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Diet and trophic niche overlap among a native waterbird and two non-native herbivores in Pampas grasslands

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ABSTRACT

The use of areas by native and non-native herbivores in sympatry has frequently led to conflicts between farmers and native animals. Dietary studies integrating methodologies usually provide a better quantified understanding of resource sharing and dietary overlap. This study aims to evaluate diet and niche overlap among three species that occur in sympatry in southern Brazil: a native herbivorous bird in South America, the southern screamer (*Chauna torquata*), domestic sheep (*Ovis aries*) and cattle (*Bos taurus*). Three complementary methodologies were used: microhistology and metabarcoding of faeces and stable isotope analysis (SIA) in faeces and whole blood. Bipartite networks were constructed for the three dietary assessments. No significant isotopic niche overlap was found between the native bird and the domestic herbivores by SIA in blood, while the three methods applied to faecal samples showed limited overlap between the bird and the non-native ungulates. Conversely, SIA in faecal samples revealed similarity in the feeding habits and niches of domestic herbivores. The complementary use of the three different techniques allowed the evaluation of the herbivore diet from different perspectives, according to strengths and limitations of each method: while metabarcoding indicated the use of a larger number of plant groups in different proportions and identified several of the components at the species level, Cyperaceae species were only identified by microhistology. Conversely, metabarcoding performed better than microhistology in the detection of some species of Onagraceae and Poaceae. SIA in blood elucidated different proportions of resources assimilated by herbivores, while faecal SIA revealed plants with similar photosynthetic cycles in the diet of the non-native herbivores. Through bipartite network analysis, modules emerged (e.g. SIA = 0.2, 95% IC = 0.1–0.19), confirming the differential use of some groups of resources. The combination of the three techniques, enhanced by comparative bipartite network analysis, was critical for obtaining a detailed understanding of the vast range of potential plants used and interactions among herbivores. The detected patterns of dietary partitioning also confirm that the southern screamer is not competing with cattle and sheep and can even provide ecosystem services through the biological control of aquatic weeds.

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1. Introduction

Detailed information on dietary partitioning among herbivores is an important issue in ecology (Kartzinel et al., 2015). Distinct food preferences or niche displacement could allow coexistence in the environment (Hofmann, 1989; Chesson, 2000), in addition to modulating the base of the trophic chain (Boavista et al., 2019). Furthermore, extensive niche overlap under conditions of limited availability of resources could lead to competition, which may result in competitive exclusion and local extinctions (Gause, 1934). Otherwise, in an environment with abundant resources, the overlap of high trophic niches may occur without competition (Schoener, 1974).

The sharing of areas with livestock potentially decreases forage availability for native herbivores (Nicholson et al., 2001), which can result in overlap and, ultimately, competition (Pedrana et al., 2018). Although the inferred resource competition between native animals and livestock is sometimes only apparent, this can result in a conflict between farmers and wildlife. In South American grasslands, such apparent conflict resulted in a decline in goose numbers due to control by culling, although it was later shown that the geese do not interfere with livestock feeding (Summers, 1985) and can even provide many ecological benefits (Gorosábel et al., 2019).

In native subtropical grasslands of the Pampas biome, cattle and sheep are frequently raised together (Carvalho and Batello, 2009) and may occur in sympatry with herbivorous native birds. In southern Brazil, this type of situation occurs with the southern screamer (*Chauna torquata*), a large year-round resident herbivorous bird (80 cm height, 3780–5535

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g) that can form flocks of up to 800 birds (Fontana et al., 1994). As this bird shares grassland environments with livestock, it is considered by some farmers to be a potential forage competitor of sheep (*Ovis aries*) and cattle (*Bos taurus*) (Sick, 1997). In Argentinean Pampas grasslands, sheep use grass, sedges and rushes (Posse et al., 1996), showing a preference for lower pastures (Pallarés et al., 2005), whereas cattle usually consume grasses (Bontti et al., 1999). The southern screamer has been described as a herbivorous bird with a preference for grasses and plants with C3 photosynthetic cycles (García, 2015).

Resource sharing or trophic overlap among animals can be accessed through bipartite networks in which plant-herbivore interactions (links) can be determined, and the structure and modularity of these interactions can be assessed (Ponisio et al., 2019). Several methods can be used to determine the diets of herbivores, but each technique has its own strengths and shortcomings. Among dietary estimation techniques, the microhistological analysis of faeces is widely used for dietary quantification based on characteristics of the epidermal leaves of plants (Steward, 1967). Faecal analysis allows the dietary comparison of animals with negligible disturbance and minimal cost (Holeček et al., 1982). However, microhistological analysis requires a reference collection of epidermal structures of plant species and is time consuming (Holeček et al., 1982; Wegge et al., 2006). Moreover, there is a potential error related to the observer skills and differential digestion of consumed plants, which can bias the results (Holeček et al., 1982).

Faster and more accurate molecular techniques such as metabarcoding can solve some difficulties, improving the identification of digested fragments in faeces (Ando et al., 2020). However, this technique requires a molecular reference database for potentially consumed plants and the selection of an informative marker to determine the diet of herbivores (Chase et al., 2007). Moreover, there is a potential error related to differential amplification, which may provide an incomplete view of the resources consumed (Lamb et al., 2019). Even so, dietary studies using metabarcoding have provided important insights on niche partitioning in African herbivores (Kartzinel et al., 2015) and revealed unidentified resources in comparison with conventional diet analysis (Sullins et al., 2018).

In turn, the stable isotope analysis (SIA) of consumer tissues provides evidence of dietary partitioning for effectively assimilated sources (Phillips, 2012). Moreover, the use of faecal samples for SIA provides an option for determining dietary preferences, enabling inferences about resource partitioning (Codron et al., 2005; Codron and Codron, 2009). The ecological niche can also be determined by using a proxy, the isotopic niche, based on SIA, in which the occupied niche area of animals (δ -space) can be evaluated (Newsome et al., 2007). The axes of these graphs are composed by chemical elements (generally $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), providing a measure that is comparable to the n -dimensional niche (Newsome et al., 2007) and to metrics of dietary niche based on conventional diet measures.

In the current study, the diets of the native southern screamer and non-native cattle and sheep were analysed through microhistological analysis, metabarcoding and SIA to characterize the diet and niche overlap patterns among the three herbivores that occur in sympatry in grasslands and associated wetlands in southern Brazil. Along with this task, the effectiveness of each diet assessment technique was also evaluated, and the different information was integrated to perform bipartite network analyses. We hypothesize that there is greater overlap between cattle and sheep, whereas the southern screamer uses different resources and thus presents limited potential for conflict with livestock. Such results can be important to support and improve management efforts for this and other native species.

2. Materials and methods

2.1. Study area

This study was carried out in two nearby farmland areas (32°52'S; 052°54'W) (about 4 km apart) adjacent to the Taim Ecological Reserve

in the Rio Grande do Sul (RS) state, southern Brazil. The area is located on coastal plains characterized by a mosaic of natural environments such as wetlands, dunes, marshes, and lagoons (Asmus, 1998) as well as irrigated rice paddies and sheep and cattle ranching (Motta-Marques et al., 2013). The climate is subtropical-humid, with annual average temperature between 17 °C and 19 °C and annual precipitation of 1200–1500 mm (Klein, 1998). Precipitation is roughly similar throughout the year, but with frequent hydric deficits in summer due to much higher evaporation (Mota et al., 1970).

The vegetation of the study area is composed of grasslands that are part of the Brazilian Pampas (or *Campos Sulinos*) biome (Andrade et al., 2019). Members of the Poaceae, Asteraceae, Fabaceae and Cyperaceae families exhibiting both the C3 and C4 photosynthetic pathways are found in these areas (Boldrini, 2009). C3 grasses provide biomass with high quality in colder periods, while C4 grasses are more productive during warmer seasons (Andrade et al., 2019). The encountered grasslands are classified as humid Pampas and present dominance of species such as *Eleocharis* sp. and *Luziola peruviana*, as well as coastal species such as *Hidrocotyle bonariensis* (Andrade et al., 2019). The predominance of grasses makes the region important for livestock rearing, which is currently one of the main economic activities in this region (Blanco et al., 2007). Interest in livestock has also led to the cultivation of plants such as perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) (Selaive-Villaroel et al., 1997).

2.2. Studied species

The southern screamer is a large year-round resident herbivore bird, whose conservation status is classified as least concern (IUCN, 2020). The bird has adaptations such as toes and long legs that allow it to forage in marshy environments (Cramp, 1992). In Southern Brazil, a descriptive study of the diet of this species found a great consumption of *Trifolium repens*, concomitant to the use of over ten species of grasses (García, 2015). Southern screamer is an abundant bird in the area of the Taim Ecological Reserve (Fontana et al., 1994), occurring also in adjacent areas such as farms.

Among the main mammals used for livestock rearing in southern Brazil, the cattle, and the sheep are raised mainly for meat and wool (sheep). In the studied area, these ruminants forage in native or enriched pastures. The latter are improved by the cultivation of plants with better nutritional value (Nabinger et al., 2000).

2.3. Ethics procedures

This study was approved by the Committee on Ethics in the Use of Animals (P012/2019) of the Universidade Federal do Rio Grande (FURG) and by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) (License No. 59242-1). Bird capture and banding was approved and metal bands were provided by the *Centro Nacional de Pesquisa e Conservação das Aves Silvestres* (CEMAVE/ICMBio).

2.4. Sampling

Southern screamers ($n = 49$) were captured at night in fourteen expeditions conducted in 2013–2014 and 2017–2018 throughout the four seasons. Captures were performed on dark nights (i.e., new and third-quarter moon phases) by walking with a LED flashlight with a 15 W (900 lm) intensity. When a bird was spotted far from the flock, a silent approach was initiated, focusing the flashlight towards the animal to disorient it. Another researcher immobilized the animal using a hand net or a hoop net with a 1.6 m diameter covered with a 25 mm nylon mesh. A dark hood was then placed on the animal's head to reduce stress. Approximately 2 ml of blood was collected from each bird from the brachial vein using a sterile syringe and needle.

Cattle ($n = 43$) were sampled in November 2017 and May 2018, and sheep ($n = 19$) were sampled in June and December 2018. After immobilization, ~2 ml of blood was sampled from the brachial vein, the coccygeal (undertail) vein or the jugular vein.

Fresh faeces were randomly sampled at different locations to avoid collecting samples of the same individual. Blood and faeces samples are thus independent. Faeces were separated into individual dry bags and later homogenized and separated into three similar portions, which were frozen for posterior microhistological analysis, metabarcoding and SIA.

Plants with C3 and C4 photosynthetic cycles were manually collected for comparison with the diets of the animals by SIA, to build a reference database for molecular analyses ($n = 21$) and for use as a reference in microhistological analyses ($n = 32$).

2.5. Microhistological analysis

Faecal subsamples were washed with flowing water through 1.00 and 0.25 mm-mesh size sieves. The content collected in the 0.25 mm mesh was transferred to 30 ml vials, fixed with FAA (formaldehyde, alcohol, acetic acid) and bleached with 10% sodium hypochlorite (Castellaro et al., 2007) to facilitate the identification of fragments. Six replicates of each faecal sample from the southern screamer ($n = 32$), sheep ($n = 16$) and cattle ($n = 16$) were visualized under an optical microscope (10 \times) through Sedgewick-Rafter millimetric slides. The area of each fragment on each slide was estimated by measuring the length and width, which were multiplied to obtain the approximate area in mm² and counted. The epidermal identification was performed according to the diagnostic characteristics (e.g. trichomes, stomata) (Castellaro et al., 2007) by comparing with reference images of the plants of the study area, guides for herbivores diet, and checking with local botanists. The plant species were identified to the lowest taxonomic level possible and grouped into functional groups (e.g. forb, grass), according to our personal database and to the epidermic reference collection available at the Institute of Biological Sciences at FURG.

2.6. Metabarcoding analysis

First, a reference database of potentially consumed plants was constructed. For this purpose, total DNA was extracted from leaves macerated in liquid nitrogen using the Nucleospin Plant II kit (Macherey-Nagel, Nucleospin Plant®, Germany). Because a universal marker for species-specific identification for plants is not available (Chase et al., 2007), three chloroplast regions were first selected (*matK*, *rbcl* and *psbA-trnH*) and amplified with the use of primers and conditions described by Sang et al. (1997), Levin et al. (2003), Tate and Simpson (2003), Ford et al. (2009), Kress et al. (2009) and Dunning and Savolainen (2010). According to this pilot amplification test, the region of the intergenic spacer, *psbA-trnH*, amplified for the largest number of reference plants. This marker was then employed for further metabarcoding procedures.

During the construction of the reference database, a fragment of the *psbA-trnH* region was amplified, purified and sequenced from a total of 21 plant species collected at the sampling sites. These plants represent eight different families as well as the most important genera found in the faecal samples through microhistology analysis, and these data were therefore used to complement the sequences of this marker already available for other species in GenBank. The PCR reactions were performed in a final volume of 20 μ l containing 1 μ l of DNA, 1 U of Taq DNA-polymerase (Ludwig), 1 \times buffer, 2 mM MgCl₂, 0.1 μ M of each primer, 0.2 mM of each dNTP, 3% DMSO and 0.1 mg/ml BSA. Amplification was optimized with an initial denaturation step at 95 °C for 45 s, followed by 35 cycles of 30 s at 95 °C, 30 s at 61 °C and 40 s at 72 °C, with a final extension step at 72 °C for 10 min. The PCR products were stained with GelRed (Biotium) and visualized in a transilluminator after electrophoresis in 0.8% agarose gel. The amplified products were purified with 7.5 M ammonium acetate

(C₂H₇NO₂) and directly sequenced in a Perkin-Elmer ABI Prism 377 automated sequencer (Macrogen, Seoul, Korea). Electropherograms were assembled with Gap4 software in the Staden package (Staden, 1996). Additional plant species sequences available in GenBank were also added to the matrix to complete the reference database (Appendix A, Table A.1).

DNA from faeces from southern screamer ($n = 26$), cattle ($n = 12$) and sheep ($n = 15$) was also extracted using Nucleospin DNA Stool kit (Macherey-Nagel, Nucleospin®, Germany). DNA quantification was performed in a Qubit fluorometer using a dsDNA HS Assay kit (Thermo Fisher). After diluting the samples to 10 ng. μ l⁻¹, they were sent to Neoprosperta (Florianópolis, Brazil), where amplification was performed under the same PCR conditions used for amplification of the plant samples. Further NGS sequencing was performed on the Illumina MiSeq platform through a single-end strategy. The sequences were submitted to the NCBI Sequence Read Archive SRA database and are available under BioProject PRJNA721415.

After sequencing, the raw data (fastq's) were trimmed to remove non-specific sequences using Cutadapt v.1.18 (Martin, 2011), Trimmomatic v.0.38 (Bolger et al., 2014) and Prinseq v.0.20.4 (Schmieder and Edwards, 2011). In this step, reads with Phred values <25 and lengths <200 bp were removed. The quality profile of the raw and trimmed data was further checked in FastQC v.0.11.8 (Andrews, 2010). For the definition of OTUs (operational taxonomic units), identical sequences were clustered in Vsearch v.2.9.0 (Rognes et al., 2016), where chimeric sequences were withdrawn. BlastN searches of each OTU were then performed against the NCBI nucleotide database (nt/nr) and our personal molecular reference database. Only samples with >50 reads, > 5 hits, e-values <1e⁻⁵⁰ and an identity >97% were maintained.

2.7. Stable isotope analysis (SIA)

Blood samples of southern screamer ($n = 49$), cattle ($n = 43$) and sheep ($n = 19$) were dried, homogenized, weighed (0.7 mg) and encapsulated. The plants were washed with flow water, and faeces from southern screamer ($n = 42$), cattle ($n = 15$), sheep ($n = 28$) and plants were oven-dried for 48 h at 60 °C, macerated with a mortar and pestle, weighed (3.0 mg) and encapsulated.

The samples were analysed with an isotope ratio mass spectrometer (IRMS) coupled to an elemental mass analyser at the Center for Stable Isotopes at the University of New Mexico (UNM-CSI). The stable isotope composition of the samples was expressed with delta notation (δ), represented as parts per thousand (‰). This approach indicates the differences in the stable isotope values of the sample in comparison to the standards of carbon (Vienna Pee Dee Belemnite - VPDB) and nitrogen (atmospheric air) given by Eq. (1), from Bond and Hobson (2012):

$$\delta^{13}\text{C or } \delta^{15}\text{N (‰)} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \quad (1)$$

2.8. Data analysis

Whenever possible, the variations in the stable isotope blood values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ obtained from animals in different seasons were compared for each animal group through *t*-tests. Normality and homoscedasticity were tested through Shapiro-Wilk and Bartlett's tests, respectively, in the R environment. When premises were not fulfilled, the nonparametric Mann-Whitney *U* test was used.

Dietary quantification was performed by numeric counting and area measurements estimated through microhistological analysis. In addition, the frequency of occurrence (%FO) as well as the numeric prey-specific proportion (%PN) and the prey-specific proportion within the area of each fragment (%PA) were calculated, in substitution to the weight percentage (%PW) in the original equation of Brown et al. (2012). These parameters were integrated into the prey-specific index of relative importance (%PSIRI), as proposed by Brown et al. (2012).

Bayesian isotope mixing models (MMs) were used for blood samples to estimate the contribution of the food items assimilated by native and non-native herbivores with the *simmr* package (Parnell and Inger, 2016) in the R environment. Food-consumer trophic discrimination factors (TDFs) in the blood of consumers were selected based on similar diets from closely related taxonomic groups. The trophic discrimination factor (TDF) values used in the blood mixing models for cattle and sheep were $2.5 \pm 0.3\%$ and $3.70 \pm 0.2\%$ for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, respectively. These TDF values were obtained in an experimental study in domestic reindeer (*Rangifer tarandus*), an ungulate herbivore (Halley et al., 2010). The food-tissue TDF values for the southern screamer were $3.6 \pm 0.52\%$ and $-0.5 \pm 0.62\%$ for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, respectively, as obtained for Anseriformes herbivores (Hahn et al., 2012). Potential food items were grouped in plant functional groups (e.g., legume, grass) to estimate the contribution of different sources assimilated for each animal group in the mixing models. The species that compose each group are indicated in Appendix A, Table A.2.

Isotopic values from the blood and faecal samples from herbivores were compared with the SIBER package (Jackson et al., 2011). The niche overlap and the overlap area were calculated for each pair of animals by using the standard ellipse area corrected for small sample size (SEAc), and the *maxLikOverlap* function was employed to obtain the overlap percentage (Jackson et al., 2011). Faecal isotopic values were corrected for TDF discrimination. The TDF values used for faecal samples from cattle and sheep were based on estimates previously obtained for each species: $\delta^{13}\text{C} = -1.0\%$ and $\delta^{15}\text{N} = 2.0\%$ (Sponheimer et al., 2003; Steele and Daniel, 1978) and $\delta^{13}\text{C} = -0.45\%$ and $\delta^{15}\text{N} = 3.0\%$ (Sutoh et al., 1993), respectively. For the southern screamer, the values were estimated for faecal samples of birds in general ($\delta^{13}\text{C} = 1.15\%$ and $\delta^{15}\text{N} = 2.91\%$), as reviewed by Caut et al. (2009).

For metabarcoding, the relative read abundance (RRA) was used to estimate the representation of reads in the diet of herbivores, as described by Deagle et al. (2019). As the *psbA-trnH* marker is normally used in combination with other markers in diet studies (Ando et al., 2020), we tested the reliability of this individual marker through correlations between RRA of C4 plants and $\delta^{13}\text{C}$ values in faecal samples (Appendix B, Fig. B.1). The correlation was positive (Pearson $\text{cor} = 0.34$; $p < 0.05$) (Appendix B, Fig. B.1), confirming that dietary estimates of herbivores using RRA of *psbA-trnH* provides reliable information.

To evaluate the trophic niche overlap of herbivores based on the plant composition in the diet, the Morisita-Horn index was calculated

for each pair of consumers according to the equation proposed by Horn (1966) based on quantitative data from microhistology analysis and RRA. The values of this index range between 0 and 1, where values near 1 indicate higher overlap.

The differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values among groups were tested through one-way ANOVA and with the post hoc Tukey's HSD test in the R environment. These data indicated the use of resources on the same time scale as the other techniques applied to faecal samples.

Finally, the trophic interactions among the herbivores were analysed through network links using the PSIRI%, RRA and SIA results from faeces. For SIA, MMs of faeces were generated to obtain the values that were converted to network parameters (Appendix A, Table A.3) according to Bosenbecker and Bugoni (2020). Nestedness and modularity were tested for each technique according to the NODF metric and the QuanBiMo (Q) algorithm, respectively. Null models were used to test the significance of both indexes. All the trophic network interaction analyses were performed in the *bipartite* package (Dormann et al., 2008).

3. Results

3.1. Diet based on microhistology

A total of 34,967 plant fragments belonging to both Monocotyledoneae and Eudicotyledoneae were identified. Grasses (including *Leersia hexandra*, *L. peruviana* and unidentified Poaceae) were the most common category in the diet of the southern screamer, sheep and cattle (FO% = 85.3, 100 and 100, respectively), followed by sedges such as *Eleocharis* sp. (FO% = 93.7, 58.8 and 100) (Appendix A, Table A.4). For the southern screamer, 17 plant species from aquatic and terrestrial families were identified, mainly *Trifolium* sp. (PN% = 30.5) and *Eichhornia* sp. (PN% = 24.3). For cattle, 16 plant species were identified, with a major contribution of grasses and sedges such as *L. peruviana* (PN% = 33.1) and *Eleocharis* sp. (PN% = 32.9). For sheep, the main contributing taxa were *Eleocharis* sp. (PN% = 35.7) and unidentified Poaceae (PN% = 31.3), which were found in all samples analysed.

The contribution related to %PSIRI indicated Eudicotyledoneae as the main food resource for the southern screamer. Among the resources identified at the species level, *T. repens* was the main species recorded (% PSIRI = 22.1), and forbs predominated in the diet of this species (Fig. 1A). Unidentified Poaceae also presented a significant contribution

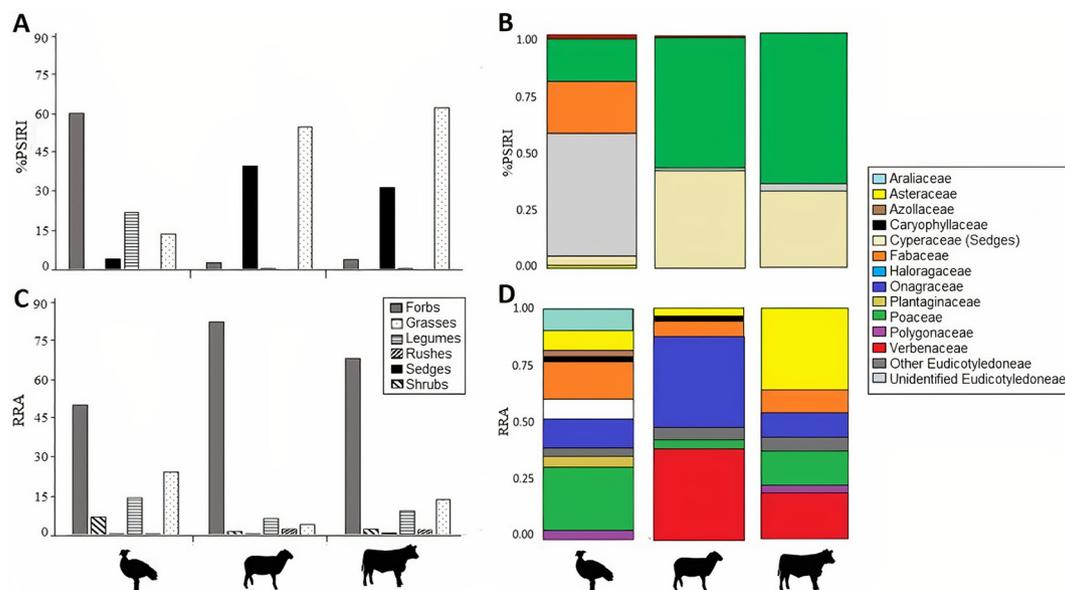


Fig. 1. Contribution of resources by functional plant groups and families of plants in the diet of the southern screamer, sheep and cattle in southern Brazil. (A and B) prey-specific index of relative importance (%PSIRI) inferred from microhistological analysis; (C and D) relative read abundance (%RRA) based on metabarcoding data.

to the diet of the southern screamer (%PSIRI = 11.7). In cattle and sheep, the sedge *Eleocharis* sp. (%PSIRI = 31.1 and 35.5, respectively) and unidentified Poaceae (%PSIRI = 21.5 and 31.6, respectively) predominated (Fig. 1A–B; Appendix A, Table A.4).

3.2. Diet inferred by metabarcoding

A total of 122 plant species belonging to 41 families were identified in all faecal samples. Metabarcoding analysis revealed greater differences among herbivores regarding the plant families consumed (Appendix A, Table A.5 and A.6; Fig. 1D). The southern screamer diet was composed mainly of Poaceae (RRA = 25.2) and Fabaceae species (RRA = 14.9), but aquatic plants were also present (e.g., *H. bonariensis*, RRA = 8.6). Onagraceae species, particularly *Ludwigia grandiflora*, were consumed by all herbivores with high predominance in the sheep diet (RRA = 38.6). In cattle, Asteraceae and Verbenaceae were the most

consumed taxa (RRA = 34.0 and 19.1, respectively). In relation to plant functional groups, forbs predominated in the diets of all herbivores (Fig. 1C), with a higher contribution in sheep (RRA = 86.2). Legumes were mostly consumed by the southern screamer and cattle (RRA = 14.8 and 9.5, respectively). In contrast, sedges and rushes presented a low contribution in all animals (Fig. 1C).

3.3. Diet inferred by SIA

The mean $\delta^{13}\text{C}$ values from blood ranged from $\delta^{13}\text{C} = -28.3$ to -23.4‰ for southern screamer, $\delta^{13}\text{C} = -24.3$ to -17.7‰ for sheep and $\delta^{13}\text{C} = -20.4$ to -17.1‰ for cattle. For nitrogen in blood, the values ranged from $\delta^{15}\text{N} = 3.2$ – 10.2‰ in the southern screamer, $\delta^{15}\text{N} = 8.2$ – 10.6‰ in sheep and $\delta^{15}\text{N} = 6.3$ – 9.2‰ in cattle (Appendix A, Table A.2). The carbon isotope values in the blood of all herbivores did not differ between sampled periods. However, $\delta^{15}\text{N}$ values

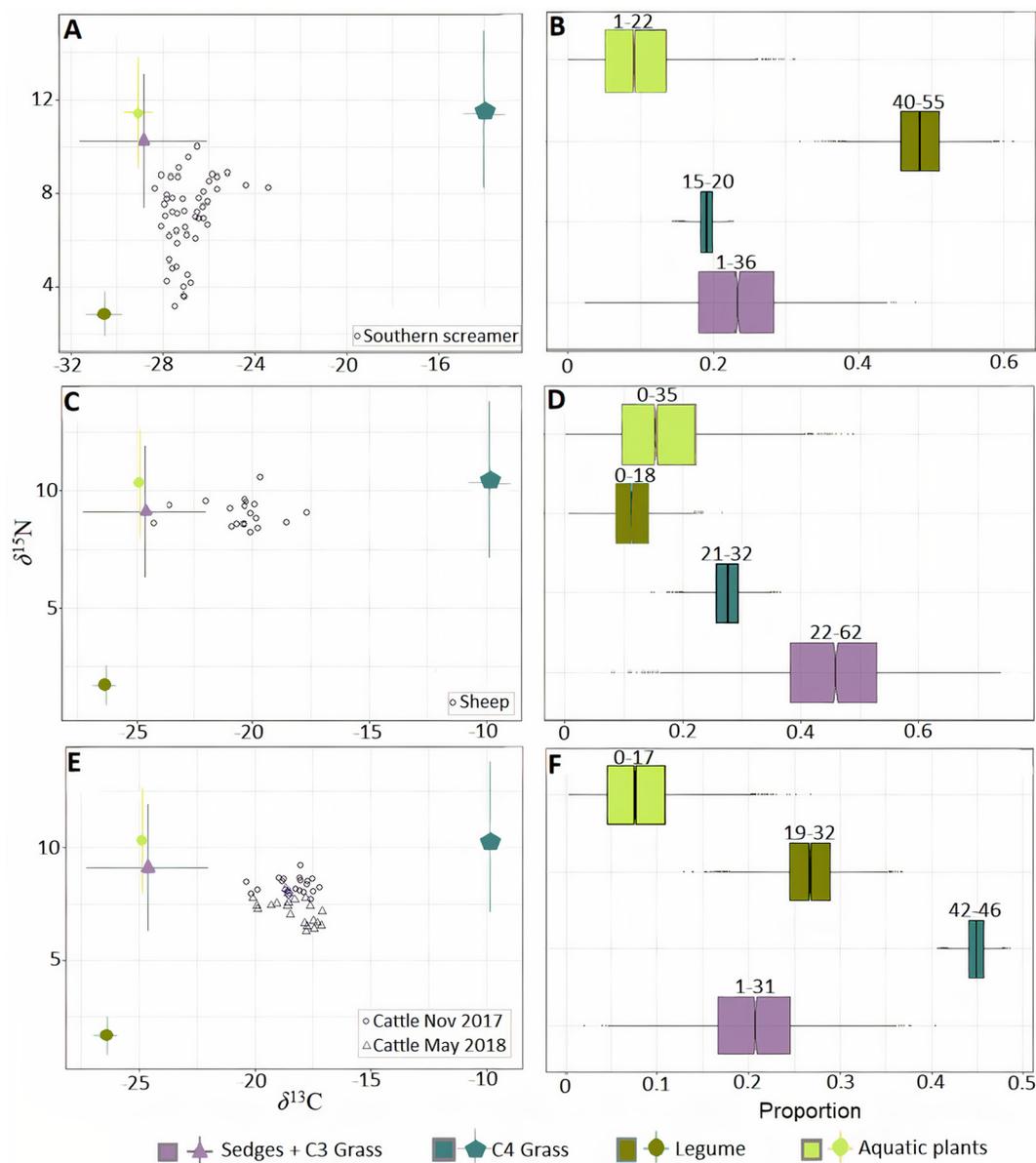


Fig. 2. Values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (‰) in the blood of the herbivorous southern screamer, sheep and cattle (empty symbols) and potential plant items (full symbols) sampled in southern Brazil (A, C, E), with their respective estimated contributions in the diet (B, D, F). The intervals of credibility of 95% (lines) and 50% (coloured symbols) were used in stable isotope mixing models generated with the *simmr* package. From the potential plants, forbs were represented by legume and aquatic plants, while sedges, C3 and C4 grass, represent the graminoids. Legumes were represented by the genus *Trifolium* sp. whereas *Eichhornia crassipes* and *Hydrocotyle bonariensis* represent aquatic plants.

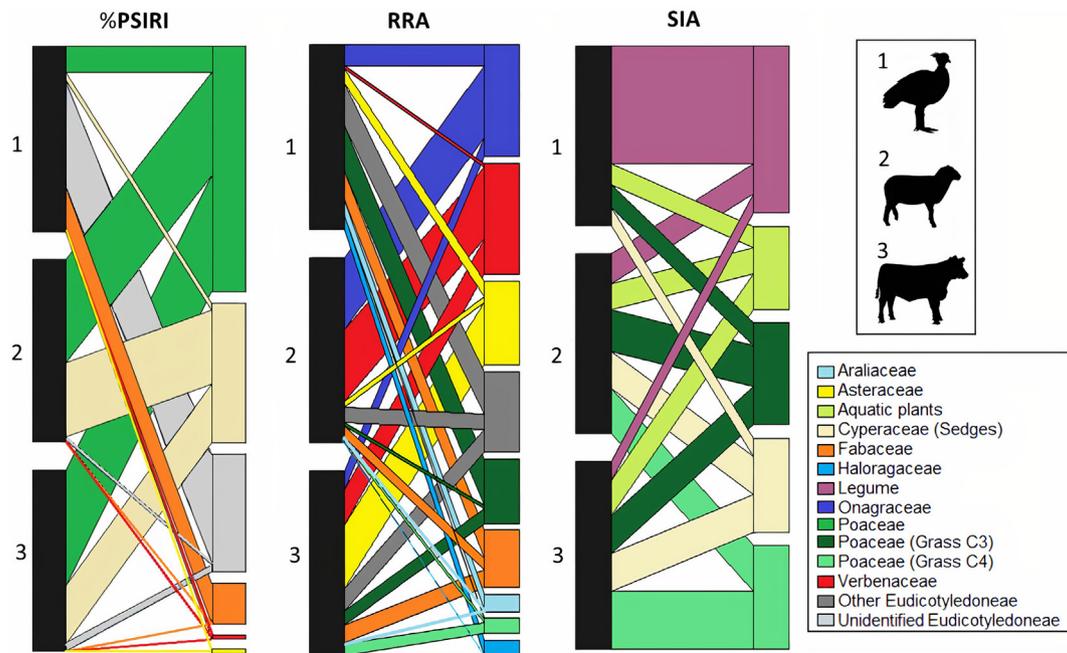


Fig. 3. Bipartite networks of the use of plants in the diet of the herbivorous southern screamer, sheep and cattle sampled in southern Brazil, as analysed by microhistological (%PSIRI), metabarcoding (RRA) and stable isotope analysis (SIA) in faeces. Colours indicate different plant families found in the diet, and the links represent the strength of the link, i.e. the use of these resources by each herbivore. Aquatic plants were represented by *Eichhornia crassipes* and *Hydrocotyle bonariensis*. Legumes encompass representatives of the genus *Trifolium* sp., while Fabaceae encompasses all members of the family, to the exclusion of *Trifolium* sp. The most consumed families by herbivores are indicated, while the remaining species were grouped under "Other".

determined in cattle blood varied between sampling seasons ($t = 6.9$; $df = 41$; $p < 0.05$; Fig. 2E), while in the southern screamer and sheep the values were similar between periods.

The Bayesian MMs indicated the importance of different food sources in the southern screamer, with legumes constituting the bulk of the assimilated food (Fig. 2A–B). A combination of sedges and C3 Poaceae represented the main resources utilized by sheep (Fig. 2C–D). In cattle, C4 Poaceae predominated in both sampling seasons (Fig. 2E–F).

In faeces, $\delta^{13}\text{C}$ values ranged from -30.9 to -24.7‰ for the southern screamer, -25.7 to -17.3‰ for sheep and -25.3 to -16.6‰ for cattle. The nitrogen content ranged from 2.7 to 10.3‰ for the southern screamer, 4.4–7.8‰ for sheep and 4.1–8.4‰ for cattle (Appendix A, Table A.2). The carbon stable isotope values in faecal samples indicated the predominant use of C3 plants by the southern screamer, while in both non-native herbivores, carbon values intermediate to those for the C3 and C4 pathways were found (Appendix B, Fig. B.2). The nitrogen values in faecal samples did not differ among the herbivores, while Tukey's HSD indicated a significant pairwise difference between the southern screamer and non-native herbivores for $\delta^{13}\text{C}$ values (ANOVA: $F = 102.7$; $p < 0.05$).

3.4. Trophic interactions and overlap between herbivores

The bipartite network of the herbivores revealed the use of species from different families by the three consumers (Fig. 3). Sheep mostly used Cyperaceae and Poaceae according to the %PSIRI-based analysis and Verbenaceae and Onagraceae according to the RRA-based analysis. The PSIRI-based network for cattle indicated that Poaceae made the highest contribution, while Asteraceae was the most important resource according to the RRA-based analysis. The network generated for the southern screamer indicated a high contribution of Eudicotyledoneae. In addition, the SIA-based bipartite network indicated the main use of legumes by the southern screamer and C4 plants by cattle. Nestedness was not found under any of the three techniques (PSIRI = 27.8, 95% IC = 23.9–35.6; RRA = 46.8, 95% IC = 34.9–64.3; SIA = 11.5, 95% IC = 8.6–39.5), whereas modularity was detected for

all techniques (PSIRI = 0.4, 95% IC = 0.02–0.07; RRA = 0.3, 95% IC = 0.07–0.2; SIA = 0.2, 95% IC = 0.1–0.19) (Appendix B, Fig. B.3).

The Morisita-Horn index based on microhistology indicated high dietary overlap between the non-native mammals (CH = 0.93) and minor overlap between the southern screamer vs. non-native animals (CH = 0.04 and 0.02). In contrast, the RRA-based analysis indicated low overlap between herbivores, with cattle and sheep presenting the highest, but still limited, overlap (CH = 0.37; Table 1).

The southern screamer exhibited nonoverlapping isotopic niches in comparison with non-native herbivores according to the blood analysis, while sheep and cattle presented limited isotopic overlap with each other. The widest isotopic niche breadth for blood samples was found for the southern screamer ($\text{SEAc} = 5.05$), followed by sheep and cattle ($\text{SEAc} = 2.85$ and 1.93, respectively), with the sheep niche presenting 44.2% overlap with that of cattle (Table 2, Fig. 4A). Regarding the isotopic-based niches detected in faecal samples, the southern screamer presented limited overlap with non-native herbivores, sharing ~13% and ~23% of the niche with cattle and sheep, respectively. Nevertheless, high overlap was found between sheep and cattle, with a 51.5% overlap area (Table 2, Fig. 4B).

4. Discussion

The food resource partitioning analysis between the native bird and two non-native herbivores showed a clear division of resources with

Table 1

Pairwise values of the Morisita-Horn index (CH) showing the range of overlap in the diet of the three herbivorous species (southern screamer, sheep and cattle) sampled on the southern Brazilian coastal plain, as inferred by microhistological quantification (lower diagonal) and metabarcoding reads (upper diagonal).

	Southern screamer	Sheep	Cattle
Southern screamer		0.30	0.16
Sheep	0.02		0.37
Cattle	0.04	0.93	

Note: Most important overlap values are in bold text.

Table 2

Standard ellipse area calculated for small sample sizes (SEAc), overlap area (%²), proportion (%) of overlap between the southern screamer, sheep and cattle and the overlap percentage (%) measured from the stable isotope values of blood and faecal samples on the southern Brazil coastal plain.

Herbivore 1 (Herb1)	Herbivore 2 (Herb2)	Tissue	SEAc	Overlap area (% ²)	% Herb1 area	% Herb2 area	% Overlap
Southern screamer	Sheep	Blood	5.05	0	0	0	0
Southern screamer	Cattle	Blood	1.93	0	0	0	0
Sheep	Cattle	Blood	2.85	5.13	44.18	29.98	21.74
Southern screamer	Sheep	Faeces	9.36	9.28	23.15	32.05	10.30
Southern screamer	Cattle	Faeces	7.23	4.01	13.85	13.65	3.47
Sheep	Cattle	Faeces	10.59	36.94	85.27	60.81	51.49

Note: Most important overlap values are in bold text.

minimum niche overlap. The combination of techniques enabled us to evaluate the diet from different perspectives, overcoming the limitations of each technique. Microhistological analysis allowed the determination of general plant contribution to the diet of each herbivore. Even so, resolution at the species level was not possible for several plant families. Conversely, metabarcoding analysis also indicated the distribution of resources but expanded the list of groups consumed, allowing resolution at the genus/species level. SIA of faecal samples showed similarity of the diet between sheep and cattle and the predominant use of C3 plants in the diet of the southern screamer. In addition, the isotopic niche based on faecal samples revealed limited overlap when the non-native herbivores were compared to the southern screamer. Finally, when the isotopic niche from effectively assimilated food was measured, cattle and sheep presented limited niche overlap. Furthermore, the isotopic values in cattle blood varied between sampling seasons.

4.1. Dietary inference in the southern screamer, cattle and sheep: the role of complementary diet techniques

The combination of methodologies revealed differences in the preferential use of resources between the native bird and two non-native

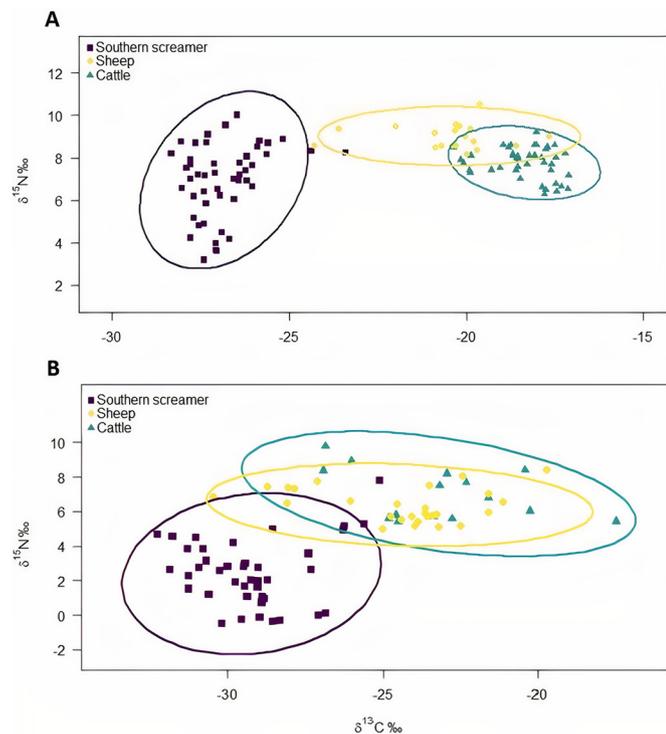


Fig. 4. Isotopic niche of the herbivorous southern screamer, sheep and cattle in southern Brazil, represented in δ -space, based on the 95% standard ellipse area for small sample size (SEAc) ellipse values, modelled using Stable Isotope Bayesian Ellipses in R (SIBER). (A) Isotopic niche based on blood values; (B) isotopic niche based on faecal values.

herbivores, indicating greater similarity between livestock species. The $\delta^{13}\text{C}$ values from faecal samples indicated that sheep and cattle used plants with values intermediate to those for the C3 and C4 pathways (indicative of a mixed diet) while in the southern screamer diet, the use of C3 plants predominated. Despite differences related to blood sampling periods of southern screamer in comparison to sheep and cattle, no significant differences were found in the isotopic values of each species in different periods. In this way, samplings carried out in different seasons of the year seemed comparable for the purpose of the current study. This result contrasts with another comparison of diet among other large herbivore rodents at the same region (Espinelli et al., 2017), which detected significant differences between seasons, and attributed this to seasonal changes in plant diversity. Thus, monitoring the diet of herbivores on a larger scale may result in eventual variations in diet among the different seasons, although this was generally not the case for the current study.

Besides the predominance of C3 plants in the diet of the southern screamer, results also indicated a limited sharing of plants with livestock throughout the year. The use of C3 grasses by the southern screamer was also inferred by other methods, which indicated a high contribution of Poaceae. This was supported, for example, by the high contribution of this group of plants in isotopic mixing models based on blood samples. Despite presenting a niche close to that of C3 plants, the southern screamer revealed to be quite generalist, although it seems to prefer plants with high nutritional quality. The low digestibility of fibre leads some Anseriformes to adopt a food throughput time strategy (Mayhew and Houston, 1993) in which animals select plants with high quality. This reduces the food transit time along the digestive tract but increases foraging time. As a member of the Anseriformes inferred to exhibit an inefficient digestive system, this strategy also seems to be used by the southern screamer.

In the southern screamer diet, forbs were the most important resource assimilated, with legumes such as *T. repens* presenting a major contribution. This type of forage is commonly cultivated for livestock feeding in the Pampas biome due to its resistance to cold winters and frost (Paim and Riboldi, 1994) and together with other C3 plants, provide pastures with high nutritional quality (Barbehenn et al., 2004). This preference for forbs was previously reported in other large birds, such as pink-footed (*Anser brachyrhynchus*) and barnacle (*Branta leucopsis*) geese, on the basis of an experimental preference test with eight plants (Owen, 1979). Similarly, it was previously reported that farms with pastures improved by *T. repens* usually attract geese, which share these environmental areas with livestock due to the availability of high-quality grasslands (Mason et al., 2018).

Grasses were also frequently identified in the microhistological and metabarcoding analyses of the southern screamer diet. The high abundance (RRA = 25.2) of Poaceae (mainly the Peruvian watergrass *L. peruviana*; Appendix A, Table A.5–6) revealed by metabarcoding analysis suggests a key role of this bird in the area: this watergrass is a native aquatic macrophyte that easily colonizes shallow ponds, where it reduces water quality due to the input of nutrients and eutrophication (Sponchiado, 2008). Irrigated rice cultivation increases the areas where this macrophyte occurs, and alternative biological control

strategies involving the introduced grass carp (*Ctenopharyngodon idella*) have been tested (Sponchiado and Schwarzbald, 2009). Data from metabarcoding analysis complemented information based on microhistological analysis, confirming the potential of the southern screamer for use in the biological control of aquatic weeds. Some Anseriformes in Argentina also consume weeds, which contributes to environmental management and improves plant quality for other herbivores, including livestock (Gorosábel et al., 2019). The use of metabarcoding has already enabled tracing the ecological role of the prairie grouse, *Tympanuchus pallidicinctus*, in North America, revealing dietary items not described by conventional analysis (Sullins et al., 2018).

The sheep and cattle diets were composed mainly of Poaceae, Cyperaceae, Verbenaceae, Onagraceae and Asteraceae in different proportions and seemed to be based on both C3 and C4 plants (i.e., grasses, sedges and forbs). For sheep, sward shape seems to be a factor in plant selection (Pallarés et al., 2005), as their diet was composed mainly of *Eleocharis* sp. (Cyperaceae) and *L. grandiflora* (Onagraceae), both of which exhibit a low height. The presence of these families was detected only because of the simultaneous use of complementary methods since Onagraceae was not identified in the microhistological analysis, while Cyperaceae was not found in the metabarcoding analysis. However, the overall pattern identified was similar under the two methods and showed selective feeding by sheep. SIA showed a high contribution of sedges and C3 grasses, while the %PSIRI results confirmed that sedges presented an important contribution to the diet of sheep.

Conversely, cattle seem to exhibit a more generalist diet, with some differences in the abundance of families in comparison with sheep, mainly including Poaceae and Asteraceae. Some non-grasses, such as forbs, complement the cattle diet, providing a protein-rich diet (Odadi et al., 2013). Thus, SIA showed a high contribution of C4 plants assimilated in blood, while some forbs were detected by other methods. In addition, the significant difference in $\delta^{15}\text{N}$ between colder vs. warmer seasons may indicate the differential use of resources throughout the year, which may be potentially related to seasonal variations in temperature and the plants growing in different periods (Espinelli et al., 2017). However, factors such as differences in root depth (Ambroise, 1991) and changes in soil nitrogen values derived from commercial fertilizers (Shearer et al., 1978) may be involved in the changes in $\delta^{15}\text{N}$ values throughout the year.

In general, the complementary use of techniques in this study allowed the elucidation of the feeding habits of three herbivores from different perspectives. Although microhistology did not allow identifying all taxons at the species level, it allowed to determine the functional group of each species. Nevertheless, the need for reference images (Castellaro et al., 2007) hinders the straightforward application of this technique. One of the main differences observed under this technique was the high representativeness of Cyperaceae, which was detected infrequently by metabarcoding. In fact, this family shows a low resolution with metabarcoding even when more established markers such as internal transcribed spacer (ITS) sequences are used (see Supplementary material in Kartzinel et al., 2015). This makes the description of the diet of herbivores feeding on Cyperaceae obtained by the use of metabarcoding alone incomplete. However, metabarcoding presents an improved resolution, allowing the identification of families and species showing poor digestive preservation or with similar epidermal structures (such as some dicots). In Poaceae for example, only this technique allowed us to infer the important ecosystem service provided by the southern screamer in southern Brazilian grasslands through the consumption of *L. peruviana* (see above). Moreover, the results revealed here show that species of Onagraceae, which were not detected through microhistology, were among the most commonly shared resources in all three herbivores, especially between the southern screamer and sheep.

Discrepancies between the results obtained by microhistology and metabarcoding were also found by Soinenen et al. (2009) and Ando et al. (2013). In these studies, the low taxonomic resolution of

microhistology was the main source of incongruence between techniques, hampering further quantitative comparisons. Nevertheless, this study also shows important limitations of metabarcoding in recovering an accurate description of the whole herbivore diet. Since the combined use of different markers is frequently mentioned as the best strategy for metabarcoding studies related to herbivores diet (Ando et al., 2013; Willerslev et al., 2014; Kartzinel et al., 2015), the sole use of *psbA-trnH* in this study may be invoked as the main source of bias. Nevertheless, the positive results of the pilot amplification test and the positive correlation between metabarcoding (RRA of C4 plants) and SIA ($\delta^{13}\text{C}$) in herbivore faeces confirms that this marker passes minimal requirements for the quantitative identification of the plant species consumed by herbivores. Moreover, the use of a single marker is justifiable under the integrative strategy adopted by this study, which benefits from a comparative approach among microhistology, metabarcoding and SIA, instead of different metabarcoding markers.

Despite the differences between the plants found by microhistology and metabarcoding, both analyses allowed the inference of resources consumed by the target species, in a way that would not be possible under the sole use of a single technique. Moreover, such a priori diet information is required to obtain precise proportions of the diet of each species under SIA, as well as to choose appropriate TDFs for each group and tissue analysed. In fact, SIA does not allow determining the species consumed (Phillips et al., 2014). Thus, our results obtained by SIA, both for blood and faeces, complement the microhistology and metabarcoding approaches providing a wider picture of diet and niche overlap patterns.

4.2. Niche partitioning between the southern screamer and non-native herbivores

The identification of the widest niche breadth for the southern screamer via SIA ($\text{SEAc} = 5.05$) indicates that this bird uses the widest range of plant resources among the herbivores analysed. Concerning patterns of niche partitioning, all techniques suggested limited overlap between the southern screamer and the non-native herbivores, with the higher values of niche overlap (of the order of 0.30) being detected by metabarcoding in the comparison between the southern screamer and sheep. This is explained by differential samplings provided by the techniques, with the most commonly shared resource, Onagraceae, being detected only by metabarcoding, and one of the most differentially consumed resources, Cyperaceae, being detected only by microhistology.

Nevertheless, in the comparisons involving sheep and cattle, microhistological analysis and SIA suggested high overlap, while metabarcoding indicated limited overlap. As different plant species may be grouped into larger taxonomic/functional groups in microhistology, or according to similar isotope values in SIA, this result is possibly related to the higher resolution provided by metabarcoding. Even so, the modularity recovered in the bipartite networks also indicates that all herbivores studied use different subsets of resources. The diversity and high quality of the forage species in the Pampas grasslands (Andrade et al., 2019) surely provide opportunities for niche segregation. Morphological factors such as body and mouth size and subtle changes in the digestive systems of ungulates (Hanley, 1982) could explain the observed differences in diet. In addition, the proximity of the studied farmlands with natural environments enables southern screamer to forage in natural areas, which could contribute to some extent to the niche segregation with livestock.

In summary, the three techniques produced low values of niche overlap between the native southern screamer and domestic grazers, and a modular pattern of the bipartite networks. In addition to differences in dietary preferences, they suggest the use of different subsets of resources by each herbivore. Studies in Argentinean farmland have shown that birds do not negatively impact grazing by livestock and may even contribute to the maintenance of environmental conditions and grassland quality (Gorosábel et al., 2019), similar to the pattern found here. Therefore, the present study showed that the integrated

use of techniques for dietary analysis revealed important ecological aspects and resolved apparent conflicts that may threaten the persistence of native species. Thus, farmers can be reassured: the negative impacts inflicted by the southern screamer on domestic animals seem to be negligible, and this species does even provide important ecosystem services through the biological control of aquatic weeds, in irrigated systems.

Thus, besides characterizing the diet and niche overlap patterns presented by three herbivores in southern Brasil, our study provided important information for the conservation of the native species. Such efforts will probably involve the scientific divulgation of findings and investment on programs of environmental information, promoting people awareness on the absence of damage to livestock and the ecosystem benefits provided by the southern screamer. Nevertheless, there are still open questions, e.g. individual specialization and ontogenetic variations in the use of resources by the southern screamer and the resource sharing with other native herbivores, aquatic and terrestrial, occurring in the region. Moreover, it would be important to compare the diet of the target species under different conditions and regions, focusing on patterns at larger ecological and geographical scales.

Authors' contributions

C.N.F., L.J.R. and L.B. designed the study. C.N.F. conducted sampling and analyses under the guidance of L.B. and L.J.R. C.N.F. wrote a first draft of the manuscript, and all authors revised and approved the manuscript.

Declaration of competing interest

We declare that there is no conflict of interest for this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fooweb.2021.e00201>.

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