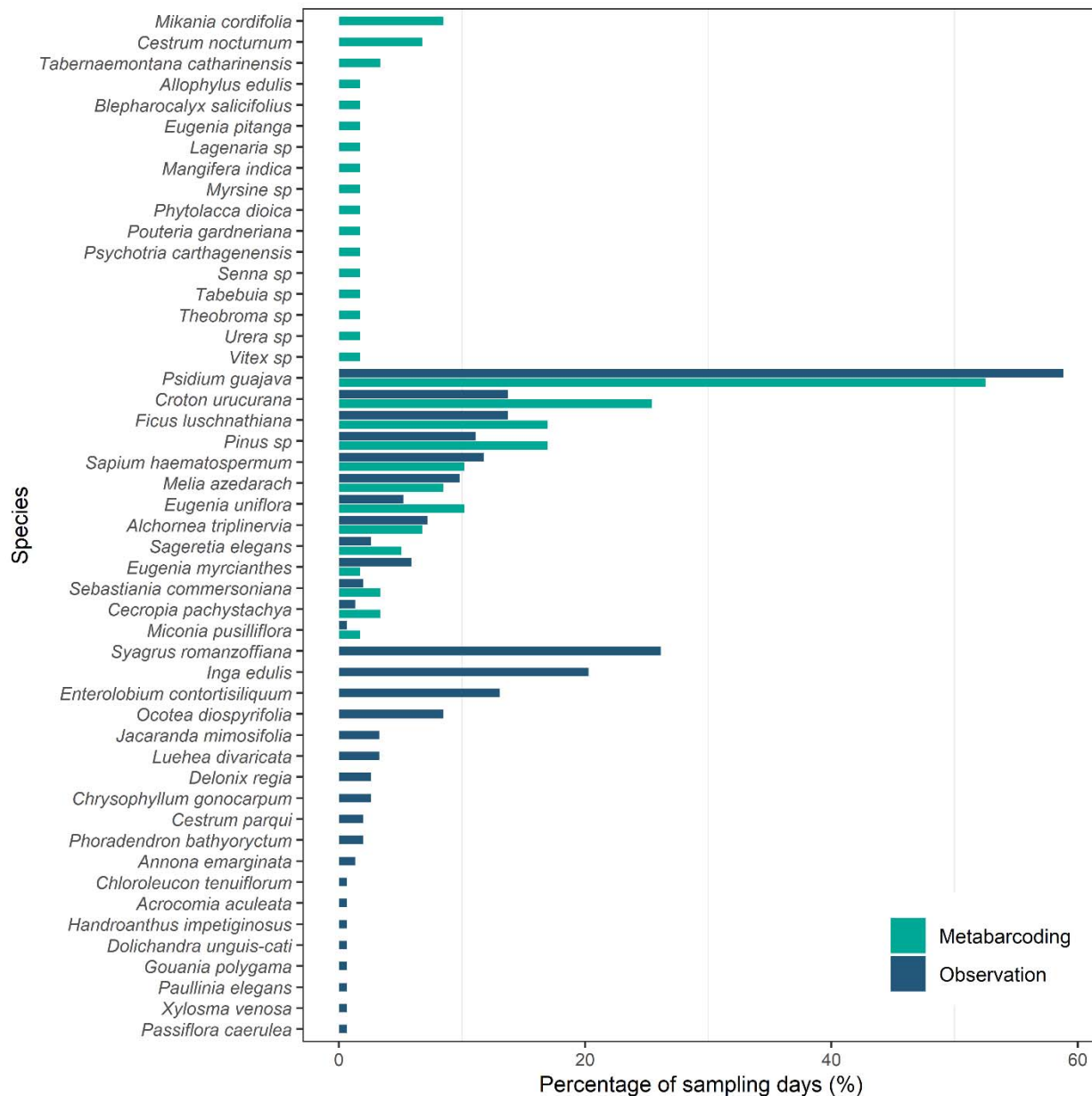
781 2018). Values above the columns in (**B**) represent the number of samples collected that month (n

782 = 83 samples). (**Resource** = wild local plants; **Provided** = commercial fruits or vegetables;

783 **Provided/Resource** = level of resolution does not allow to exclude either option;

784 **Contamination** = Accidental presence, byproduct of fecal sample collection; **Ambiguous** =

785 either of the previous categories).

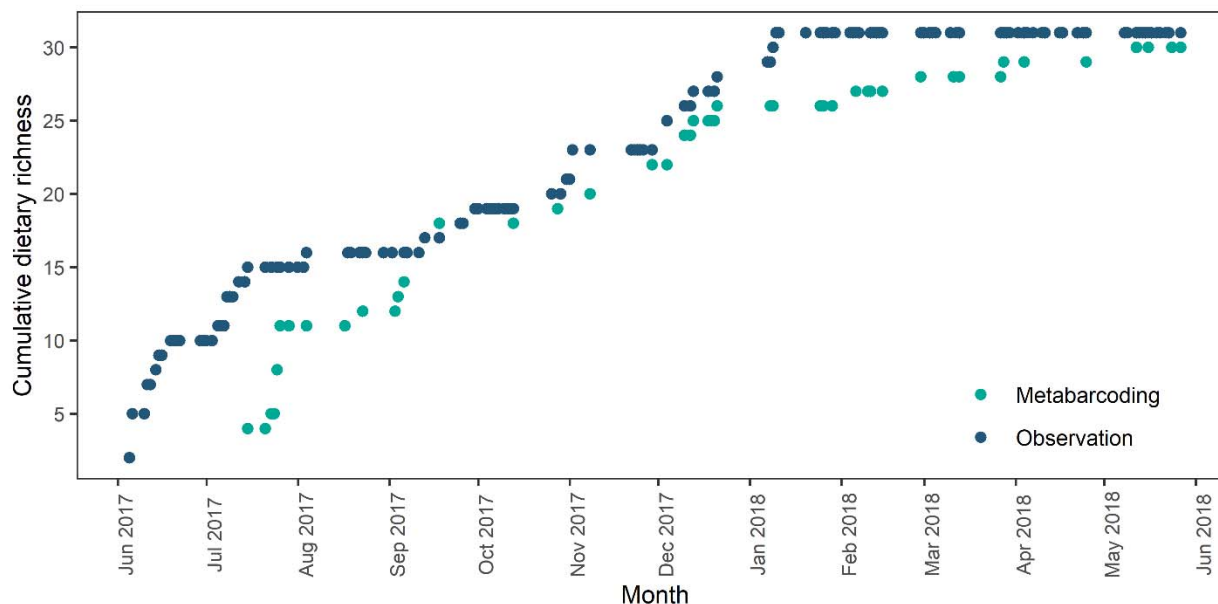


786

787 **FIGURE 5:** Plant species consumed by the Red-and-green Macaw at the Iberá Wetlands and
 788 their relative contribution to the diet (percentage of sampling days in which each species was
 789 detected) based on observational (blue; $n = 140$ days) and DNA-based data (teal; $n = 53$ days).

790

791

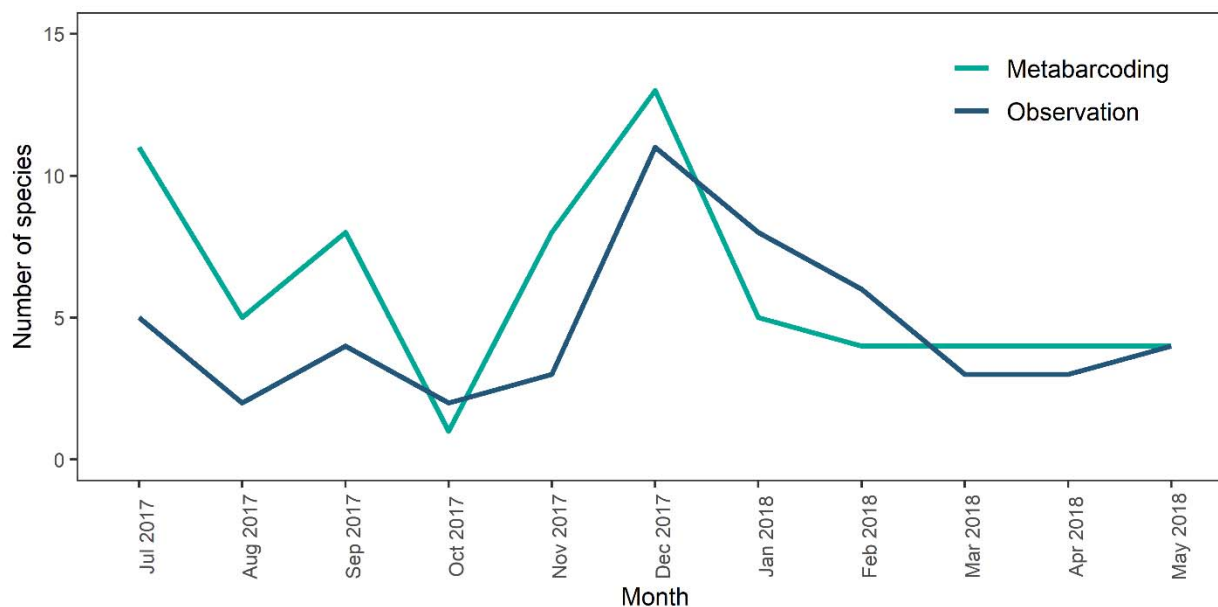


792

793 **FIGURE 6.** Cumulative number of resource plant taxa in the diet of reintroduced *Ara*

794 *chloropterus* since the day of release based on observational (blue; $n = 140$ days) and

795 metabarcoding (teal; $n = 53$ days) data.



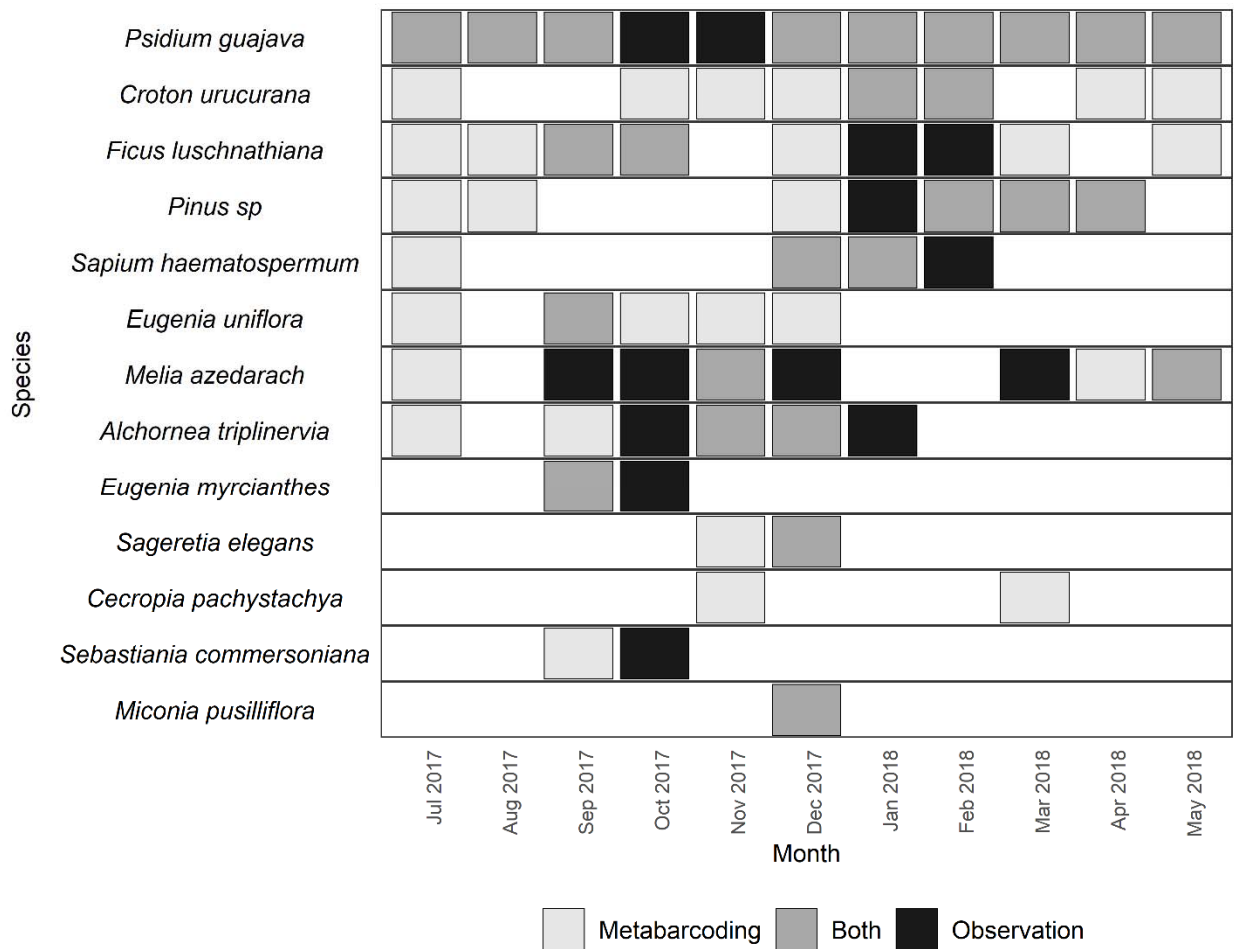
796

797 **FIGURE 7.** Number of resource species consumed by released Red-and-green Macaws each

798 month, based on observational (blue; $n = 219$ feeding events) and metabarcoding (teal; $n = 61$

799 samples) data collected on the same date ($n = 39$ days).

800



801

802 **FIGURE 8.** Monthly occurrence of the 13 species present in both observational ($n = 252$ feeding

803 events) and metabarcoding datasets ($n = 75$ samples) in the diet of released Red-and-green

804 Macaws. Only records collected within the same time frame area included. (Light grey =

805 presence detected only by barcode, Black = presence detected only through observation, Dark

806 grey = presence detected by both methods)

807

808