Uncovering trophic interactions through metabarcoding to understand community assembly of insular terrestrial vertebrates

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Dedicated to Manuel Pinho

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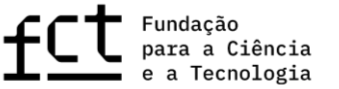

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Resumo

Os sistemas insulares de zonas áridas remotas são modelos excepcionais para o estudo de processos ecológicos e evolutivos. São geralmente sistemas simplificados que albergam taxa únicos com características ecológicas e morfológicas notáveis. No entanto, estas espécies estão frequentemente ameaçadas e são pouco conhecidas, o que as torna mais vulneráveis à extinção, principalmente devido às alterações climáticas e à intervenção humana. Por conseguinte, o estudo das redes ecológicas das mesmas é importante para o desenvolvimento de planos de conservação precisos. O *metabarcoding* de ADN, com capacidade de fornecer dados de dieta com alta resolução de forma eficiente e rápida, é particularmente poderoso e valioso para estudar ecossistemas remotos que são mais vulneráveis a estas ameaças, como as zonas áridas. Por conseguinte, o principal objectivo desta tese foi utilizar o poder desta técnica para revelar a composição da dieta de taxa de vertebrados terrestres a fim de descobrir interacções tróficas. Ao fazê-lo, esta tese almeja explorar os padrões evolutivos envolvidos na formação de comunidades em sistemas insulares e fornecer dados para a conservação sustentável de espécies ameaçadas.

O arquipélago de Cabo Verde serve como um laboratório natural simplificado, albergando pares de espécies de répteis endémicos descendentes de um único ancestral comum, com espécies simpátricas exibindo grande variação de tamanho e competindo por recursos limitados. Neste contexto, esta tese começa por usar o *metabarcoding* de ADN para estudar as relações tróficas e os mecanismos de coexistência de dois pares de espécies: um das osgas *Tarentola gigas* e *Tarentola raziana* nas ilhas do norte (ilha de Santa Luzia e ilhéu Raso) e um dos escincídeos *Chioninia delalandii* e *Chioninia vaillantii* nas ilhas do sul (ilha do Fogo e ilhéu de Cima). Isto revelou o papel da competição, e possivelmente do deslocamento de caracteres, na formação da variação da dieta e da morfologia destas espécies únicas. Ao mesmo tempo, o estudo das dietas das osgas produziu dados inesperados sobre a biodiversidade, fornecendo o primeiro registo de pseudoescorpiões para as ilhas Desertas. Além disso, esta tese realçou ainda mais o potencial do *metabarcoding* de ADN quando conteúdos digestivos antigos e degradados de espécimes de museu foram usados para descrever eficazmente a dieta do emblemático e extinto lagarto gigante de Cabo Verde *Chionina coctei* e para discutir as possíveis causas da extinção do mesmo.

Finalmente, a aplicabilidade destas técnicas para o estudo das interacções tróficas foi alargada a outros grupos e sistemas insulares. Foi avaliado o impacto do rato doméstico em Cabo Verde, mostrando o elevado impacto negativo deste na agricultura e nas

espécies de plantas nativas e a necessidade urgente de implementar medidas de controlo deste invasor no arquipélago. Além disso, para compreender se os mecanismos de partilha de recursos observados em Cabo Verde são comuns a outros sistemas áridos e isolados, a composição da dieta da comunidade de répteis de Wadi Ashar, na Arábia Saudita, foi estudada. Esta área enfrenta várias ameaças à biodiversidade, com o desenvolvimento recente a resultar numa acentuada destruição do habitat adequado para a herpetofauna. Este estudo revelou, mais uma vez, a influência significativa do tamanho do corpo dos répteis na escolha das presas e na partição dos recursos alimentares nesta comunidade vulnerável.

Em geral, este trabalho ajudou a preencher várias lacunas de conhecimento, fornecendo informações importantes sobre os mecanismos de coexistência de espécies em ambientes insulares áridos e as adaptações tróficas e morfológicas que lhes estão subjacentes. Forneceu também novos registos de biodiversidade, um novo entendimento sobre a ecologia de espécies anteriormente pouco estudadas e/ ou ameaçadas de áreas remotas, discutiu possíveis ameaças que podem contribuir para o aumento do risco de extinção das espécies e destacou o impacto negativo de espécies invasoras de mamíferos em ecossistemas insulares. Em última análise, esta tese fornece informações importantes para o desenvolvimento de estratégias de gestão sistemática para a conservação em zonas áridas, remotas e insulares. Por exemplo, considerações para a reintrodução sustentável de *T. gigas* em Santa Luzia, informações sobre os possíveis factores de extinção que actuam sobre as espécies de répteis existentes em Cabo Verde, a importância de erradicar o rato doméstico em pequenas áreas protegidas e elementos-chave para a gestão sustentável da comunidade de répteis de Wadi Ashar.

No entanto, e tendo em conta as várias limitações do presente trabalho, muito há ainda a fazer nestes fascinantes ecossistemas que oferecem oportunidades ímpares para explorar dinâmicas ecológicas e evolutivas com relevância global mais alargada. São necessários estudos a longo prazo abrangentes para preencher as lacunas no conhecimento ecológico básico das espécies apresentadas e de outras que também estão em risco de extinção. Isto é essencial para proteger a biodiversidade única destas áreas.

Palavras-chave: Genética da conservação, evolução, ecologia trófica, análise da dieta, *metabarcoding*, deslocamento de caracteres, competição, partição de recursos, morfologia, répteis, espécies introduzidas, rato doméstico, Macaronésia, Áreas Protegidas, áreas remotas, Cabo Verde, Arábia Saudita.

Abstract

Insular systems from remote arid areas are exceptional models for the study of ecological and evolutionary processes. They are often simplified systems harbouring unique taxa with remarkable ecological and morphological characteristics. However, these species are often threatened and poorly known, making them more vulnerable to extinction, mainly due to climate change and human intervention. Therefore, the study of their ecological networks is important for the development of accurate conservation plans. DNA metabarcoding, with its ability to provide high-resolution dietary data efficiently and rapidly, is particularly powerful and valuable for studying remote ecosystems that are more vulnerable to these threats, such as arid areas. Therefore, the main objective of this thesis was to use the power of this technique to reveal the dietary composition of terrestrial vertebrate taxa in order to uncover trophic interactions. In doing so, this thesis aimed to explore the evolutionary patterns involved in shaping community assembly in insular systems and to provide data for the sustainable conservation of threatened species.

The Cabo Verde Archipelago serves as a simplified natural laboratory, hosting a groups of endemic reptile species descended from a single common ancestor, with sympatric species exhibiting wide size variation and competing for limited resources. In this context, this thesis starts by using DNA metabarcoding to study the trophic relationships and coexistence mechanisms of two species pairs: one of the geckos *Tarentola gigas* and *Tarentola raziana* in the northern islands (Santa Luzia Island and Raso Islet) and one of the skinks *Chioninia delalandii* and *Chioninia vaillantii* in the southern islands (Fogo Island and Cima Islet). This revealed the role of competition and the possibility of character displacement in shaping the dietary and morphological variation of these unique species. At the same time, the study of gecko diets has yielded unexpected biodiversity data, providing the first record of pseudoscorpions for the Desertas Islands. Moreover, this thesis further highlighted the potential of DNA metabarcoding when old and degraded digestive contents from museum specimens were used to effectively describe the diet of the emblematic extinct Cabo Verde giant skink *Chionina coctei* and to discuss the possible causes of its extinction.

Finally, the applicability of these techniques to study trophic interactions was extended to other groups and island systems. The impact of the house mouse in Cabo Verde was assessed, showing its high negative impact on agriculture and native plant species, and the urgent need to implement measures to control this invader in the archipelago. In addition, to understand whether the resource partitioning mechanisms observed in Cabo

Verde are common to other arid and isolated systems, the dietary composition of the reptile community of Wadi Ashar in Saudi Arabia was studied. This area faces several threats to biodiversity, with recent development resulting in severe destruction of suitable habitat for the herpetofauna. This study revealed, again, the significant influence of reptile body size on prey selection and the partition of food resources within this vulnerable community.

Overall, this work has helped to fill several knowledge gaps, providing important insights into the mechanisms of species coexistence in arid insular environments and the trophic and morphological adaptations behind them. It also provided new biodiversity records, a new understanding of the ecology of previously poorly studied and/ or threatened species from remote areas, discussed possible threats that can contribute to increased species extinction risk, and highlighted the negative impact of invasive mammalian species within insular ecosystems. Ultimately, this thesis provides important information for the development of systematic management strategies for conservation in arid, remote and insular areas. For example, considerations for the sustainable reintroduction of *T. gigas* to Santa Luzia, information on the possible extinction drivers acting on extant reptile species in Cabo Verde, the importance of eradicating the house mouse in small protected areas, and key elements for sustainable management of the Wadi Ashar reptile community.

However, and considering the several limitations of the present work, much still remains to be done in these fascinating ecosystems, which offer unparalleled opportunities to explore ecological and evolutionary dynamics that have broader global relevance. Long-term, comprehensive studies are needed to fill gaps in basic ecological knowledge for the species presented and others that are also at risk of extinction. This is essential to protect the unique biodiversity of these areas.

Keywords: Conservation genetics, evolution, trophic ecology, diet analysis, metabarcoding, character displacement, competition, resource partitioning, morphology, reptiles, introduced species, house mouse, Macaronesia, Protected Areas, remote areas, Cabo Verde, Saudi Arabia

Table of Contents

Acknowledgements ... ii

Resumo ... iv

Abstract ... vi

List of Tables ... x

List of Figures ... xii

List of Abbreviations ... xviii

Chapter 1. General introduction ... 1

1.1. Insular systems as models in ecology and evolution ... 2

1.2. True Island system: Macaronesia as a model ... 4

1.2.1. The Cabo Verde Archipelago ... 5

1.3. Island-like system: analogous models ... 11

1.4. Metabarcoding as a biodiversity survey tool ... 13

1.5. General objectives and thesis structure ... 16

References ... 20

Chapter 2. Metabarcoding as a tool to study trophic interactions ... 38

Article I. Table for two: diet composition differences of allopatric and sympatric populations of island geckos ... 39

Article II. Smaller islands, bigger appetites: evolutionary strategies of insular endemic skinks ... 75

Article III Diet study of geckos reveals the first records of pseudoscorpions on Desertas Islands (Cabo Verde) ... 119

Chapter 3. Metabarcoding as a tool to explore vertebrate extinction drivers ... 124

Article IV. Digging in a 120 years-old lunch: What can we learn from collection specimens of extinct species? ... 125

Chapter 4. Metabarcoding diet analysis of terrestrial vertebrates for conservation ... 147

Article V. Trust your guts? The effect of gut section on diet composition and impact of Mus musculus on islands using metabarcoding ... 148

Article VI. Green matters: Dietary assessment of a reptile community using DNA metabarcoding ... 170

Chapter 5. General Discussion 191

5.1. Main results and conclusions 192
5.2. Conservation implications 196
5.3. Limitations 198
5.4. Future studies 200
References 202

Appendix A – Supplementary Articles 209

Article A. Big and small: how do we fit all? Morphological evolution of sympatric and allopatric populations of small and giant island geckos 210

Article B. Diet of the exotic Madeiran wall lizard: first insights into trophic interactions in an Atlantic seabird sanctuary 241

Article C. Aliens on Boats? The Eastern and Western Expansion of the African House Gecko 250

Appendix B – Supplementary Reports 273

Report A. Medição do impacto do rato-doméstico nas plantas de Cabo Verde usando sequenciação de nova geração 274
Report B. Monitoring the environmental impact of the wind farm of Selada de Flamengo on reptiles of the insular plate of São Vicente – Final report 291

List of Tables

Chapter 2

Article I

Table 1. Results of univariate tests. The prey items that significantly ($p < 0.05$) contributed to the differences in diet composition between allopatric (Tz_ra vs Tz_SL) and sympatric (Tz_ra vs Tg_ra) populations, considering three taxonomic levels (Order, Family and MOTUs) are given.

Article II

Table 1. Results of pairwise comparisons. Multivariate comparisons in diet composition between the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s*, C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima), considering three taxonomic levels (Order, Family and MOTUs). Significant p-values are in bold and Dev stands for deviance values.

Article III

Table 1. Pseudoscorpion diversity of Cabo Verde Archipelago. Taxonomic details, distribution, values of pedipalpal femur length (FL) and width (FW), patella length (PL) and width (PW), chela length with pedicel (CL), chela width (CW), and fixed finger length (FiL) in mm, location – São Vicente (SV), Raso (Ra), Branco (Br), Santa Luzia (SL), São Nicolau (SN), Sal (S), Boavista (BV), Santiago (ST), Fogo (F), Brava (B), the mitochondrial marker sequenced and Morphobank and GenBank codes are also given. Samples marked with an (*) correspond to juveniles. Old records were retrieved from Arechavaleta et al. (2005), Vasconcelos *et al.* (2009), and Harvey (2013).

Chapter 3

Article IV

Table 1. Study specimens. Details of the *Chioninia coctei* vouchers rediscovered in 'Institut Océanographique' Fondation Albert Ier, Prince de Monaco. All specimens were collected on Branco Islet in 1901.

Chapter 4

Article VI

Table 1. Sampled herpetofauna of Wadi Ashar. List of taxa sampled ordinated by family and their codes, number of sampled individuals (N), of collected pellets (Np), of successfully sequenced pellets (Ns), average snout-vent length (SVL), body size category (class), activity period (Activity) and previous knowledge on diet composition (Diet) based on the literature: [1]Al-Sadoon et al. (2020); [2]Arnold (1980); [3]Arnold (1993); [4]Carranza et al. (2021); [5]Cottone and Bauer (2009); [6]Ibrahim and Busais (2016); [7]Ibrahim and El-Naggar (2013); [8]Kalboussi and Nouira (2004); [9]Ribeiro-Júnior et al. (2022); [10]Roobas and Feulner (2013); [11]Taylor et al. (2012); [12]Tsairi and Bouskila (2004); [13]Yadollahvandmiandoab *et al.* (2018). *Ptyodactylus* sp1 x sp3 and *Ptyodactylus* sp1 refer to two new species of the *Ptyodactylus hasselquistii* species complex in the process of description.

List of Figures

Chapter 1

Figure 1. The study area, the Cabo Verde Archipelago. A) Geographical location of the archipelago within Macaronesia. B) Map of the archipelago showing the islands clusters, elevation, and bathymetry (GEBCO Compilation Group, 2021). C) Island ages in million years (My) and the distribution of the reptile species and subspecies across the islands (Geographic Coordinate System, Datum WGS84).

Figure 2. The study reptile species of Cabo Verde. Map of the archipelago showing the geographic location and elevations of the islands (Geographic Coordinate System, Datum WGS 84). A) The *Tarentola* species pair from the Desertas. B) The *Chioninia* species pair from Rombo's Islets, Fogo and Santiago. All species are represented showing their size discrepancies. Dashed lines represent the species with the largest distribution of the pair and filled areas correspond to the sympatric areas of each species pair.

Figure 3. Map of the study area of Wadi Ashar. A) The geographic location of the Kingdom of Saudi Arabia and B) the AlUla County, with the location Wadi Ashar represented by a red dot. C) Wadi Ashar boundary is in red (Geographic Coordinate System, Datum WGS84). D) Representation of the different habitat types and anthropogenic structures found in this area (photographs by C.J. Pinho, R. Vasconcelos, S. Carranza and J. Šmíd).

Figure 4. Simplified overview of the metabarcoding workflow. A) Arid landscape representing examples of samples used for eDNA biodiversity surveys (Image generated using Wepik AI image generator); B–E) Laboratory procedures; F–G) Bioinformatic analyses and interpretation of the obtained data.

Chapter 2

Article I

Figure 1. Study area and species. A) Geographic location of the Cabo Verde Archipelago. B) Desertas Islands, depicting elevation and bathymetry. C) Habitat types and distributions of the three *Tarentola* populations (*T. raziana* from Santa Luzia in light blue, Tz_SL, from Raso in dark blue, Tz_ra, and *T. gigas* from Raso, Tg_ra in pink),

showing the number of faecal samples (N) extracted, and used in the analyses, respectively (Geographic Coordinate System, Datum WGS84).

Figure 2. Diet composition results for the three *Tarentola* populations (*T. raziana* from Santa Luzia, Tz_SL, from Raso, Tz_ra, and *T. gigas* from Raso, Tg_ra). A) Rank abundance curve plots, showing the percentages of the three more abundant diet item families for each population and the common prey items among them. Distinct colours are used to indicate families exclusively present in each population, white if shared by all populations and summed colour if shared by two of them. B) Accumulation curves depicting observed (full line) and extrapolated (dashed line) family richness with their 95% confidence intervals. C) Non-metric multidimensional scaling ordination (NMDS) of the diet composition. Points closer together correspond to samples with a more similar dietary composition. Ellipses represent the standard error of the dietary composition centroids at 95% confidence intervals.

Figure 3. Bipartite network representing associations among the three *Tarentola* populations (*T. raziana* from Santa Luzia, Tz_SL, from Raso, Tz_ra, and *T. gigas* from Raso, Tg_ra), and their diet items. The predators are on the left and arthropod (yellow), plant (green) and vertebrate (orange) prey families on the right. The width of the connecting bars represents the frequency of occurrence of each family in the pellets of each population. The symbol (*) indicates the families that contributed significantly to differences between sympatric (Tz_ra vs Tg_ra) and (¥) in both *T. raziana* (Tz_ra vs Tz_SL) populations. Only family names with a frequency of occurrence above one are mentioned.

Article II

Figure 1. Study area and study taxa. A) Geographic location of the Cabo Verde Archipelago (Geographic Coordinate System, Datum WGS84). B) Islands of Cabo Verde, depicting their elevation and indicating the two island groups: Windward (Santo Antão, São Vicente, Santa Luzia, São Nicolau, Sal, and Boavista) and Leeward (Maio, Santiago, Fogo, and Brava). C) Geographical distributions of the five sampled *Chioninia* populations (Cd, *C. delalandii* in green tones; Cvx, *C. vaillantii* in blue tones). Reptile taxa are represented showing their size discrepancies. Dashed lines represent the species with the largest distribution of the pair and filled areas correspond to their sympatric areas. The number of faecal samples (N) extracted, and used in the analyses, respectively, are also shown for each of the five populations (Cd.F.s, *C. delalandii* and

Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima).

Figure 2. Linear measurements taken for both *Chinonina* species. The measurements of the head included head length (HL), width (HW), and height (HH), eye-snout distance (ES), eye-ear distance (EE), and eye diameter (ED). Body measurements included snout-vent length (SVL), trunk length (TL), front (FLL), and hind limb length (HLL). The photos given as examples correspond to specimens of *Chioninia delalandii* (photographs by R. Vasconcelos).

Figure 3. Bipartite network representing associations among the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima) and their diet items. The populations are on the left and arthropod (yellow), plant (green) and vertebrate (orange) prey families are on the right. The width of the connecting bars represents the frequency of occurrence of each family in the pellets of each population. The symbol (*) indicates the families that contributed significantly to differences among populations. Only family names with a frequency of occurrence above two percent are mentioned. NI stands for non-identified families.

Figure 4. Diet composition results for the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima). A) Adjacency matrix summarizing the population subgroups according to their identified diet item orders. The species are represented proportionally according to their sizes along the vertical axis, and plant, arthropod and vertebrate orders are shown along the horizontal axis. Subgroups are delineated by yellow boxes. The darker the yellow squares the more frequent the association between a diet item and its predator. B) Accumulation curves depicting observed (full line) and extrapolated (dashed line) family richness with their 95% confidence intervals. C) Non-metric multidimensional scaling ordination (NMDS) of the diet composition at the family level. Points closer together correspond to samples with a more similar dietary composition. Ellipses represent the standard error of the dietary composition centroids at 95% confidence intervals.

Figure 5. Morphological analysis results considering the snout-vent length (SVL) adjusted values for the five *Chioninia* populations (Cd.F.s, C. delalandii and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima). A) Principal component analysis (PCA) of the SVL-adjusted linear measures. Each individual is represented by a circle in the PCA

space, and ellipses represent the 95% confidence intervals for each population. The contribution of the variables for the PCs is also represented. B) Results from Tukey's Honest Significant Difference (HSD) tests in SVL-adjusted linear measures (SVL, snout-vent length; HL, head length, HW, width and HH, height; ES, eye-snout distance; EE, eye-ear distance; ED, eye diameter; TL, trunk length; FLL front, and HLL, hind limb length) among populations. The horizontal bars represent the 95% confidence intervals for each comparison and the symbol (*) indicates the significant differences in mean levels among populations.

Article III

Figure 1. Pseudoscorpion diversity of Cabo Verde. (A) Map of the archipelago showing the previously recorded (circles) and new occurrences (other symbols) of pseudoscorpions across the islands; dashed ellipse highlights our study site, the Desertas Islands; occurrences of *Parolpium minor* not shown (see Table 1); (B) Maximum likelihood phylogenetic tree (GTRþG model) of available Olpiidae sequences; black dots indicate bootstrap values. 70%; (C) Photos of the pseudoscorpion specimens found on Desertas, Cabo Verde in 2016 and 2017 (colours and shapes on the left corner correspond to taxa and islands in the map): (I) *Garypus* cf. *saxicola* (found within a faecal pellet) from Raso Islet, Chã Branca (16.6177238N, 24.6022398W; specimen Tg_ra_07.80), dorsal view, (II) ventral view and (III) size detail of same specimen; (IV) *Olpium pallipes* from Raso Islet, Chã do Castelo (16.6171108N, 24.5866278W; specimen ra_Inv_91) dorsal view, (V) ventral view and (VI) pedipalp detail; (VII) *Olpium* sp. from Branco Islet, Ponta Parede – Tope Berta (16.650718N, 24,666078W; specimen Br_Inv_58), dorsal view; (VIII) *Olpium pallipes* from Santa Luzia Island (16.7648N, 24.7438W; SL_Inv_105), dorsal view, (IX) ventral view and (X) pedipalp detail; (XI) nymphs of *Olpium pallipes* from Santa Luzia Island (specimens SL_Inv_106 I and II), dorsal and (XII) ventral view.

Chapter 3

Article IV

Figure 1. Extant and extinct (†) *Chioninia* species of Cabo Verde Islands. Distribution map of the seven different endemic *Chioninia* species, with illustrations showing their remarkable size variation.

Figure 2. Ventral views of *Tachigonetria* nematodes identified from *Chioninia coctei*'s digestive contents (photos by V. Roca). A) *T. longicollis,* male caudal end (only *T. l. longicollis* is represented as differences between subspecies are difficult to depict); B) *T. numidica*, female; C) *T. macrolaimus,* male caudal end; D) *T. conica*, female.

Figure 3. Diet items detected in the stomach contents of the *Chioninia coctei* vouchers (photo by M. Dagnino). Items are represented until the higher taxonomic identification possible to obtain. Plant and invertebrate MOTUs are represented in green and yellow, respectively. NI describes non-identified families.

Chapter 4

Article V

Figure 1. Map of Cabo Verde showing the geographic location of the islands and the sampled sites (yellow circles). (a) Two islands were sampled in the Windward group, Santo Antão (Paúl) and São Vicente (Mindelo). (b) One island was sampled in the Leeward group, Santiago (São Lourenço dos Órgãos). N stands for the number of sequenced samples per island.

Figure 2. Metabarcoding results per gastrointestinal section. Frequencies of occurrence of plants (in green) and invertebrates (in yellow) in the *Mus musculus* stomach and intestines (small and large) samples.

Figure 3. Results of the similarity percentage analysis. Frequency of occurrence of 30 Molecular Operational Taxonomic Units (MOTUs) with the highest contribution to differences between the stomach and intestine of *Mus musculus*. Plant MOTUs are represented in green and invertebrates in yellow. Magnitude of significance levels shown with asterisks: *** $p< 0.001$; ** $p< 0.01$; * $p < 0.05$.

Article VI

Figure 1. Map of the study area and study site. A) Geographic location of the Kingdom of Saudi Arabia and B) the AlUla County, with the location of the study site represented by a red dot. C) Wadi Ashar boundary in red, and the 10 sampled squares (Geographic Coordinate System, Datum WGS84).

Figure 2. Bipartite network representing associations between the reptile community of Wadi Ashar on the left and arthropod (yellow) and plant (green) prey orders on the right.

The width of the connecting bars represents the frequency of occurrence of each order for each reptile species. Reptile species are represented proportionally according to their sizes, and with darker colour boxes indicating their increasing body size category. Information on reptiles' activity pattern (diurnal versus nocturnal) is also given.

Figure 3. Dietary composition of the reptile community of Wadi Ashar according to the body size categories. A) Adjacency matrix summarizing the reptile community subgroups according to their identified diet item orders. Reptile species are represented proportionally according to their sizes along the vertical axis with darker colour circles indicating their increasing body size category, and plant and arthropod orders are shown along the horizontal axis. Subgroups are delineated by brown boxes. The darker the brown squares the more frequent the association between a diet item and its predator. Information on reptiles' activity pattern (diurnal versus nocturnal) is also given. B) Non-metric multidimensional scaling (NMDS) of the prey composition of the Wadi Ashar reptile community by body size categories. Points closer together correspond to individuals with a more similar diet item composition. Ellipses represent the standard error of the dietary composition centroid for each body size category at 90% confidence interval.

Chapter 5

Figure 1. Integrated overview of general discussion of the thesis showing the main results and conclusions.

List of Abbreviations

ANOVA	Analyses of variance
asl	Above Sea Level
BLAST	Basic Local Alignment Search Tool
bp	base-pair
ºC	degree Celsius
Ca.	Circa
CL	Chela Length
COI	Cytochrome C Oxidase I
CW	Chela Width
DNA	Deoxyribonucleic Acid
ED	Eye diameter
EE	Eye-ear distance
e.g.	exempli gratia, for example
ES	Eye-snout
Fig.	Figure
FiL	Fixed Finger Length
FL	Femur Length
FLL	Front limb length
FO	Frequencies of Occurrence
FW	Femur Width
GLM	Generalized Linear Model
GPS	Global Positioning System
HSD	Honest significant difference
HH	Head height
HL	Head length
HLL	Hind limb length
HW	Head width
IUCN	International Union for Conservation of Nature
km	kilometre
km2	square kilometre
LI	Large Intestine
m	Meters
Ma	Million years ago
My	Million years
min	minute
ML	Maximum Likelihood
mm	millimetre
MOTU	Molecular Operational Taxonomic Units

MUHNAC	National Museum of Natural History and Science of Lisbon
N	Number
NCBI	National Center of Biotechnology Information
NGS	Next Generation Sequencing
NI	Non-identified Families
nM	nanomolar
NMDS	Non-metric Multidimensional Scaling
O	Overlap
ONG	Non-Government Organisation
PC	Principal components
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PL	Patella Length
PW	Patella Width
rDNA	Recombinant Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
s	second
SI	Small Intestine
SVL	Snout-Vent Length
TL	Trunk length
TrnL	Transfer RNA Asparagine-like
µl	Microlitre
µm	Micrometre

Chapter 1

General Introduction

1.1. Insular systems as models in ecology and evolution

Islands stand out as exceptional models for studying ecological, evolutionary, and biogeographic processes (Warren *et al.*, 2015). These ecosystems are frequently regarded as 'natural laboratories' due to their geographical isolation and clearly defined boundaries, offering discrete, manageable, and replicable microcosms of biological communities (MacArthur *et al.*, 1963; Whittaker *et al.*, 2017). The importance of evolutionary processes on islands was first recognised in the seminal studies of Charles Darwin on the Galapagos Islands, which gave rise to the Theory of Evolution by means of natural selection (Darwin, 1859). Over the last decades, research has focused on multiple facets of islands, contributing to the establishment of ecological and evolutionary theories (Gravel *et al.*, 2011; MacArthur *et al.*, 1963, 1967; Steinbauer *et al.*, 2016; Warren *et al.*, 2015; Whittaker *et al.*, 2007, 2017).

Although islands tend to have a low number of species compared to mainland systems, when considering that their total area comprehends only 6.7% of the emergent land of the world (Sayre *et al.*, 2019), they appear as exceptional hotspots of biodiversity, hosting approximately 20% of the biodiversity of the world (Kier *et al.*, 2009). However, due to the synergetic influence of genetic and demographic factors, this biodiversity is more vulnerable than mainland species (Frankham, 1997). Islands contribute significantly to globally threatened or extinct species, with nearly half of all species classified as threatened in any of the IUCN's red list categories (Fernández-Palacios *et al.*, 2021). This proportion further increases to around 75% when considering documented extinctions since the European expansion worldwide (Fernández-Palacios *et al.*, 2021). This significance is particularly pronounced in certain taxa, with birds representing a staggering 94.4% and reptiles 89.7% of world extinctions within their respective groups (Fernández-Palacios *et al.*, 2021).

Evolution on islands often promotes the occurrence of unusual biological characteristics, described as insular syndromes (Adler *et al.*, 1994). These characteristics often offer species advantages in untouched island ecosystems, such as gigantism or dwarfism (Lomolino *et al.*, 2012; Meiri *et al.*, 2011), flightlessness (McNab, 1994; Wright *et al.*, 2016), diet specialization (DeMiguel, 2016; Pérez-Mellado *et al.*, 1993; Sagonas *et al.*, 2014), reduced anti-predator behaviour (Berger *et al.*, 2007; Blumstein *et al.*, 2005) and tameness (Cooper Jr *et al.*, 2014). Nevertheless, these unique traits that characterize island species also make them more susceptible to extinction, primarily due to human interference (Berger *et al.*, 2007; Fernández-Palacios *et al.*, 2021; Russell *et al.*, 2019;

Tingley *et al.*, 2013). Human-induced factors such as habitat destruction, introduction of invasive species, direct hunting, or overexploitation of essential resources, along with indirect impacts like climate change, significantly impact island biota (Fernández-Palacios *et al.*, 2021). Consequently, the study and conservation of island biodiversity assume paramount importance.

Oceanic islands are of particular interest to understand speciation and community assembly, as they have a volcanic origin and are thus geographically isolated from continental areas. Such islands serve as blank canvases, presenting unexplored ecological opportunities for migrant lineages. With limited ecological competitors and predators coupled with new unexplored resources available, colonizers have the potential for rapid speciation (Losos *et al.*, 2009), resulting in significant *in situ* ecological diversification (Schluter, 2000). Subsequent isolation from mainland source areas limits gene flow, fostering geographical isolation and population differentiation, consequently contributing to typically high rates of endemism (Whittaker *et al.*, 2007). The richness of terrestrial species on islands varies as a function of isolation, island area, age, and shape, habitat diversity, distance to other islands and the mainland, taxon biology and human influence (Triantis *et al.*, 2003; 2017; Whittaker *et al.*, 2008). As a consequence, islands often exhibit more simplified ecological networks characterized by a disharmonic biota, particularly noticeable in the more remote islands (Frankham, 1997; Lopes *et al.*, 2019; Warren *et al.*, 2015). In this way, oceanic islands represent simplified models for studying ecological networks, as species inhabit more confined areas with a limited number of interactions, facilitating more comprehensive sampling. Ultimately, this can allow for obtaining thorough data with significantly less effort compared to mainland systems.

Insular systems include not only true islands (isolated portions of land surrounded by water) but also island-like systems (Itescu, 2019). Island-like systems refer to any isolated habitats that despite being part of a larger landmass, exhibit characteristics resembling those found on true islands (e.g., lakes, streams, caves, mountain tops, human-generated fragmented habitats, protected areas, and even living animals; (Itescu, 2019). Even though MacArthur *et al.* (1967) noted that island biogeography theories can be applied to any isolated habitat, most studies are focused on true islands. However, numerous additional island-like systems hold significance in evolutionary and ecological studies, offering considerable prospects for research.

1.2. True Island system: Macaronesia as a model

The Macaronesia biogeographical region, located in the North Atlantic Ocean off the European and African coasts (Figure 1A), has become of particular interest for ecological and evolutionary studies due to its exceptional conditions (Florencio *et al.*, 2021). It comprises five archipelagos of volcanic origin (Figure 1A) and is thought to be the product of several geological hotspots, Azores, Madeira, Selvagens, Canary Islands, and Cabo Verde (Fernández-Palacios *et al.*, 2011; Whittaker *et al.*, 2007). These oceanic archipelagos comprise a total of 40 islands and islets larger than 1 km^2, with a broad range of geological ages and geographical isolation from continental areas (Fernández-Palacios *et al.*, 2011; Florencio *et al.*, 2021).

The latitudinal variation of the Macaronesian archipelagos results in a great disparity of climates, from the oceanic temperate climate of the Azores to the warm arid climate of Cabo Verde, including the Mediterranean climates of Madeira, Selvagens and the Canaries (Fernández-Palacios *et al.*, 2002, 2010). In addition, due to their different sizes, altitudes and climates the Macaronesian islands present a wide variety of habitats, ranging from the desert and semi-arid vegetation of Cabo Verde and Canary Islands to the humid laurel forests present on the Azores, Madeira and the Canary Islands (Fernández-Palacios, 2010). This region presents an outstanding endemic biota, particularly in the terrestrial realm (Florencio et al., 2021), providing an exceptional framework for the study of speciation, adaptive radiation, and genetic diversity.

The archipelagos of Macaronesia are relevant for the study of community assembly and species interactions. The absence of some functional groups on these islands often leads to disharmonic community compositions (e.g., seed dispersal networks in the Canaries, González-Castro *et al.*, 2012; Nogales *et al.*, 2016), making them particularly vulnerable to disturbance (Pombo *et al.*, 2010; Simberloff, 2000). Anthropogenic activities pose a significant threat to the endemic Macaronesian biota, having caused extensive habitat loss and subsequent changes in community composition, within a relatively short period (Castilla-Beltrán *et al.*, 2021; Triantis *et al.*, 2010). Contemporary human-mediated introduction of alien species poses one of the main threats to biodiversity in Macaronesia. For example, in the Azores more than 60% of vascular plants are introduced (Silva *et al.*, 2008). Therefore, protecting Macaronesian endemic species is crucial, and involves numerous measures, such as protecting native habitats, control of exotic flora and fauna, and mitigating the effects of human activity (Caujapé-Castells *et al.*, 2010; Florencio *et al.*, 2021; Romeiras *et al.*, 2016; Vasconcelos *et al.*,

2012a). Despite the collective efforts of systematists, ecologists and biogeographers to collect comprehensive data on the terrestrial and marine biodiversity of Macaronesia (e.g., Arechavaleta *et al.*, 2005; Borges *et al.*, 2008; Caujapé-Castells *et al.*, 2017; Fernández-Palacios *et al.*, 2011; Freitas *et al.*, 2019; Lobo *et al.*, 2010), there are still gaps in ecological and evolutionary knowledge for all archipelagos and some taxonomic groups that limit conservation efforts. In particular, the Cabo Verde Archipelago, despite its ecological and evolutionary importance, has received considerably less research attention than the other four archipelagos. This disparity is often attributed to historical factors, the remoteness of the archipelago, and the difficulty of accessing certain habitats (Neto *et al.*, 2020; Romeiras *et al.*, 2020). This thesis seeks to contribute to closing this gap, by placing Cabo Verde as the primary focus of this thesis.

1.2.1. The Cabo Verde Archipelago

Cabo Verde is a young mid-developing country located approximately 1500 km southwest of the Canary Islands and 570 km west of the African coast. It comprises ten main islands and several islets of volcanic origin, never connected to the mainland (Mitchell-Thomé, 1976), that are arranged in a deep arc with its concavity facing westwards (Figure 1B). These islands are commonly divided into two groups, Windward (Santo Antão, São Vicente, Santa Luzia, São Nicolau, Sal and Boavista) and Leeward (Maio, Santiago, Fogo and Brava), but can also be subdivided into three clusters (Figure 1B): North-western (Santo Antão, São Vicente, Santa Luzia, Branco and Raso islets and São Nicolau); Southern (Maio, Santiago, Fogo and Brava) and Eastern (Sal and Boavista). The islands of the archipelago are very different in size and landscape: Santiago is the largest (about 1000 km^2) and the most populated island, while Santa Luzia is the smallest (about 34 km^2) and currently uninhabited (Duarte *et al.*, 2009); Fogo is the highest (about 2829 m above sea level) and the only island with recent volcanic activity (Mata *et al.*, 2017). The north-western and the southern islands, in particular Santo Antão, São Nicolau, Santiago and Fogo, are characterized by steep, high mountains and deep river valleys providing a wide range of habitats over relatively short distances (Duarte *et al.*, 2009). The eastern islands, and the southern island of Maio, having experienced more erosion, are flatter, drier, and less diverse in habitats (Duarte *et al.*, 2009). The age of the islands ranges from around 0.21 to around 17 million years (My) with islands closer to the mainland being the oldest (Figure 1C; references therein Florencio *et al.* 2021). The islands of the Desertas group (Santa Luzia, Branco and Raso islets) and São Vicente are thought to have been connected during low sea levels in the

Pleistocene (Ancochea *et al.*, 2015). On the contrary, the remaining islands were highly unlikely to have been connected in the past, due to the deep-water channels among them (Figure 1B).

The archipelago belongs to the African Sahelian arid and semi-arid climatic region. It experiences a climate ranging from tropical dry to semi-desert, mainly controlled by the Azores anticyclone, the Intertropical Convergence Zone (ITCZ) and the mid-Atlantic air mass movements caused by their seasonal changes in location (Duarte *et al.*, 2009). Rainfall is concentrated in the rainy season between July and October, with annual precipitation ranging from 80–300 mm in the arid coastal areas to 1200–1600 mm in the highlands exceeding an altitude of 2829 m (Duarte *et al.*, 2009; Mitchell-Thomé, 1976). Temperatures are stable throughout the year, ranging from 23–27°C in coastal areas to 18–20°C in the highlands, but can reach maximum values of 35–40°C in the interior of the arid eastern islands (CCKP, 2021).

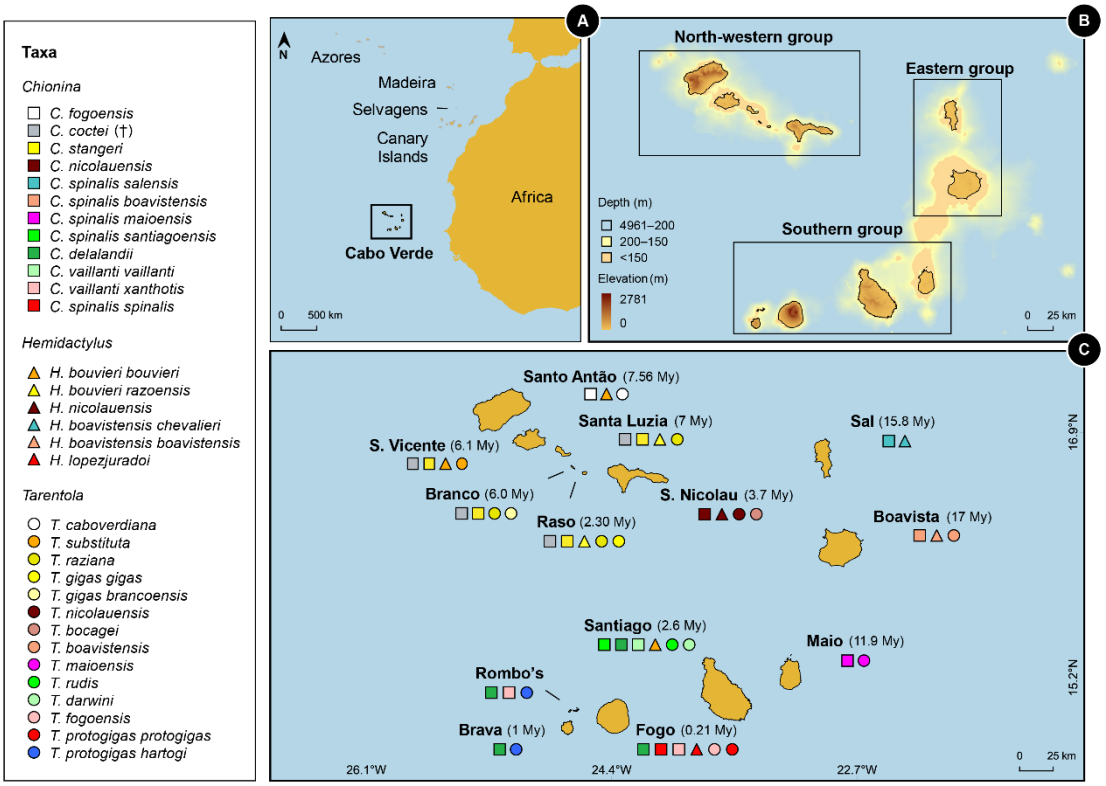

Figure 1. The study area, the Cabo Verde Archipelago. A) Geographical location of the archipelago within Macaronesia. B) Map of the archipelago showing the islands clusters, elevation, and bathymetry (GEBCO Compilation Group, 2021). C) Island ages in million years (My) and the distribution of the reptile species and subspecies across the islands (Geographic Coordinate System, Datum WGS84).

The Cabo Verde archipelago hosts a rather poor terrestrial vertebrate fauna, except for endemic reptiles, which present the greatest diversity in the Macaronesian region (Vasconcelos *et al.*, 2013). Terrestrial mammals are represented by only five species of bats, probably all non-endemic (Borloti *et al.*, 2020), and several introduced species (e.g., green monkey and house mouse, Arechavaleta *et al.*, 2005). Cabo Verde is also an important breeding area for several bird species (Clarke, 2020).

Reptiles are more prevalent on islands (Pincheira-Donoso *et al.*, 2013) and emerge as exceptional models for evolutionary and ecological studies due to their unique characteristics. With low metabolic rates and high resistance to desiccation and salinity in some groups, particularly in geckos, reptiles are the vertebrates most capable of colonising oceanic islands, after birds (Carranza *et al.*, 2000). They can play important roles within ecosystems as seed dispersers, pollinators (Borzí, 1911; Elvers, 1977; Hansen *et al.*, 2006; Nyhagen *et al.*, 2001; Whitaker, 1987) and in a few cases even as top predators (Alcover *et al.*, 1994; Miranda, 2017), more often than in mainland systems. In Cabo Verde in particular, there are 32 endemic terrestrial reptile taxa from three genera: *Chioninia* (Scincidae), *Hemidactylus* (Gekkonidae) and *Tarentola* (Phyllodactylidae) (Arnold *et al.*, 2008; Miralles *et al.*, 2011; Vasconcelos *et al.*, 2012b, 2013, 2020). Although these species are well-studied taxonomically and phylogeographically (Arnold *et al.*, 2008; Miralles *et al.*, 2011; Vasconcelos *et al.*, 2012b, 2013) and despite their highly threatened status, their ecological roles remain largely underexplored. Hence, understanding the ecology of these unique species in Cabo Verde can potentially provide valuable insights into their specialized adaptations. In turn, this knowledge may contribute to an improved understanding of evolutionary patterns not only in Cabo Verde but also in similar insular systems. Ultimately, this data is also fundamental for developing sustainable management plans within these fragile ecosystems. Ecological studies are further facilitated in this archipelago by the manageable magnitude of the diversity of reptiles, their typically high local abundances, ease of handling and the high potential for non-invasive sampling (Vasconcelos, 2010).

The *Chioninia* skinks are thought to have reached Cabo Verde from a single colonization event from West Africa, some 26 My ago (Pereira *et al.*, 2017). There are six extant species and seven subspecies of *Chioninia* throughout the archipelago (Figure 1C), and one extinct species, the Cabo Verde giant skink *Chinionia coctei* Duméril & Bibron, 1839 (Miralles *et al.*, 2011). The genus *Hemidactylus* is also inferred to have an origin in West Africa, somewhere between 10–18 My ago (Arnold *et al.*, 2008), with four endemic species and four subspecies (Arnold *et al.*, 2008; Vasconcelos *et al.*, 2020; Figure 1C).

The *Tarentola* geckos are thought to have originated from a single colonization event from the Canary Islands, approximately 7.7 My ago, giving rise to 12 endemic species and four subspecies (Carranza *et al.*, 2000; Vasconcelos *et al.*, 2010; Figure 1C).

Both *Chioninia* and *Tarentola* derive from a medium-sized ancestral species, from which some developed larger body sizes (e.g., *Tarentola gigas* (Bocage, 1875) and *Chioninia vaillantii* (Boulenger, 1887)), while others retained the ancestral size (e.g., *Chioninia delalandii* (Duméril & Bibron, 1839)) or became smaller (e.g., *Tarentola raziana* Schleich, 1984; Miralles *et al.*, 2011; Pereira *et al.*, 2017; Vasconcelos *et al.*, 2012b). A few developed extreme body sizes, such as the extinct *C. coctei* (Bocage, 1873), and an increased rate in the evolution of this trait was proven (Pereira *et al.*, 2017). Character displacement – an evolutionary process whereby two resource competitors diverge in phenotype and resource utilization, facilitating coexistence (Brown *et al.*, 1956) – has been suggested to partially explain these discrepancies for both genera (Carranza *et al.*, 2000, 2001), but has not been explored in detail. Remarkably, Cabo Verde presents a unique opportunity to explore sister taxa of contrasting sizes coexisting within the same islands, sharing scarce food resources and habitats (Vasconcelos *et al.*, 2013). Given that related species tend to exhibit similar feeding habits and distribution patterns this creates potential for competition. Syntopic reptile species have been found to rapidly diverge in ecological niches by shifting ranges, altering morphology or diet preferences to cope with competitors and habitat change (Amorim *et al.*, 2017; Dufour *et al.*, 2018a, 2018b). These ecological differences should favour the long-term coexistence of species and thus the diversity of the communities (Pianka, 2011). In this way, understanding dietary niche partitioning among evolutionary and ecologically related and co-occurring species is important for the development of a general understanding of community assembly and co-evolution (Arrizabalaga-Escudero *et al.*, 2018; Kartzinel *et al.*, 2015; Schmack *et al.*, 2021). This is greatly facilitated within systems where it is possible to characterise and compare dietary habits between sympatric and allopatric populations of one or both species. Such a sampling framework allows for a greater understanding of the extent to which the diet composition of geographically overlapping species is influenced by competition.

One notable case exists in the northern islands, with the giant wall gecko *T. gigas*, locally abundant on Raso and Branco islets, but recently extinct on Santa Luzia Island, and the Raso wall gecko *T. raziana*, occurring across all islands of the Desertas Island group (Figure 2A; Vasconcelos *et al.*, 2013). These geckos represent the largest and smallest species in the archipelago, respectively (Vasconcelos *et al.*, 2012b) and belong to sister

clades that diverged approximately 4.5 My ago (Vasconcelos *et al.*, 2010). The *T. gigas* clade comprises tendentially larger species than the inferred ancestral species, whereas species in the *T. raziana* clade are generally similar in size to the ancestral species (Vasconcelos *et al.*, 2012b). Therefore, building from a historical phylogenetic signal for size differentiation, both species most likely further diverged in opposite directions accentuating this size discrepancy. Both species are nocturnal and syntopic on Raso and Branco (Figure 1A; Vasconcelos *et al.*, 2013), having access to the same limited resources and being subjected to the same eco-geographical pressures, thus creating the potential for competition between them.

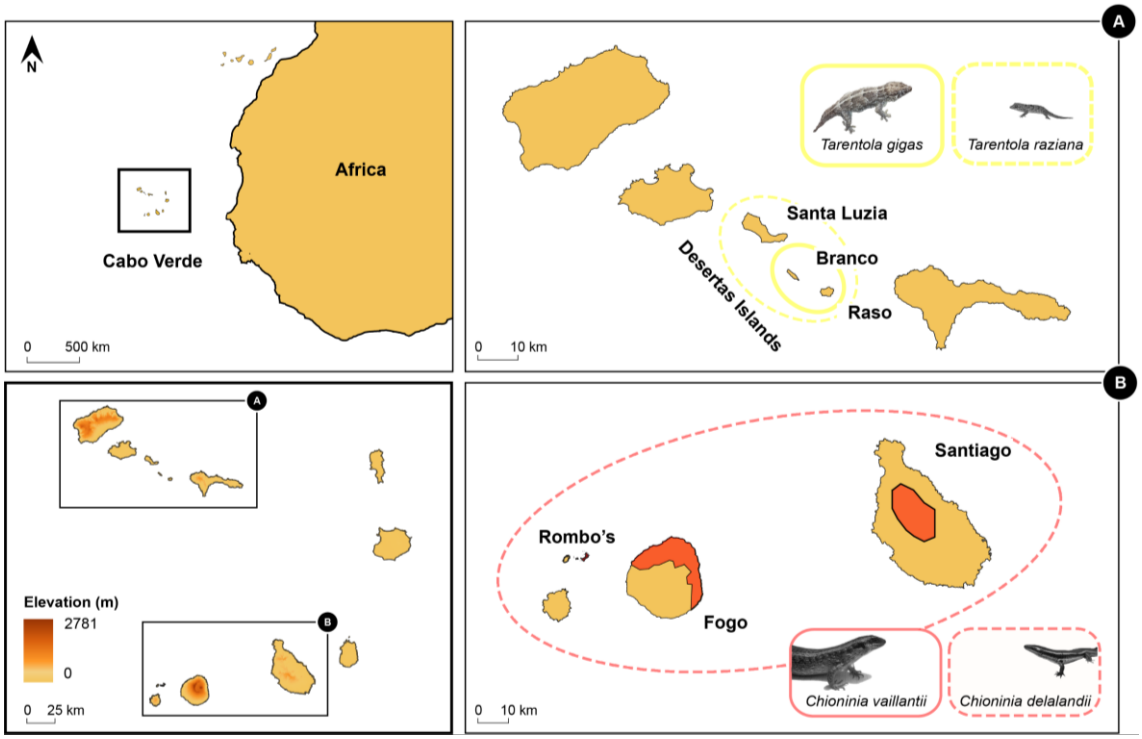

Figure 2. The study reptile species of Cabo Verde. Map of the archipelago showing the geographic location and elevations of the islands (Geographic Coordinate System, Datum WGS 84). A) The *Tarentola* species pair from the Desertas. B) The *Chioninia* species pair from Rombo's Islets, Fogo and Santiago. All species are represented showing their size discrepancies. Dashed lines represent the species with the largest distribution of the pair and filled areas correspond to the sympatric areas of each species pair.

Similarly, in the southern islands, the Vaillant's skink *C. vaillantii*, represented by two subspecies, *C. vaillantii vaillantii* (Miralles *et al.*, 2011) on Santiago Island and *C. vaillantii xanthotis* (Miralles *et al.*, 2011), on Fogo Island and Cima Islet (Figure 1C), co-occurs with the smaller Delalande's skink *C. delalandii* (Miralles *et al.*, 2011; Vasconcelos *et al.*, 2013). The Delalande's skink is widespread on the leeward islands of Santiago, Fogo, Brava and Rombo's Islets, and, unlike *C. vaillantii*, occurs also in allopatry with the latter species on these islands (Figure 2B; Miralles *et al.*, 2011; Vasconcelos *et al.*, 2013). They

are sister species that diverged between 6.9 and 5.9 My ago (Miralles *et al.*, 2011), with *C. vaillantii* showing an increase in body size compared to *C. delalandii*, which retained the inferred intermediate ancestral size (Miralles *et al.*, 2011). Both species are diurnal and syntopic at high altitudes on Fogo and Santiago and on the entire extent of Cima Islet (Figure 1B; Vasconcelos *et al.*, 2013), leading to the potential for competition between them. Due to the restricted area and increased ecological constraints on Cima Islet (e.g., limited resources and introduced species), competitive pressure between these species is expected to be higher than on Fogo, which may be reflected in further behavioural, dietary and morphological divergences. Thus, Cabo Verde provides a relevant framework to investigate evolutionary responses to competitive pressures for these two genera (Figure 2).

While intricate ecological interactions among reptile species in Cabo Verde shed light on the evolutionary responses to competitive pressures, their ecological equilibrium faces significant threats. Invasive plant species stand as the primary cause of degradation and loss of plant biodiversity in the region (Caujapé-Castells *et al.*, 2010; Romeiras *et al.*, 2016). Alarmingly, around 60% of the described flora in Cabo Verde comprises introduced species (Arechavaleta *et al.*, 2005). Analogously, mammal invaders such as cats *Felis catus* L. and rodents have had a notable impact, contributing to the extinction of endemics, such as the Cabo Verde giant skink *Chioninia coctei*, and the extirpation of the giant wall gecko *Tarentola gigas* on Santa Luzia Island (Mateo *et al.*, 2005; Medina *et al.*, 2020). Of the rodent invaders, the house mouse *Mus musculus* L. has achieved widespread distribution across both inhabited and uninhabited islands within the archipelago (Hazevoet *et al.*, 2011). Similar to what has been observed in other island ecosystems (Bunce *et al.*, 2009), mice have likely have significant negative impacts on Cabo Verdean native fauna and flora. Additionally, these rodents can greatly affect agricultural activities, which can lead to substantial economic losses in Cabo Verde. Therefore, this topic is of great importance for the Cabo Verdean governmental authorities that are actively trying to address this issue. An eradication plan for invasive mammals is already taking place on Santa Luzia Island (Alho *et al.*, 2022). Nevertheless, the ecological damage caused by these invaders in Cabo Verde has never been quantified. The introduction and spread of invasive species likely pose severe threats to the survival of endemic reptile species, exacerbating competition and ecological pressures. It is thus fundamental to understand and address these impacts to conserve biodiversity and maintain ecological dynamics in Cabo Verde.

1.3. Island-like system: analogous models

Anthropogenic activities and climate change are causing the extinction of species at a rate faster than they can be discovered, raising global concern about how to stop the current loss of biodiversity (Kristensen *et al.*, 2020; Tollefson, 2019). This biodiversity crisis is accompanied or even preceded by the disappearance of natural assemblages of species and their particular ecological interactions (Tollefson, 2019; Valiente-Banuet *et al.*, 2015). This affects species functionality and ecosystem services at a faster rate than species extinctions (Valiente-Banuet *et al.*, 2015). The current unprecedented human impact is resulting in the loss of contiguous habitats, leading to habitat fragmentation and the creation of island-like habitats which, like true islands, are more prone to the loss of ecological links and species extinctions (Erwin, 1991; Reed, 2004). While typically not surrounded by water, these habitats frequently exhibit ecological traits such as isolation, limited resources, distinct boundaries, and unique ecosystems (Itescu, 2019). Island-like systems provide opportunities to study aspects of ecology and evolution similar to those found on real islands, allowing for the study of patterns of biodiversity, species interactions and adaptations in relatively confined and discrete environments (Dawson, 2016; Itescu, 2019; MacArthur *et al.*, 1967). By understanding and protecting these island-like systems, we not only protect unique biodiversity but also gain valuable knowledge that can be applied to conservation management strategies for larger and more complex ecosystems worldwide.

In the context of island-like ecosystems, Wadi Ashar is a valley of approximately 30 km^2 within the Harrat Uwayrid UNESCO Biosphere Reserve surrounded by sandstone mountains (Figure 3 B–C). It is composed of different habitat types such as dunes and sandy areas, gravel plains, rocky slopes, gullies, and agricultural areas (Figure 3D). The area is substantially influenced by overgrazing, and it holds several tourist resorts (Figure 3D). Recent development has resulted in severe habitat destruction and fragmentation in the area (Figure 3D). Currently, little is known about the biodiversity of this arid valley and its trophic interactions, but it provides the opportunity for valuable insights into how communities are impacted in disturbed island-like systems. The valley is situated in Saudi Arabia, precisely in AlUla County, located 1,100 km northwest of Riyadh in the Medina region (Figure 3A–B). AlUla covers a vast area of 22,561 km^2, including desert valleys, sandstone mountains and several ancient heritage sites, and is the site of several management projects being developed by local authorities. The Kingdom of Saudi Arabia (Figure 3A), covering more than 2.15 million km^2, encompasses a wide range of climates and terrestrial habitats, from mesic, cool, high mountains to arid

deserts and hot, semi-arid coastal plains, and thus holds a rich biodiversity (AbuZinada *et al.*, 2004; Vincent, 2008). In particular, reptiles are the terrestrial vertebrate group that contains the highest number of endemic species and, therefore, are very important from a biodiversity point of view (Cox *et al.*, 2012). The terrestrial reptile fauna of Saudi Arabia includes approximately 118 species (80 lizards, 36 snakes and two freshwater turtles), eight of which are endemic to the Kingdom (Gasperetti *et al.*, 1993; Šmíd *et al.*, 2017a, 2017b, 2021, 2023; Wilms *et al.*, 2010). However, to effectively protect this unique reptile biodiversity, much work remains to be done to fully understand its diversity, distribution, and ecology (Aloufi *et al.*, 2019).

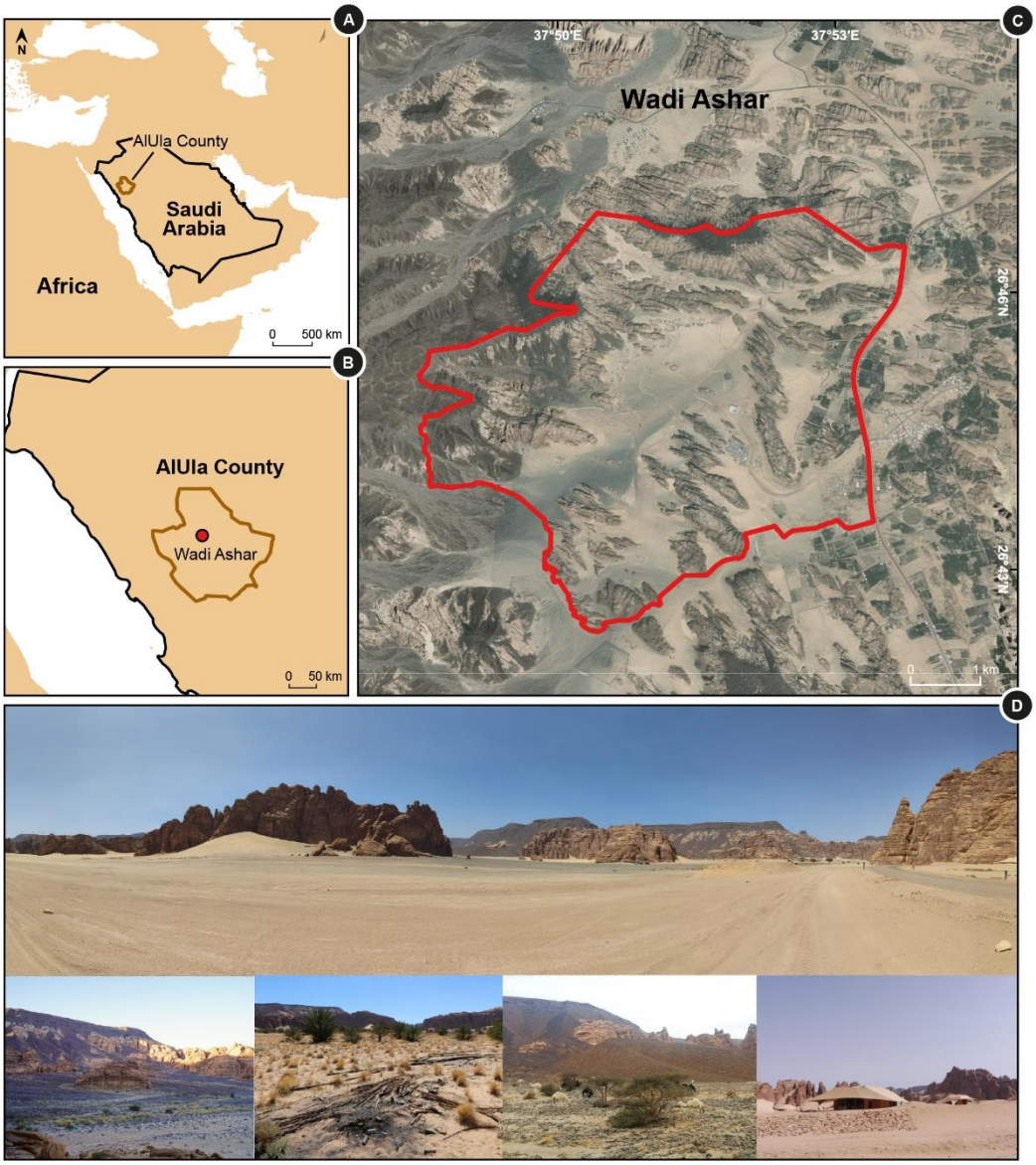

Figure 3. Map of the study area of Wadi Ashar. A) The geographic location of the Kingdom of Saudi Arabia and B) the AlUla County, with the location Wadi Ashar represented by a red dot. C) Wadi Ashar boundary is in red (Geographic Coordinate System, Datum WGS84). D) Representation of the different habitat types and anthropogenic structures found in this area (photographs by C.J. Pinho, R. Vasconcelos, S. Carranza and J. Šmíd).

1.4. Metabarcoding as a biodiversity survey tool

Traditional methods of morphological species characterisation for biodiversity assessment have been revolutionised by DNA-based species identification. DNA barcoding is a molecular technique standardised by Hebert *et al.* (2005) that allows species to be identified using short, standardised DNA fragments known as DNA barcodes. This DNA-based identification relies on the prior establishment of strong reference libraries. Metabarcoding extends DNA-based species identification to complex samples allowing a large-scale taxonomic identification (Pompanon *et al.*, 2012), relying on high-throughput sequencing of DNA using general or group-specific primers. With the power of next-generation sequencing (NGS) methodologies, this multispecies identification approach is becoming faster and more cost-effective each day (Shendure *et al.*, 2008). This tool is revolutionising the way biodiversity surveys are used to answer ecological and evolutionary questions across a wide range of organisms (e.g., bacteria, plants, invertebrates, vertebrates), ecosystems (e.g., terrestrial, marine, freshwater) and sample types (e.g., soil, water, faeces) (Figure 4A; Forsman *et al.*, 2022; Ruppert *et al.*, 2019). Metabarcoding is being used to reconstruct ancient ecosystems (e.g., Pedersen *et al.*, 2013; von Hippel *et al.*, 2022), for the premature detection of invasive species (e.g., Brown *et al.*, 2016; Hatzenbuhler *et al.*, 2017), characterization of invertebrate communities (e.g., Gleason *et al.*, 2023; Watts *et al.*, 2019), understanding ecosystem response to pollution (e.g., Bell *et al.*, 2014; Frontalini *et al.*, 2018), air quality monitoring (e.g., Leontidou *et al.*, 2018; Oh *et al.*, 2014), microbiome studies (e.g., Çelik *et al.*, 2022; Ficetola *et al.*, 2019) and to study species interactions such as plant-pollinator networks (e.g., Bell *et al.*, 2017; Pornon *et al.*, 2017) and diet analyses (e.g., Ando *et al.*, 2020; Pinho *et al.*, 2018).

Metabarcoding is gaining particular attention in the field of trophic ecology (Ando *et al.*, 2020; de Sousa *et al.*, 2019), as it allows dietary assessments to be made with higher taxonomic resolution than traditional methods (Gil *et al.*, 2020; Pompanon *et al.*, 2012; Taberlet *et al.*, 2012). With this technique, it is possible to obtain a large amount of data with less effort and time (Taberlet *et al.*, 2012), which is particularly useful for studying the diets of threatened animals from remote areas. Moreover, compared to classical methods, this technique is less reliant on taxonomic expertise (Gil *et al.*, 2020; Pompanon *et al.*, 2012), and allows the detection of soft, small and difficult-to-detect prey that become fully assimilated post-digestion (Symondson, 2002; Tomé, 2013). Metabarcoding typically utilizes samples such as faeces or gut contents for diet characterization (Jakubavičiūtė *et al.*, 2017; Thuo *et al.*, 2019). Faecal samples are

particularly advantageous for threatened species as they can be obtained with minimal or no impact on individual fitness (Ferreira *et al.*, 2018). Although metabarcoding of faecal samples is gaining popularity in understanding dietary partitioning strategies between co-occurring species (e.g., Arrizabalaga-Escudero *et al.*, 2018; Lopes *et al.*, 2015; Westeen *et al.*, 2023), to our knowledge, no studies have used this approach to investigate complex evolutionary questions. Furthermore, few studies have used this technique to understand trophic interactions of whole communities (Ingala *et al.*, 2021; McShea *et al.*, 2019), and to our knowledge none on reptile communities. On the other hand, gut contents often yield higher-quality DNA and are commonly employed in diet assessments of invasive species (Robeson II *et al.*, 2018; Siegenthaler *et al.*, 2019). While studies have explored prey DNA detectability in mammalian stomachs versus faeces (Egeter *et al.*, 2015), none has examined how analysing distinct gastrointestinal tract sections influences prey DNA detectability. This is particularly important to omnivorous species that consume items with different tissue cell density, digestion rate, and DNA quality decay after digestion (Pompanon *et al.*, 2012). These factors can significantly influence the outcomes derived from DNA metabarcoding approaches.

These features make this method advantageous and easy to apply in a variety of ecological studies, but some methodological implications need to be considered in the different steps of the metabarcoding workflow – sampling, laboratory processing, bioinformatic analysis and data interpretation (Figure 4; Alberdi *et al.*, 2019; Cuff *et al.*, 2022). Firstly, field sampling should be conducted to avoid possible sources of DNA contamination. This issue should also be considered during DNA extraction, and negative controls should be implemented (Bohmann *et al.*, 2022; Taberlet *et al.*, 2018). Marker choice also needs special attention, as the primers should be able to amplify the range of expected prey without amplifying the host or untargeted groups (Piñol *et al.*, 2015). The taxonomic resolution of the marker region should also be considered when selecting a primer set (Deagle *et al.*, 2014; Pompanon *et al.*, 2012). Furthermore, interpreting read counts obtained from sequencing demands caution, as they may not precisely reflect relative abundance or consumed biomass (Deagle *et al.*, 2018; Krehenwinkel *et al.*, 2017; Nichols *et al.*, 2018). Several technical and biological factors influence sequenced reads, including PCR amplification bias, random sampling during sequencing, prey size, digestibility rates, tissue cell densities, and variation in gene copy number (Cuff *et al.*, 2022; Deagle *et al.*, 2018; Jarman *et al.*, 2013; Pompanon *et al.*, 2012; Tomé, 2013). For dietary studies using faecal pellets, another implication arises as the extracted DNA is highly degraded, therefore the DNA from the predator is highly dominant compared to the prey DNA (Vestheim *et al.*, 2008). Oligonucleotides can be

used to block the amplification of the non-target DNA and prevent the decrease in the sequencing depth of the fragments of interest (Piñol *et al.*, 2015; Vestheim *et al.*, 2008).

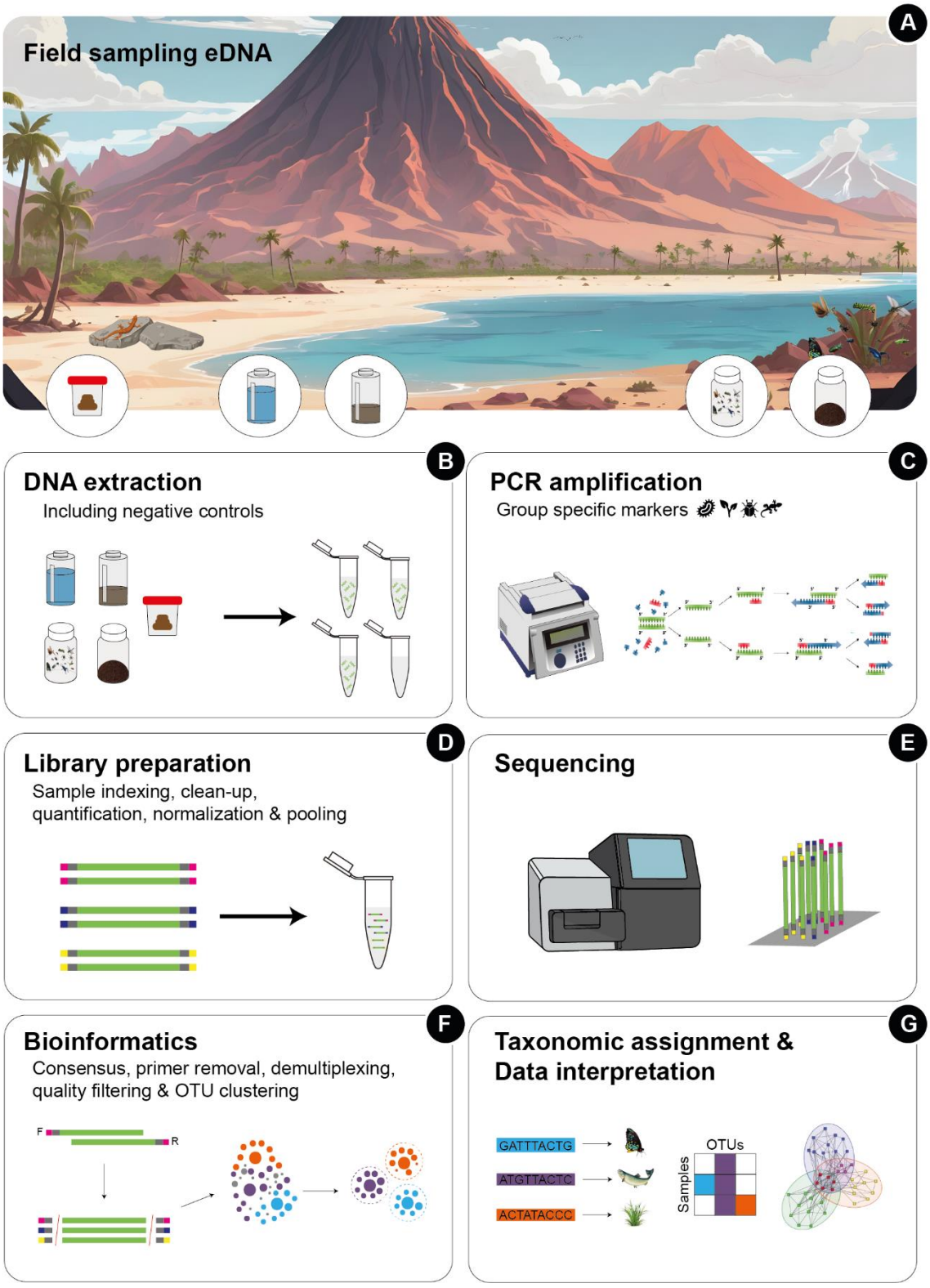

Figure 4. Simplified overview of the metabarcoding workflow. A) Arid landscape representing examples of samples used for eDNA biodiversity surveys (Image generated using Wepik AI image generator); B–E) Laboratory procedures; F–G) Bioinformatic analyses and interpretation of the obtained data.

Nevertheless, DNA metabarcoding is foreseen as the most powerful tool to understand complex trophic interactions and reconstruct ecological networks (Casey *et al.*, 2019; Cuff *et al.*, 2022; de Sousa *et al.*, 2019; Mata *et al.*, 2021; Roslin *et al.*, 2016; Russo *et al.*, 2023). The characterization of species diets unveils trophic networks, which are one of the main vectors shaping natural community assemblies and ecosystem processes (Thébault *et al.*, 2005). With the advancement of the metabarcoding approach, diets of many species have been re-examined and new patterns of resource partitioning, competition and diet overlap revealed (Arrizabalaga-Escudero *et al.*, 2018; Gordon *et al.*, 2019; Kartzinel *et al.*, 2015; Nakashima *et al.*, 2022; Zurdo *et al.*, 2023). Such data holds increasing relevance in biodiversity conservation efforts, as a comprehensive understanding of food webs, dietary habits and trophic niches is essential for effectively achieving that goal. Understanding these aspects becomes indispensable for assessing ecological responses to environmental changes, evaluating ecosystem health, and assessing the impact of invasive species on resource competition with native ones. Particularly in remote and arid areas where resources are scarce or facing imminent anthropogenic impacts, the loss of even a single resource can be catastrophic for community stability (Lopes *et al.*, 2019; McNeely, 2003). Moreover, this approach facilitates rapid surveys for understudied taxa on remote islands that are difficult to access, which conventional methods cannot accomplish (Gil *et al.*, 2020). The large amount of data provided within a short time span can significantly help to increase the success of biodiversity conservation actions (Ji *et al.*, 2013). However, dietary studies focusing on reptile species still lag behind compared to other groups such as mammals (Ando *et al.*, 2020). In general, reptiles, particularly those inhabiting remote arid regions, often remain overlooked in insular and arid systems, resulting in insufficient knowledge about their ecological associations. As the effects of climate change are expected to be even more pronounced in arid and semi-arid regions, such as Cabo Verde and Saudi Arabia (Allan & Douville, 2024), it is imperative to study the biodiversity of these areas, which is particularly facilitated by this technique.

1.5. General objectives and thesis structure

The main aim of this thesis was to use the power of DNA metabarcoding techniques to reveal the dietary composition of terrestrial vertebrate taxa and to uncover trophic interactions. In doing so, this thesis aimed to explore the evolutionary patterns involved in shaping community assembly in insular systems and to provide data for the sustainable conservation of threatened species. This thesis consists of five chapters which contain six articles.

Chapter 1 is the present general introduction, in which the conceptual basis of the thesis is presented. It emphasises the importance of island systems in ecological and evolutionary studies, with a particular focus on the Cabo Verde archipelago. It explores island-like systems as analogous models and highlights the power of metabarcoding in biodiversity surveys. The chapter concludes by outlining the general aims and structure of the thesis.

In **Chapter 2**, entitled "Metabarcoding as a tool to study trophic interactions", the main goal was to explore trophic relationships among sympatric and allopatric populations and investigate the role of competition and resource partitioning on species coexistence. Ultimately it was aimed to contribute to a comprehensive understanding of the ecological dynamics within the Cabo Verde Archipelago. The expected results should improve the understanding of the ecological requirements of those species and thus inform targeted conservation efforts in the country. This chapter includes three articles, whose specific objectives are detailed below.

In **Article I**, entitled "Table for two: diet composition differences of allopatric and sympatric island geckos"**,** the Cabo Verde system was used as a framework to investigate ecological and evolutionary responses to competition in endemic geckos. Employing DNA metabarcoding of faecal pellets, this study aimed to investigate diet composition differences between the sympatric species *Tarentola gigas* and *Tarentola raziana* of Raso Islet, and the allopatric population of *T. raziana* from Santa Luzia Island (Figure 2A). By assessing dietary habits in allopatric contexts, this study explores the resource partitioning mechanisms that favour the long-term coexistence of these species of considerable size discrepancy. Similarly, **Article II**, entitled "Smaller islands, bigger appetites: evolutionary strategies of insular endemic skinks", examined the same pattern of striking size variation in endemic skinks from Cabo Verde. Also using DNA metabarcoding of faecal pellets, the diet composition of the sister species *Chioninia vaillantii* and *Chioninia delalandii* was investigated in two sympatric contexts (Figure 2B). On Fogo, where this species pair occurs in humid high elevation areas with abundant vegetation, and on Rombo's Islets (Cima Islet), where competitive pressures are expected to be higher. The diet composition of the allopatric population of *C. delalandii* inhabiting lowland areas of Fogo was also assessed. In addition, linear morphological measures were compared among populations to explore how competitive pressure has shaped the evolution of body morphology in these species. Ultimately, both **Articles I & II** aimed to discuss the ecological and evolutionary mechanisms that facilitate the

coexistence of these sister species in Cabo Verde. **Article I** is already submitted to Global Ecology and Conservation and **Article II** is in preparation for submission.

Article III, entitled "Diet study of geckos reveals the first records of pseudoscorpions on Desertas Islands (Cabo Verde)", reveals an unexpected outcome of studying the diet of these endemic reptiles. In addition to inferring trophic interactions, dietary studies can also reveal unknown biodiversity data. This article presents the first record of pseudoscorpions for the Desertas Islands. These specimens were collected during a dietary study of *T. gigas*. One pseudoscorpion was found in a fresh faecal pellet of a gecko captured on Raso. The remaining six pseudoscorpions were caught in pitfall traps set to build up a reference collection of possible invertebrate prey from these islands. This article has already been published in the Journal of Arachnology (Santos *et al.*, 2022).

Chapter 3, entitled “Metabarcoding as a tool to explore vertebrate extinction drivers”, is comprised of **Article IV**, entitled “Digging in a 120 years-old lunch: What can we learn from collection specimens of extinct species?”. This article took advantage of the power of DNA metabarcoding technologies to shed light on the enigmatic ecology of the extinct endemic Cabo Verde giant skink *Chinionia coctei*. Particularly, this work aimed to learn more about the health status and diet of the last three known specimens of giant skink collected in 1901 on Branco Islet by the Princess Alice II expedition and which, according to public knowledge, have never been examined by scientists so far. In addition, the possible reasons that may have contributed to its extinction were discussed, which may help to develop conservation measures for the extant threatened reptile species of Cabo Verde. This study demonstrated the potential of NGS metabarcoding to recover valuable information from collection specimens. This article has been published in the journal PLOS One (Pinho *et al.*, 2022b).

Lastly, **Chapter 4**, entitled “Metabarcoding diet analysis of terrestrial vertebrates for conservation”, aimed to explore the applicability of the DNA metabarcoding techniques employed throughout this thesis to study trophic interactions in other species and other insular systems, in order to advise responsible authorities on the development of conservation actions. This chapter is composed by two articles whose specific objectives are detailed below.

Article V, entitled “Trust your guts? Diet composition differences of *Mus musculus* gastrointestinal tract sections using metabarcoding”, aimed to investigate for the first time the impact of the invasive species *Mus musculus* in Cabo Verde using a DNA

metabarcoding approach. Such studies are important since this invader has the potential to affect the already threatened biodiversity and economy of the country. With this study, it was also intended to investigate a methodological aspect of metabarcoding techniques, namely whether there are differences in dietary estimates depending on the section of the gastrointestinal tract analysed, and which section(s) provided a better representation of the diet. To do this, the gut contents of the house mouse were examined, considering three sections: stomach, small intestine and large intestine. In addition, it was aimed to provide a framework to infer the ecological/ economic impact of invaders on an island ecosystem, especially in agricultural areas. This article has been published in the journal Ecology and Evolution (Pinho *et al.*, 2022a).

Lastly, in **Article VI**, entitled “Green matters: Dietary assessment of a reptile community using DNA metabarcoding”, a non-invasive approach was implemented, taking advantage of the metabarcoding of faecal pellets to investigate the dietary composition and the possibility of resource segregation by the reptile community of Wadi Ashar located in AlUla County, north-western Saudi Arabia. Alongside, a biodiversity survey was carried out to assess the diversity of reptile species in the community. The ultimate goal was to explore ecological patterns through prey items consumed and contribute to a better understanding of the trophic networks involving plants, arthropods, and reptiles at a community level in this restricted area. This data is expected to inform local authorities about which elements are central to the sustainable management of the reptile community and its composition. This article is already published in the Global Ecology and Conservation journal (Pinho *et al.*, 2023).

Chapter 5 presents a general discussion of the main results and conclusions of the thesis. It highlights the main findings and common patterns and processes observed across this work, the implications of the thesis results for the conservation of the species studied, the main constrains faced along this work, and future research perspectives.

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Chapter 2

Metabarcoding as a tool to study trophic interactions

Article I

Table for two: diet composition differences of allopatric and sympatric populations of island geckos

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Abstract

Oceanic islands are exciting models for studying how evolutionary processes and environmental variables can jointly contribute to speciation and community assembly over time. In this context, the Cabo Verde archipelago serves as a simplified natural laboratory, harbouring a group of endemic reptile species that are descended from a single common ancestor, with sympatric species exhibiting wide morphological variation, particularly for size, and competing for limited food resources. This framework allows for the exploration of how the diet composition of closely related and spatially overlapping species is influenced by competition. This is exemplified by the two endemic wall geckos, *Tarentola gigas* and *Tarentola raziana,* which occur in sympatry on Raso Islet, with the latter also occurring in allopatry on Santa Luzia Island, due to the human-mediated extinction of *T. gigas*. DNA metabarcoding of faecal pellets was used to compare the diets of both sympatric and allopatric populations. It revealed significant differences in diet composition between both populations of *T. raziana*, with significantly lower prey richness and niche breadth in the Santa Luzia population. Differences observed in sympatric species were due to a higher incidence of vertebrates and plants in the diet of *T. gigas*, while differences between the allopatric populations of *T. raziana* were due to a higher incidence of plants and a lower incidence of invertebrates in the population of Santa Luzia, compared to Raso. These results suggest that competition has played an important role in structuring the stable coexistence of these species in sympatry, promoting extreme morphological discrepancies that allowed resource partitioning. In addition, this study reveals unexpected dietary differences within the allopatric population of *T. raziana* on Santa Luzia, suggesting a niche shift in response to the absence of a direct competitor that may influence the reintroduction planning of *T. gigas*. Hence, this work highlights the importance of understanding the mechanisms of resource partitioning for conservation efforts and management, especially for fragile island ecosystems.

Keywords: Cabo Verde; DNA metabarcoding; ecological processes; endemic reptiles; Macaronesia

Introduction

Trophic interactions are one of the main factors shaping natural communities and population dynamics (Gravel *et al.*, 2011; Warren *et al.*, 2015). In small and isolated ecosystems such as oceanic islands, typically characterised by extreme environmental conditions and unique habitats, these trophic networks are often simplified with a low number of species and limited trophic links (Gil *et al.*, 2020; Lopes *et al.*, 2019; Warren *et al.*, 2015). In the case of islets, their small size can even lead to closer links between terrestrial and marine trophic webs (Lopes *et al.*, 2019). Moreover, islands typically host a high number of endemic and often threatened species with unique adaptations, that coexist and coevolved in such restricted space, by occupying distinct ecological niches (Dufour *et al.*, 2018; Tamar *et al.*, 2019). Compared with mainland areas, island communities are typically more vulnerable to species-level extinctions due to environmental and habitat changes, loss of genetic diversity, introduced species, and the limited potential and realised range sizes of insular species. Thus, small alterations, such as a decrease in the availability of prey species, may drastically affect the equilibrium of the ecosystem (Whittaker *et al.*, 2017). This makes oceanic islands excellent models for studying evolutionary and ecological dynamics.

Cabo Verde is an arid oceanic archipelago of ten islands and several islets within the Macaronesia biogeographical region (Figure 1A), with ages between 2–26 million years, My (Ancochea *et al.*, 2015). This archipelago, within the Mediterranean biodiversity hotspot (Mittermeier *et al.*, 2011), hosts a remarkable reptile biodiversity, including a total of 32 endemic terrestrial reptile taxa from three genera (Vasconcelos *et al.*, 2013, 2020). Among these, geckos within the genus *Tarentola* are estimated to have originated from a single colonization event from the Canary Islands approximately 7.7 My ago, followed by the beginning of radiation around 6 My ago (Carranza *et al.*, 2000; Vasconcelos *et al.*, 2010). Their diversification within Cabo Verde has resulted in 12 endemic species and four subspecies (Vasconcelos *et al.*, 2010, 2012), some of which co-occur in pairs across the islands of the archipelago. Although the Canary Island ancestor was most likely of medium size, in Cabo Verde there is a very marked size difference between sympatric species (Carranza *et al.*, 2000; Vasconcelos *et al.*, 2012). This contrast is particularly evident in the northern islands with the case of the giant wall gecko *Tarentola gigas* (Bocage, 1875), locally abundant on Raso and Branco islets, but recently extinct on Santa Luzia Island, and the Raso wall gecko *Tarentola raziana* Schleich, 1984, occurring across all islands of the Desertas Island group (Santa Luzia Island, Branco and Raso islets; Vasconcelos *et al.*, 2013), the two species representing the largest and

smallest geckos in the archipelago, respectively (Vasconcelos *et al.*, 2012). These belong to two sister clades that diverged approximately 4.5 My ago (Vasconcelos *et al.*, 2010). The *T. gigas* clade comprises tendentially larger species than the ancestral species, whereas species in the *T. raziana* clade are generally similar in size to the ancestral species (Vasconcelos *et al.*, 2012). Therefore, building from a historical phylogenetic signal for size differentiation, the two species most likely further diverged in opposite directions accentuating this size discrepancy. Both species are nocturnal and syntopic on Raso and Branco (Vasconcelos *et al.*, 2013), having access to the same limited resources and being subjected to the same climatic pressures, thus creating the potential for competition between them.

The competitive exclusion hypothesis suggests that two coexisting species cannot completely overlap in their ecological niche (Gause, 1934). Thus, it has been suggested that syntopic reptile species should rapidly diverge in their ecological niche as a response to competition (Amorim *et al.*, 2017; Dufour *et al.*, 2017). This is often emphasised by variations in morphological features that allow differential resource use and subsequent resource partitioning (Schoener, 1974; Williams, 1983). This can be achieved through, either consuming different food items (e.g., Crested Butte bumblebees (Pyke, 1982)) or using the same limiting resource in different ways (e.g., Bimini Island *Anolis* (Schoener, 1968)). These ecological differences should favour the long-term coexistence of species and thus the diversity of the communities (Pianka, 2011). Understanding dietary niche partitioning among evolutionary and ecologically related and co-occurring species is important for the development of a general understanding of community assembly and co-evolution (Arrizabalaga-Escudero *et al.*, 2018; Kartzinel *et al.*, 2015; Schmack *et al.*, 2021). This is greatly facilitated within systems where it is possible to characterise dietary habits within allopatric populations of one or both species. Such a sampling framework allows for a greater understanding of the extent to which the diet composition of geographically overlapping species is influenced by competition. On the island of Santa Luzia, the Raso wall gecko occurs without the presence of the giant wall gecko, providing an appropriate comparative framework for interpreting the diet of *T. raziana* when there is no competitor. Previous morphological analyses of faecal contents have suggested that *T. raziana* feeds mainly on arthropods (Mateo *et al.*, 2016; Schleich, 1987), while *T. gigas* appears to feed on a mix of arthropods, plants, birds, reptiles and fish remains (Mateo *et al.*, 2016; Schleich, 1980, 1987). However, the trophic competition between them has never been explored in detail. The diet of *T. gigas* has recently been characterised on the islands of Branco and Raso through metabarcoding of faecal

pellets, highlighting a strong trophic link with seabirds, and revealing the importance of plant species within its diet (Lopes *et al.*, 2019; Pinho *et al.*, 2018).

Large reptiles are often prone to a more herbivorous diet in insular ecosystems (Meiri, 2008; Van Damme, 1999), and a shift to plant consumption may also lead to changes in the structure and function of the digestive system, such as longer gastrointestinal tracts and the development of cecal valves (Pafilis *et al.*, 2016; Sagonas *et al.*, 2015). These morphological adaptations are even observed in omnivore species when compared to strictly insectivorous reptile species (Herrel *et al.*, 2004). Elongation of the intestine allows for slower gut passage times and longer exposure to digestive enzymes, thus increasing the digestive efficiency and nutrient absorption of fibrous plant items (Dearing, 1993; Pafilis *et al.*, 2016), which can be difficult to digest due to their thick cell walls when compared to animal items (Holland *et al.*, 2020). These adaptations may reflect the evolutionary responses of island reptiles to optimise energy intake from limited resources and enhance survival in resource-limited island habitats (Sagonas *et al.*, 2015), which can be the case for *T. gigas*.

DNA-based non-invasive studies can provide a large amount of data at different trophic levels with less time investment, disturbance and more cost-effectively, compared to alternative approaches (Pompanon *et al.*, 2012; Taberlet *et al.*, 2012). Additionally, compared to conventional methods, DNA-based approaches provide for improved taxonomic resolution, detection of rare prey species and detection of soft, small prey that can be undetectable by classical methods after digestion (Symondson, 2002). This is particularly important when studying endangered or near-threatened species such as the Cabo Verdean *Tarentola* geckos. Besides highlighting specific trophic relationships, such data can additionally shed light on species coexistence and the partitioning of food resources. Here we use DNA metabarcoding of faecal pellets to investigate how diet composition varies among sympatric populations of *T. gigas* and *T. raziana* on Raso Islet, and the allopatric populations of *T. raziana* from Santa Luzia Island and Raso. In the context of competition theory, we predict that *T. gigas* and *T. raziana* will have moderate dietary overlap and will compete for some resources, but with evidence for resource partitioning and dietary specialization. Furthermore, we predict that any specialisation toward plants will be accompanied by gastrointestinal adaptations, thus providing stronger support for dietary differences as an evolved response. Between the two allopatric populations of *T. raziana,* we expect to observe a signal of ecological release with the Santa Luzia population through dietary niche expansion, in response to the absence of the competitor. Moreover, by contributing to the general understanding of competition theory, the knowledge gained on the ecological needs of these two

species can support local conservation actions, such as guiding the reintroduction of *T. gigas* on Santa Luzia.

Methods

Study area

The Desertas island group is located within the northwest of the Cabo Verdean archipelago and is composed of Santa Luzia Island, together with the Branco and Raso islets (Figure 1). These islands are currently uninhibited and were designated as nature reserves in 1990 and marine protected areas in 2003. The Desertas, together with São Vicente, are thought to have been connected during lower sea levels in the Pleistocene (Figure 1B; Ancochea *et al.*, 2015) and share elements of their fauna and flora (Romeiras *et al.*, 2019; Vasconcelos *et al.*, 2015). Santa Luzia has an area of approximately 35 km^2, with a landscape of arid lowlands, mountainous areas, streams and floodplains, and sandy areas (Figure 1C; Diniz *et al.*, 1994). Raso, with an area of less than 6 km^2, is characterized by flat and low-altitude arid zones, small intermittent streams (Figure 1C), and vegetation mainly composed of patches of grasses (Freitas *et al.*, 2015; Gomes *et al.*, 2023).

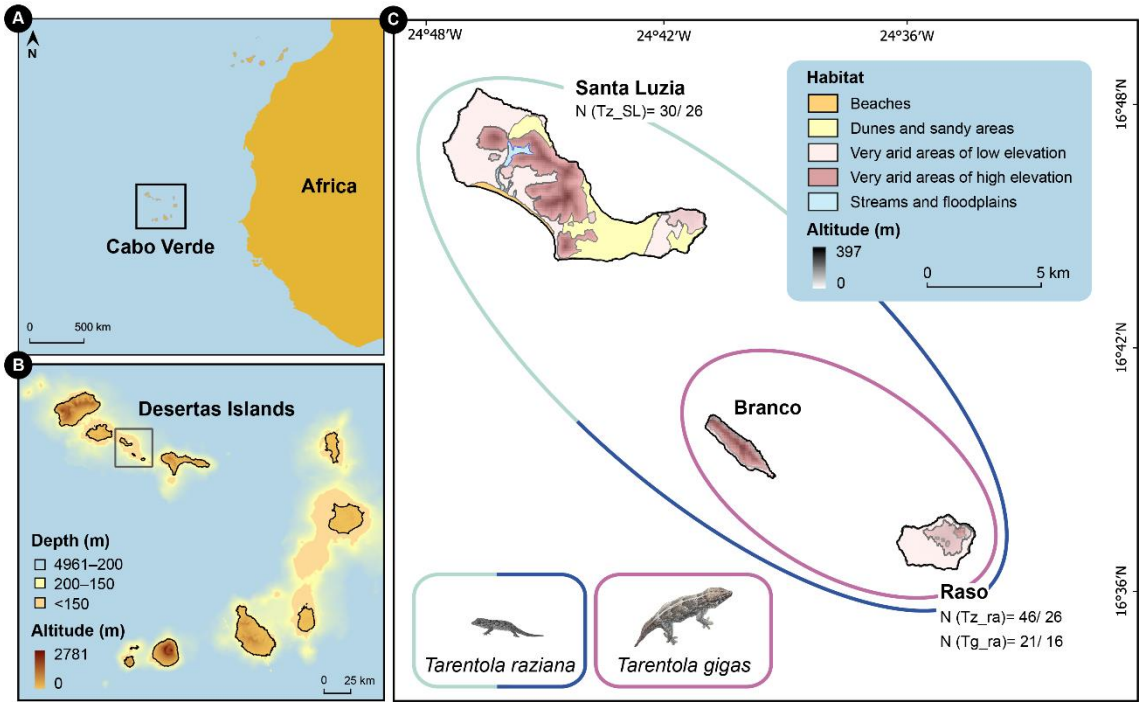

Figure 1. Study area and species. A) Geographic location of the Cabo Verde Archipelago. B) Desertas Islands, depicting elevation and bathymetry. C) Habitat types and distributions of the three *Tarentola* populations (*T. raziana* from Santa Luzia in light blue, Tz_SL, from Raso in dark blue, Tz_ra, and *T. gigas* from Raso, Tg_ra in pink), showing the number of faecal samples (N) extracted, and used in the analyses, respectively (Geographic Coordinate System, Datum WGS84).

Study species

The Cabo Verde giant wall gecko *Tarentola gigas,* currently one of the largest geckonids in the world (maximum snout-vent length, SVL of 155 mm), is classified as Endangered in the International Union for Conservation of Nature (IUCN) Red List of Threatened species, primarily due to its restricted distribution and exploitation of its prey (Vasconcelos, 2013a). It is divided into two morphologically distinct subspecies, *Tarentola gigas brancoensis* (Schleich, 1984) (mean SVL= 94.80 ± 1.79 mm), found solely on Branco Islet, and *Tarentola gigas gigas* (Bocage, 1875) (mean SVL= 105.47 ± 1.91 mm), found exclusively on Raso Islet (Delgado *et al.*, 2021; Joger, 1984; Schleich, 1984; Vasconcelos *et al.*, 2012). The Raso wall gecko *Tarentola raziana*, the smallest Cabo Verdean wall gecko (mean SVL= 49.30 ± 4.00 mm), is classified as Near Threatened in the IUCN Red List of Threatened species and can typically be found in rocky areas in Santa Luzia, and across the Branco and Raso Islets (Vasconcelos, 2013b). This species is distinguishable from *T. gigas* by its smaller size, its particularly pointed snout and concave forehead, with no tubercles between the eye and the ear opening, and its dark banded dorsal colouration (Vasconcelos *et al.*, 2012). Both species are oviparous, and ground-dwelling (Vasconcelos, 2012, 2013a, 2013b).

Sampling

Sampling took place during the dry season on Raso (June to August 2016) and Santa Luzia (May 2021). On Raso, transects were performed to cover the island area and a total of 21 and 46 faecal samples were collected for *T. gigas* and *T. raziana*, respectively. On Santa Luzia, transects were carried out in the northwest of the island, where the habitat is mainly characterized by arid lowlands, analogous to Raso (Figure 1C) and a total of 30 samples were collected for *T. raziana*. The individuals were captured by hand and an abdomen massage was performed to obtain fresh pellets, which were immediately preserved in 96% ethanol to avoid contamination. Samples were refrigerated at 4 °C as soon as possible and then transported to the CIBIO research centre in Portugal and stored at -20ºC until further processing. Specimens were sexed, measured (SVL to the nearest mm) and photographed to corroborate species identification. Animals were kept in individual tissue bags while others were being processed, to minimize their stress and georeferenced using a GPS device before being released at the same site of their capture.

To further develop a local DNA barcode reference library, an additional 56 samples of invertebrates and 16 of plants from Santa Luzia Island to those characterised by Pinho *et al.* (2018), were collected and processed, following protocols described in Pinho *et al.* (2018).

DNA extraction and sequencing

DNA extraction of faecal pellets was carried out, including negative controls, using the Stool DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada), following the manufacturer's instructions. All DNA samples were amplified with three previously validated genetic markers (Pinho *et al.*, 2022; Pinho *et al.*, 2018): IN16STK-1F-mod/IN16STK-1R-mod primers, targeting the mitochondrial 16S gene (Pinho *et al.*, 2018) for invertebrates; g/h primers targeting the short P6-loop of chloroplast trnL (UAA) gene for plants (Taberlet *et al.*, 2007); and the primer pair 12sv5F/12Ssv5R, targeting V5-loop within the mitochondrial 12S gene for vertebrates (Riaz *et al.*, 2011). To avoid the amplification of *T. gigas* and *T. raziana* DNA, a blocking primer for the 12S, previously designed for *T. gigas*, was used (Pinho *et al.*, 2018). Its effectiveness for *T. raziana* was previously validated by comparing PCR amplification with and without the blocking primer, using DNA extracted from the tail tissues with the EasySpin™ kit, following the manufacturer's instructions. All PCRs were performed, including negative controls, with the conditions described by Pinho *et al.* (2018). Libraries were prepared following the Illumina MiSeq protocol ‘16S Metagenomic Sequencing Library Preparation’ (Illumina, 2013). Samples were sequenced in a MiSeq sequencer (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit V2 (Illumina, San Diego, CA, USA) for an expected average of 20,000 paired-end reads per PCR product.

Plant DNA for the reference collection was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following modifications described in Romeiras *et al.* (2015). Invertebrate DNA for the reference library was extracted from legs or wings, using saline extraction methods following Carranza *et al.* (1999). Invertebrate DNA from the reference collection was amplified using two primer sets. The IN16STK primer set, allowing the match with diet items, and the LCO1490/HC02198 primer set, targeting the animal barcode region of the cytochrome oxidase I (COI). PCR conditions used for the latter are described in (Folmer *et al.*, 1994). Barcode sequencing was undertaken to assist with taxonomic assignments. Plant DNA was amplified for the chloroplast TrnL (UAA) using the primer set c/d (Taberlet *et al.*, 2007) to allow comparison with diet items. All individual-based amplicons were Sanger sequenced.

Bioinformatics filtering

The software package OBITtools was used for general sequence processing, which includes the alignment of the sequences obtained and the filtering of sequencing errors, to obtain molecular operational taxonomic units (MOTUs), using pipelines described in Pinho *et al.* (2018). Based on PCR blank counts, all PCR products with less than 300 reads were removed and within the remaining samples, MOTUs representing less than 1% of the total reads of that PCR product were also excluded. The remaining MOTUs were then compared against known reference sequences in the NCBI Nucleotide Database and our reference collection, using the BLAST+ software. Our local database was created using *makeblastdb* and compared against obtained diet sequences using *blastn*. Sequences that had less than 85% of percent identity value with known species were classified only to the class level, the ones with values between 85% to 90% to the order level, while sequences with values between 90% to 95% were classified to the family level. Sequences with similarity values above 95% that match to a single species were classified to the species level. If a sequence matched more than one species with equal similarity values (>95%) all belonging to the same genus, a genus-level assignment was considered. When a sequence matched more than one species or genus with similar probabilities, only species or genera known to occur in the archipelago were considered, otherwise a higher ranking would be attributed (e.g., family). If more than one MOTU corresponds to the same taxon, a number would be attributed to each one of them (e.g., Poaceae_1 and Poaceae_2). Lastly, MOTUs detected in extraction blanks were identified as contaminations and removed from the corresponding batch of samples (e.g., human, fungi).

Data analysis

The frequencies of occurrence (FO) of each of the three diet item groups (plant, invertebrate, vertebrate) were estimated for the two sympatric populations from Raso (*T. gigas* – Tg_ra, and *T. raziana* – Tz_ra from hereafter) and *T. raziana* in allopatry on Santa Luzia (Tz_SL from hereafter). To test for potential differences in proportions of each diet item group between populations, FOs were compared using two-proportion z-tests with the function *prop.test* in R software version 4.2.2 (R Core Team, 2023).

Since it was not possible to achieve species-level taxonomic resolution for several MOTUs, family-level identification was used for the subsequent analysis. The FOs of each prey family were calculated for each population. To visualize and compare the

relative abundance and evenness of families present in the diet of each gecko population, rank-abundance curve plots were generated using the *rankabundance* function from the BiodiversityR package (Kindt *et al.*, 2005). To visualize the dietary networks at the family level for the three populations bipartite networks were generated using the package bipartite v2.17 (Dormann *et al.*, 2008).

Resource richness and niche breadth were compared among the three populations, at the family level, through rarefaction curves. Richness values were estimated for the double of species with the lower sample size and considered to significantly differ if there was no overlap in the 95% confidence intervals (Chao *et al.*, 2020). This was performed and visualized using the functions *iNEXT* and *ggiNEXT*, respectively, from the INEXT package (Hsieh *et al.*, 2022). Dietary niche overlap at the family level between the allopatric population of *T. raziana* (Tz_ra vs Tz_SL) and sympatric populations of *T. gigas* and *T. raziana* (Tg_ra vs Tz_ra) were calculated using Pianka's index (Pianka, 1973). This index ranges from 0 to 1, where 0 indicates no common resources and 1 complete overlap in resource use. To test if the obtained dietary niche overlap differed from what would be expected by chance, null models were performed based on the RA3 randomization algorithm (Lawlor, 1980), generating 10,000 null matrices that were to compare with our observed data, using the EcoSimR package (Gotelli *et al.*, 2015). Additionally, to evaluate prey-use differences between the three populations, non-metric multidimensional scaling (NMDS) was used. Samples were ordinated according to diet similarity in the two-dimensional space using the function *metaMDS* from the vegan package (Oksanen *et al.*, 2022) based on a Jaccard distance matrix.

Lastly, generalized linear models (GLM) were carried out to test for significant differences in diet composition between sympatric (Tg_ra vs. Tz_ra) and allopatric (Tz_ra vs. Tz_SL) populations, at the MOTU, family, and order levels. GLMs for multivariate presence/absence data were fitted by implementing the *manyglm* function of the mvabund package (Wang *et al.*, 2022). The complementary log-log distribution presented the best fit and was used for subsequent testing. The significance of the GLMs was tested using the *anova.manyglm* function. The same function was then implemented with the argument p.uni= 'adjusted' to perform univariate tests, identifying which prey items were responsible for differences in diet composition between species and populations.

Gastrointestinal comparisons

To conduct preliminary comparisons of gut length between *T. raziana* and *T. gigas*, five individuals of each species were opened from the collections of the National Museum of Natural History and Science of Lisbon (MUHNAC), with the permission of the curator (Table S1). These specimens were originally collected on Raso Islet in 1970. The SVL of the individuals was measured to the nearest mm, as well as the length of the small intestine (SI) and large intestine (LI).

The normality of the data was assessed using the Shapiro–Wilk test (Shapiro *et al.*, 1965) with the function *shapiro.test* from the stats package (R Core Team, 2023). To test for correlations between SVL and SI and LI lengths for each species the Pearson correlation coefficient (r) was calculated using *cor.test* function of the stats package (R Core Team, 2023). To test for significant differences between correlation values of the two species a Fisher's r-to-z comparison was used by applying the *paired.r* function of package psych (Revelle, 2023).

Results

Diet composition

The final dataset comprised 68 samples (Tz_SL= 26, Tz_ra= 26, Tg_ra= 16) with a total of 279,007 reads. Across all samples from both species, a total of 114 MOTUs were identified (Tz_SL= 30, Tz_ra= 68, Tg_ra= 55) from eight taxonomic classes (Tz_SL= 6, Tz_ra= 6, Tg_ra= 8), 42 orders (Tz_SL= 18, Tz_ra= 27, Tg_ra= 29) and 66 families (Tz_SL= 20, Tz_ra= 41, Tg_ra= 38; Table S2).

For Tz_SL, the most frequent families identified were Zygophyllaceae and Fabaceae (Figure 2A), namely the MOTUs *Tetraena simplex* (L.) Beier & Thulin (FO = 65.38%) and *Lotus brunneri* (FO= 38.46%). For the diet of Tz_ra, the most frequent family observed was Rhinotermitidae (Figure 2A), namely the MOTU *Reticulitermes* sp. (FO= 57.69%). The most frequent families in the diet of Tg_ra were Plumbaginaceae and Fabaceae (Figure 2A), namely *Limonium brunneri* (Webb ex Boiss.) (FO= 75%) and *Lotus brunneri* Webb (FO= 56%) MOTUs, respectively.

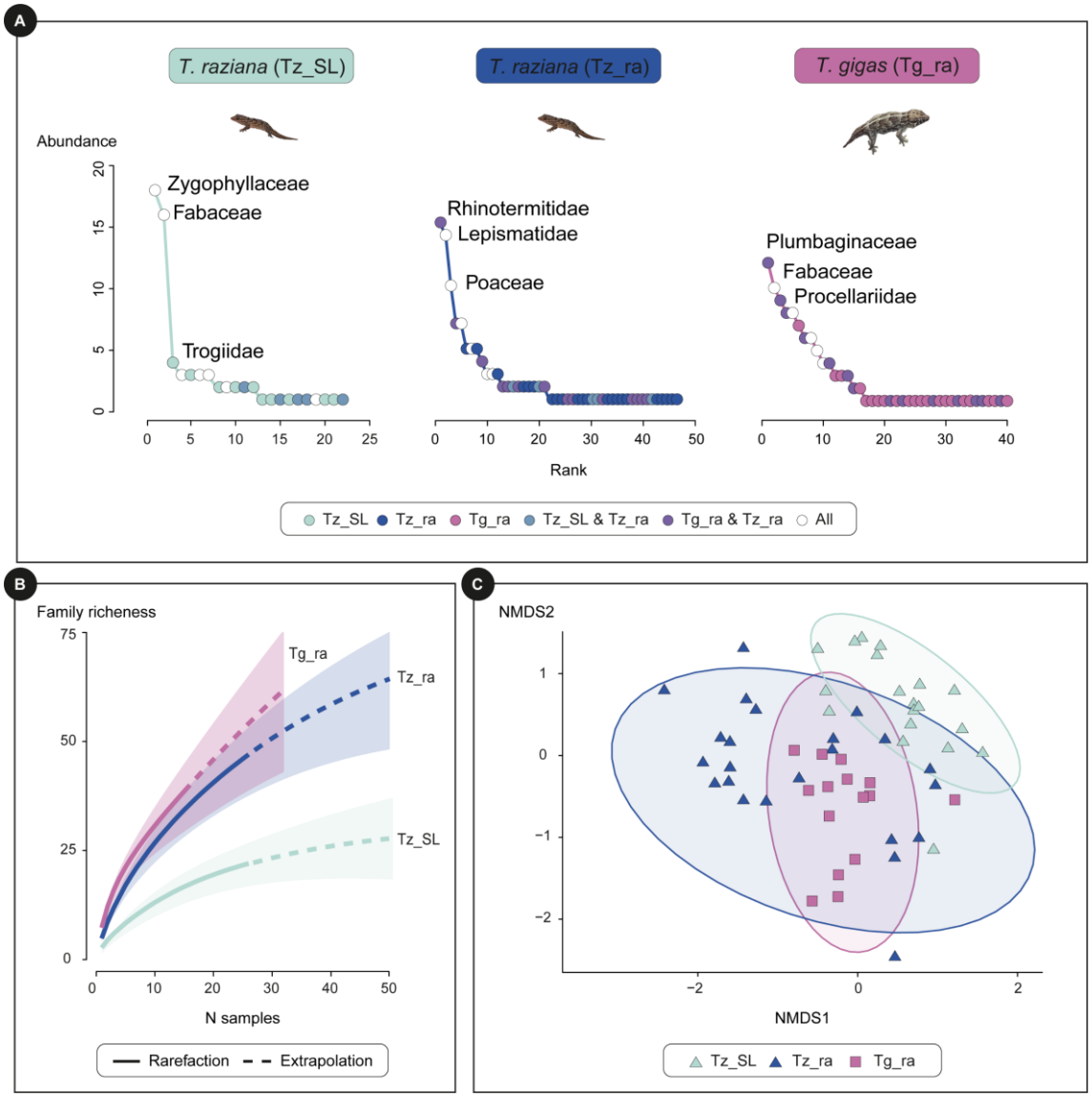

Figure 2. Diet composition results for the three *Tarentola* populations (*T. raziana* from Santa Luzia, Tz_SL, from Raso, Tz_ra, and *T. gigas* from Raso, Tg_ra). A) Rank abundance curve plots, showing the percentages of the three more abundant diet item families for each population and the common prey items among them. Distinct colours are used to indicate families exclusively present in each population, white if shared by all populations and summed colour if shared by two of them. B) Accumulation curves depicting observed (full line) and extrapolated (dashed line) family richness with their 95% confidence intervals. C) Non-metric multidimensional scaling ordination (NMDS) of the diet composition. Points closer together correspond to samples with a more similar dietary composition. Ellipses represent the standard error of the dietary composition centroids at 95% confidence intervals.

The occurrence of the three taxonomic diet groups (plants, invertebrates, vertebrates) differed between species and populations. Plants proportions differed significantly between populations ($\chi^2_{Tz_ra\ vs\ Tz_SL}$= 11.53, p= 0.001; $\chi^2_{Tz_ra\ vs\ Tg_ra}$= 4.67, p= 0.03), with higher frequencies in the diets of Tz_SL (FO_{Tz_SL} = 100%) and Tg_ra (FO_{Tz_ra} = 94%) than in Tz_ra (F_{Tg_ra} O= 58%). Invertebrates had similar frequencies in the sympatric populations (FO_{Tz_ra}= 62% FO vs $_{Tg_ra}$= 50%) and lower frequency for *T. raziana* in

allopatry (FO $_{Tz_SL}$= 35%), however none were significant (χ^2 $_{Tz_ra\ vs\ Tz_SL}$= 2.77, *p*= 0.09; χ^2 $_{Tz_ra\ vs\ Tg_ra}$= 0.17, *p*= 0.679). Vertebrate prey taxa were more frequent in the diet of the giant (FO $_{Tg_ra}$= 94%) than in both Raso wall gecko populations (FO $_{Tz_SL}$= 12%, FO $_{Tz_ra}$= 23%), with significant differences for the species in sympatry but not between *T. raziana* allopatric populations (χ^2 $_{Tz_ra\ vs\ Tz_SL}$= 0.53, *p* = 0.463; χ^2 $_{Tz_ra\ vs\ Tg_ra}$= 17.06, *p*= 3.61e^{-05}).

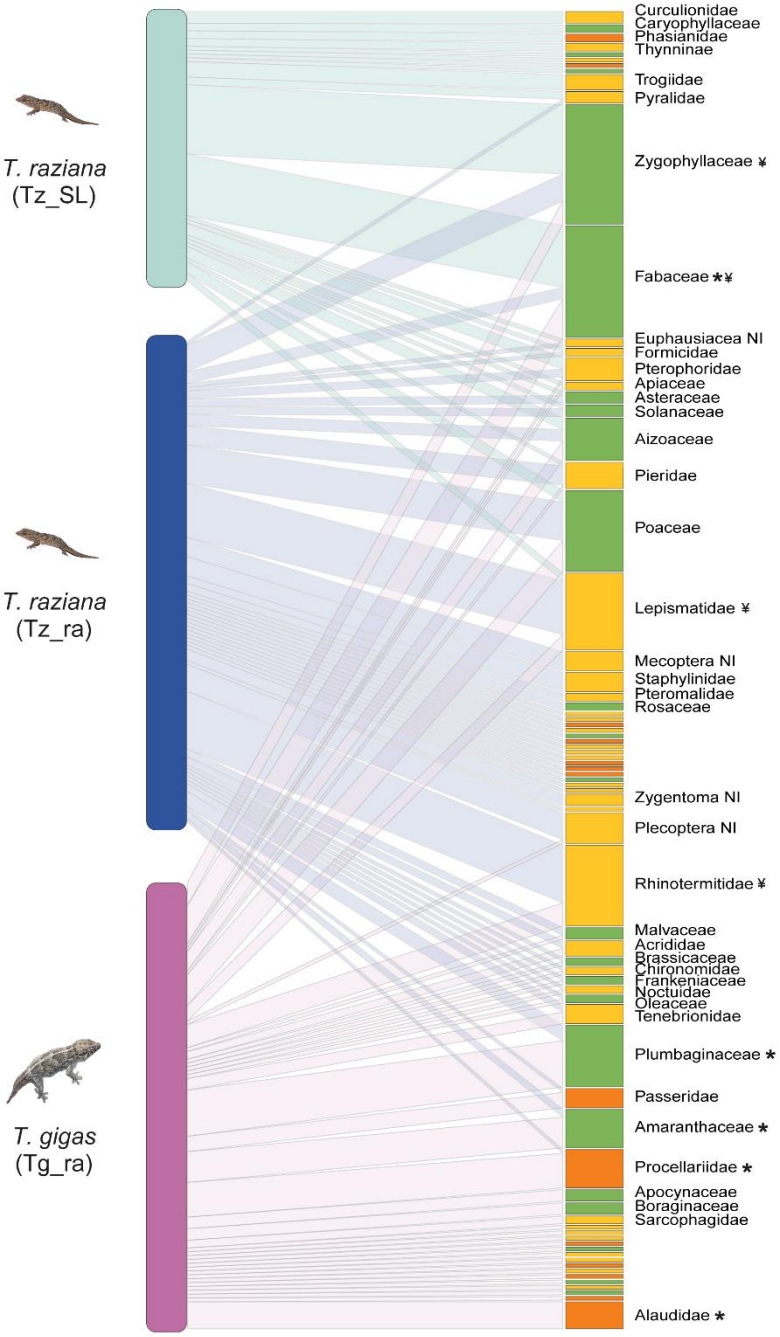

Figure 3. Bipartite network representing associations among the three *Tarentola* populations (*T. raziana* from Santa Luzia, Tz_SL, from Raso, Tz_ra, and *T. gigas* from Raso, Tg_ra), and their diet items. The predators are on the left and arthropod (yellow), plant (green) and vertebrate (orange) prey families on the right. The width of the connecting bars represents the frequency of occurrence of each family in the pellets of each population. The symbol (*) indicates the families that contributed significantly to differences between sympatric (Tz_ra vs Tg_ra) and (¥) in both *T. raziana* (Tz_ra vs Tz_SL) populations. Only family names with a frequency of occurrence above one are mentioned.

The two species living in sympatry (Tg_ra and Tz_ra) presented no differences in family-level richness, both observed and extrapolated, while *T. raziana* in allopatry presented significantly lower richness values (Tz_SL; Figure 2B). In sympatry, diet niche overlap (O) at the family level was significantly higher than expected by chance ($O_{Tz_ra\ vs\ Tg_ra}$= 0.57, p< 0.001; Figure 2C) and higher than overlap between allopatric *T. raziana* populations ($O_{Tz_ra\ vs\ Tz_SL}$= 0.36, not significant).

The multivariate analysis revealed significant differences in the diet composition of the two populations of *T. raziana* at the MOTU, family, and order levels (all with p= 0.001). Univariate tests showed that differences in diet composition between both populations of *T. raziana* were due to four MOTUs (*Reticulitermes*_1, *Tetraena simplex*, Plecoptera_1 and *Thermobia domestica* (Packard, 1837)), four families (Fabaceae, Rhinotermitidae, Lepismatidae and Zygophyllaceae; Figure 3) and five orders (Blattodea, Fabales, Zygentoma, Plecoptera and Zygophyllales), with invertebrate items present exclusively in the Tz_ra diet and plant items in the Tz_SL diet. Diet composition differences between sympatric populations (Tz_ra vs Tg_ra; p= 0.001) were mainly due to vertebrate prey taxa (*Alauda razae*, *Bulweria bulwerii* (Jardine & Selby, 1828)) and plants species (*Chenopodiastrum murale* (L.) S.Fuentes, Uotila & Borsch, *Limonium brunneri*, *Lotus brunneri*, *Patellifolia procumbens* (Chr.P.Sm.) A.J.Scott, Ford-Lloyd & J.T.Williams), all either consumed exclusively or in higher frequencies by *T. gigas*. The families that contributed significantly to these differences were Alaudidae, Procellariidae, Plumbaginaceae, Fabaceae and Amaranthaceae (Figure 3) and the Procellariiformes, Fabales and Passeriformes orders. The p-value results for the univariate tests are presented in Table 1.

Table 1. Results of univariate tests. The prey items that significantly (p< 0.05) contributed to the differences in diet composition between allopatric (Tz_ra vs Tz_SL) and sympatric (Tz_ra vs Tg_ra) populations, considering three taxonomic levels (Order, Family and MOTUs) are given.

Comparisons	**Order**	***p***	**Family**	***p***	**MOTUs**	***p***
Tz_ra vs Tz_SL	Blattodea	0.001	Fabaceae	0.001	Reticulitermes_1	0.001
	Fabales	0.003	Rhinotermitidae	0.001	*Tetraena simplex*	0.009
	Zygentoma	0.006	Lepismatidae	0.003	Plecoptera_1	0.017
	Plecoptera	0.017	Plecoptera_1	0.014	*Thermobia domestica*	0.050
	Zygophyllales	0.024	Zygophyllaceae	0.018		
Tz_ra vs Tg_ra	Procellariiformes	0.003	Alaudidae	0.003	*Alauda razae*	0.003
	Fabales	0.007	Plumbaginaceae	0.003	*Bulweria bulwerii*	0.003
	Passeriformes	0.007	Procellariidae	0.003	*Chenopodiastrum murale*	0.003
			Fabaceae	0.011	*Limonium brunneri*	0.003
			Amaranthaceae	0.033	*Lotus brunneri*	0.011
					Patellifolia procumbens	0.043

Gastrointestinal comparisons

Snout-vent length (SVL) and intestinal length (SI, LI) of museum specimens are shown in Table S1. The *T. raziana* and *T. gigas* individuals had an average SVL and standard deviation of 48.90 ± 4.04 and 105.30 ± 11.8, SI of 3.20 ± 0.27 and 11.3 ± 3.14 and LI of 1.62 ± 0.13 and 4.74 ± 0.44, respectively. There was no significant correlation in the relationship between SVL and the two intestine portions for *T. raziana* (r_{SI}= 0.2, p= 0.74; r_{LI}= -0.32, p= 0.6; Figure S1). For *T. gigas*, there was a significant positive correlation in the relationship between SVL and SI (r_{SI}= 0.88, p= 0.05; Figure S1), indicating an increase in SI length with body size, but no significant correlation was observed between SVL and LI length (r_{LI}= 0.72, p= 0.17; Figure S1). For LI the Pearson correlation value of *T. gigas* was significantly higher than that of *T. raziana* (p= 0.03), however, for SI the results were not significant (p= 0.19).

Discussion

The present study reveals significant differences in diet composition between *T. raziana* and *T. gigas* living in sympatry on Raso Islet, as well as between the two allopatric populations of *T. raziana* present on Santa Luzia and Raso. These results show that competition had an important role in structuring the stable coexistence of these sympatric species, promoting extreme morphological discrepancies that allowed differential resource use and subsequent resource partitioning. Additionally, this study reveals an unexpectedly high degree of dietary specialization in the allopatric population of *T. raziana* on Santa Luzia, indicating a niche shift as a response to the absence of a direct competitor.

Allopatric populations

On Santa Luzia, under reduced interspecific competition, *T. raziana* would be expected to exploit previously unused resources, consistent with ecological release and subsequent niche expansion (Pianka, 2011). However, in contrast to our initial hypothesis, the population from Santa Luzia demonstrated niche contraction rather than expansion, as evidenced by a significantly less diverse diet when compared to the Raso population. This indicates that, without its direct competitor, the Santa Luzia population has narrowed its dietary niche to focus on a smaller set of resources. One potential explanation for this could be in the context of an improved cost-benefit ratio, either

because food items are easier to access, more nutritious or have greater energy potential. The significantly higher frequencies of plants in the diet of Tz_SL, when compared to the Raso population, as well as the plant items (Fabales and Zygophyllales) contributing to the diet composition differences, reflect this specialisation. For instance, pollen and nectar-rich plants such as *Tetraena simplex*, highly frequent in the Tz_SL diet, may represent less demanding, easily digestible, and more nutritious food options than ground-dwelling invertebrates (e.g., termites and firebrats, two dietary items that differ significantly between Tz populations), and are consequently favoured by the Santa Luzia population. Although exhibiting similar frequencies of consumed vertebrates to the SL population, the Raso population shows a greater variety of items (eight for Tz_ra and two for Tz_SL) including seabird MOTUs (*Calonectris edwardsii* (Oustalet, 1883) and *Puffinus boydi* Mathews, 1912) and several fish species, indicating a diet link with seabird communities and different feeding habits. This most likely results from the ingestion of seabird regurgitations and faeces, a behaviour already reported for *T. gigas* (Schleich, 1980). On the contrary, the vertebrates found in the diet of the Santa Luzia population are certainly of anthropogenic origin (e.g., chicken from waste left by fishermen/ rangers), reflecting mostly opportunistic habits of this population rather than active foraging. The observed results are in line with optimal foraging theory which predicts that as the availability of preferred resources decreases, the dietary niche breath should expand (Perry *et al.*, 1997). Particularly on Raso, given the low resource availability, the preferred food items of *T. raziana* may be further restricted due to competition for resources with *T. gigas*. Thus, *T. raziana* individuals may have been forced to expand their diet to include previously overlooked prey items that may be less nutritious (Bonesi *et al.*, 2004), leading to niche expansion with increased competition. In contrast, individuals on Santa Luzia are expected to have easier access to preferred resources, resulting in a more restricted diet, by preying on a small set of high-value resources while avoiding those with an opportunity cost exceeding their value (Parent *et al.*, 2014; Wilson *et al.*, 1994). These results justify the reintroduction of *T. gigas* on Santa Luzia on different areas of *T. raziana* occurrence to prelude mutual exclusion.

It is important to note that there may also be alternative competitive pressures acting within Santa Luzia, such as introduced mammals, which are known to be a major threat to reptiles on the island (Geraldes *et al.*, 2016; Medina *et al.*, 2020). The recent eradication of cats on Santa Luzia has led to an exponential increase in the density of mice, which share activity patterns and refuges with *T. raziana* (Pinho & Vasconcelos, pers. comm.). This could pose an additional threat to this already near threatened endemic species, through competition for resources, or even predation. Consequently,

it may be of future interest to characterise the diet of the introduced mouse populations on Santa Luzia and evaluate the level of dietary overlap and predation intensity on *T. raziana*.

Sympatric populations

The two sympatric populations on Raso showed moderate dietary overlap, even though sharing the same limited resources on the small islet of Raso. This suggests that although there is resource competition, the species were able to establish a stable coexistence by exploiting different resources, confirming our initial hypothesis. As stated by Pianka (1973), species cannot overlap in all basic niche dimensions. On Raso, *T. raziana* and *T. gigas* show high overlap in two dimensions, activity pattern and distribution (Vasconcelos *et al.*, 2013). Therefore, to effectively reduce interspecific competition, it would appear that they have reduced overlap in the trophic dimension through resource partition.

Both species showed similar richness and niche breadth values, indicating that the variety of items consumed is similar. However, diet composition was significantly different between the two, which further supports dietary niche segregation as a mechanism through which competition is avoided (du Preez *et al.*, 2017; Razgour *et al.*, 2011; Westeen *et al.*, 2023). In particular, several plant and vertebrate items contributed significantly to differentiating the diet of the two sympatric species, by presenting significantly higher proportions in the diet of *T. gigas*. Only two (*Passer iagoensis* (Gould, 1838) and *Calonectris edwardsii*) out of the 14 vertebrate MOTUs found were shared between species. Similarly, despite invertebrates showing similar proportions in both diets, only 12% of the invertebrate MOTUs were shared between the species when in sympatry, highlighting their distinct exploitation of the islet resources. On the other hand, half of the plant MOTUs were shared between the two species, which may reflect the high availability of these food items on the islet. However, although both species may feed on plants, they can consume different parts of the plant and with different nutritional values, or at different rates or times of the day to avoid competition (Schoener, 1974; Williams, 1983), a pattern that unfortunately cannot be distinguished through DNA metabarcoding.

The marked body size difference between the two species likely influences prey selection and facilitates resource partitioning. For instance, *T. gigas* presents a stronger trophic link with terrestrial and marine birds and fish, as previously reported by Lopes *et al.* (2019), than *T. raziana*, and this is most likely favoured by its larger size and expected

stronger bite force (Donihue *et al.*, 2023; Herrel *et al.*, 2001). Additionally, this may indicate a strong influence of seabird colony densities of Raso on the body size evolution of *T. gigas*, as suggested by Stadler *et al.* (2023), promoting the selection of continually larger individuals in the direction of gigantism. Likewise, plants were demonstrated to have a significant role in the diet of *T. gigas* (Pinho *et al.*, 2018), most of which are succulent plants or with succulent parts such as fleshy stems or leaves and fruits (e.g., *Limonium bruneri*). These should be easily consumed by *T. gigas* due to its expected stronger bite, whereas *T. raziana*, most likely relies on more nutritious and easier to consume options such as pollen and nectar. Furthermore, the most frequent invertebrate families in the diet of *T. raziana* on Raso, Rhinotermitidae (Blattodea) and Lepismatidae (Zygentoma), are both mainly composed of small and ground-dwelling individuals. This suggests that *T. raziana* individuals may be more likely to target smaller sized prey, as suggested by Arnold *et al.* (2008), whereas *T. gigas* requires prey with a higher energetic value to meet its nutritional needs (Pough, 1973).

Morphological variations

A shift towards herbivory is a common adaptation of insular reptiles of large size, which have proportionately larger intestinal sizes that allow easier plant digestion (Carretero, 2004; Pafilis *et al.*, 2016; Van Damme, 1999). The present results further support this hypothesis, by showing a significant positive relationship between small intestine length and body size for *T. gigas* which is not observed in *T. raziana*. Additionally, the total average ratio between body size and intestinal length was proportionally almost twice in *T. gigas* than in *T. raziana*. This suggests that intestinal length is part of the ontogeny of this species (Herrel *et al.*, 2008; Pafilis *et al.*, 2016; Vervust *et al.*, 2010). This allows for longer intestinal transit times and the digestion of difficult items such as vertebrates and plants, which are highly important in the diet of adult *T. gigas*. This adaptation maximises energy yield, which is essential for large reptiles with high energy requirements (Pafilis *et al.*, 2016; Sagonas *et al.*, 2015). Unfortunately, and as both are protected species, we were only allowed to sample five vouchers of each species, which does not provide strong statistical power. However, there is a clear trend that should be investigated further with larger sample sizes. Additionally, differences in diet composition are most likely related to other morphological differences between the two species, that enabled their coexistence (Dufour *et al.*, 2018; Hutchinson *et al.*, 1959; Schoener, 1970). Our results provide evidence that apart from the phylogenetic signal for size differentiation, reflected in the presumed size discrepancy between the ancestors of the two sister clades (Carranza *et al.*, 2000; Vasconcelos *et al.*, 2010), competition might have

favoured character displacement, selecting for individuals with extreme sizes for both *T. gigas* and *T. raziana*. This indicates that, as the availability of preferred resources is reduced due to intraspecific competition, selection might favour individuals that can explore very distinct resources, thereby releasing competitive pressure (Martin *et al.*, 2009). In the context of ecological specialization and resource competition, our data suggest that *T. gigas* individuals were most likely selected to be larger than, and *T. raziana* smaller than, their respective ancestral forms (Grant, 1972). This could explain why *T. gigas* and *T. raziana* are the largest and the smallest species within their respective clades, and the only species from these clades that occur in sympatry, representing a possible case of ecological character displacement (Dufour *et al.*, 2017; Grant, 1972). An interesting avenue of future research would be to explore if other morphological features of the head differ among the three populations, which may further explain differences in their trophic niche. Larger reptiles have larger heads, which is inherently related to higher bite force enabling the ingestion of larger and harder prey (Sagonas *et al.*, 2014).

Novel dietary records for *T. raziana*

The results presented here provide improved knowledge of the dietary composition of the Raso wall gecko. On Raso, the presence of invertebrates and plant material in the faeces of *T. raziana* had already been reported (Schleich, 1987), however, taxonomically identification of the plant diet items was not possible. More recently, Mateo *et al.* (2016), described this species to feed exclusively on terrestrial arthropod species. Our results suggest the presence of both invertebrates and plants in the diet, although some items could also be the result of secondary detection (da Silva *et al.*, 2019; Tercel *et al.*, 2021). In this particular case, Mateo *et al.* (2016) revealed Coleoptera to be the most frequent order in the diet of *T. raziana*, contrary to the present results showing Blattodea and Zygentoma as main items. One possible explanation for this difference could sampling bias by the presence of easily identifiable chitinous exoskeletons in faeces using a magnifying lens (Brown *et al.*, 2012; Gil *et al.*, 2020). Overall, Zygophyllaceae, Fabaceae and Poaceae were the most frequent plant families in the diet of *T. raziana* on Santa Luzia and Raso populations, corresponding to plants that are abundantly available on the Desertas Islands (Gomes *et al.*, 2023; Lobin, 2015). Most of the invertebrates found in the total diet of *T. raziana* were terrestrial and some were phytophagous. In fact, it was observed that about a third of *T. raziana* samples, on Santa Luzia and Raso, contained both plants and invertebrates, so there is a possibility of indirect consumption of plant items by these geckos (Cuff *et al.*, 2022). On the other hand, two-thirds of Santa Luzia

and one-third of Raso samples contained only plant DNA, indicating the importance of direct consumption of these items by this species. The present study also added new arthropod groups to the diet of *T. raziana*, such as Lepidoptera and Psocoptera. Moreover, the present results showed for the first time the consumption of vertebrates by this small gecko, albeit at a lower frequency when compared to its larger competitor. The low frequencies may indicate that vertebrates, or their remains (e.g., regurgitations), are ingested only occasionally and opportunistically and are not a main component of the diet of this species. Analogous behaviour was already observed in *T. gigas* (Schleich, 1980) and other similar-sized species, such as the Selvagens gecko *Tarentola (boettgeri) bischoffi* (Joger, 1984), in the remote Selvagem Grande (Gil *et al.*, 2020).

Conclusion

In conclusion, the present study demonstrates resource partitioning facilitating the long-term coexistence of generalist species, and provides valuable insights into the ecological dynamics in island ecosystems. In addition to highlighting specific trophic relationships, these data will help to ensure the sustainable management of the plant, invertebrate and vertebrate communities as a whole. In small and isolated islands these studies are of particular importance, since due to the limited resources the loss of one particular resource can impact the whole network (Lopes *et al.*, 2019; Pinho *et al.*, 2018). Therefore, understanding the mechanisms and outcomes of resource partitioning can provide valuable information for conservation efforts and management (e.g., Nakashima *et al.*, 2022; Zurdo *et al.*, 2023), particularly for fragile island ecosystems (Pinho *et al.*, 2018; Schmack *et al.*, 2021).

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Supplementary material Article I

Appendix 1. Abstract in Portuguese

As ilhas oceânicas são modelos estimulantes para estudar a forma como os processos evolutivos e as variáveis ambientais podem contribuir conjuntamente para a especiação e o estabelecimento de comunidades ao longo do tempo. Neste contexto, o arquipélago de Cabo Verde serve como um laboratório natural simplificado, albergando um grupo de espécies endémicas de répteis que descendem de um único ancestral comum, com espécies simpátricas exibindo uma ampla variação morfológica, particularmente em termos de tamanho, e competindo por recursos tróficos limitados. Este enquadramento permite explorar a forma como a composição da dieta de espécies muito próximas e com a mesma distribuição é influenciada pela competição. Isto é exemplificado por duas osgas endémicas, *Tarentola gigas* e *Tarentola raziana*, que ocorrem em simpatria no ilhéu Raso, com esta última a ocorrer também em alopatria na ilha de Santa Luzia. O *metabarcoding* de ADN de amostras fecais foi utilizado para comparar as dietas de ambas as populações simpátricas e alopátricas. Este revelou diferenças significativas na composição da dieta entre as duas populações de *T. raziana*, com uma riqueza de presas e amplitude de nicho significativamente menores na população de Santa Luzia. As diferenças observadas nas espécies simpátricas foram devidas a uma maior incidência de vertebrados e plantas na dieta de *T. gigas*, enquanto as diferenças entre as populações alopátricas de *T. raziana* deveram-se a uma maior incidência de plantas e uma menor incidência de invertebrados na população de Santa Luzia, em comparação com a do Raso. Estes resultados sugerem que a competição desempenhou um papel importante no estabelecimento de uma coexistência estável destas espécies em simpatria, promovendo grandes discrepâncias morfológicas que permitiram a partição dos recursos. Adicionalmente, este estudo revela diferenças inesperadas na dieta da população alopátrica de *T. raziana* em Santa Luzia, sugerindo uma mudança de nicho em resposta à ausência de um competidor directo. Além disso, este trabalho destaca a importância de entender os mecanismos de partição de recursos para esforços de conservação e gestão, especialmente em ecossistemas insulares frágeis.

Table S1. List of specimens of *Tarentola raziana* and *Tarentola gigas* sampled for intestinal length analysis. Original voucher codes from the National Museum of Natural History and Science of Lisbon (MUHNAC), species name (*T. raziana* from Raso, Tz_ra, and *T. gigas* from Raso, Tg_ra), and measurements (in mm) of snout-vent length (SVL), small intestine length (SI) and large intestine length (LI) are given with the respective average and standard deviation values.

Voucher code	Species	SVL	SI	LI
232-1970	Tz_ra	51.61	3.5	1.7
233-1970	Tz_ra	53.63	3.3	1.6
234-1970	Tz_ra	49.00	2.8	1.5
235-1970	Tz_ra	46.99	3.1	1.5
237-1970	Tz_ra	43.26	3.3	1.8
		48.90 ± 4.04	3.2 ± 0.3	1.6 ± 0.1
409-1970	Tg_ra	121.97	14.4	4.9
437-1970	Tg_ra	111.60	14.5	5.4
438-1970	Tg_ra	103.43	10.8	4.7
412-1970	Tg_ra	96.85	7.3	4.3
413-1970	Tg_ra	92.52	9.5	4.4
		105.30 ± 11.80	11.3 ± 3.1	4.7 ± 0.4

Table S2. List of the taxonomic identified MOTUs to the maximum resolution obtained. MOTU's frequency of occurrence is provided for each *Tarentola* population (*T. raziana* from Santa Luzia, Tz_SL, from Raso, Tz_ra, and *T. gigas* from Raso, Tg_ra). The symbol (*) indicates the orders, families and MOTUs that contributed significantly to differences between sympatric (Tz_ra vs Tg_ra) and (¥) in allopatric (Tz_ra vs Tz_SL) populations. NI stands for non-identified orders or families.

Phylum	Class	Order	Family	MOTU	Tz_SL	Tz_ra	Tg_ra
Tracheophyta	Liliopsida	Arecales	Arecaceae	Arecaceae_1	0.04	0.00	0.00
		Poales	Poaceae	Poaceae_1	0.04	0.04	0.00
				Poaceae_2	0.04	0.08	0.25
				Poaceae_3	0.00	0.04	0.06
				Poaceae_4	0.00	0.04	0.00
				Aristida adscensionis	0.00	0.04	0.19
				Chloris virgata	0.04	0.15	0.19
				*Festuca*_1	0.00	0.12	0.13
				Zea mays	0.00	0.00	0.06
	Magnoliopsida	Apiales	Apiaceae	Apiaceae_2	0.04	0.00	0.06
		Asterales	Asteraceae	Asteraceae_1	0.04	0.08	0.00
		Boraginales	Boraginaceae	Boraginaceae_1	0.00	0.00	0.19
		Brassicales	Brassicaceae	Brassicaceae_1	0.00	0.04	0.06
		Caryophyllales	Aizoaceae	*Aizoon canariense*	0.12	0.12	0.31
			Amaranthaceae *	*Chenopodiastrum murale*	0.00	0.00	0.44
				Patellifolia procumbens *	0.00	0.08	0.50
			Caryophyllaceae	Caryophyllaceae_1	0.04	0.00	0.00
				Paronychia illecebroides	0.08	0.00	0.00
			Frankeniaceae	*Frankenia ericifolia*	0.00	0.04	0.06
			Plumbaginaceae *	*Limonium brunneri* *	0.00	0.15	0.75
			Tamaricaceae	*Tamarix*_1	0.00	0.04	0.00
		Ericales	Ericaceae	Ericaceae_1	0.00	0.04	0.00
		Fabales * ¥	Fabaceae * ¥	Fabaceae_1	0.23	0.04	0.06
				*Arachis*_1	0.08	0.00	0.00
				*Cicer*_1	0.04	0.00	0.00
				*Desmodium*_1	0.04	0.00	0.00
				Lotus brunneri *	0.38	0.08	0.56
		Gentianales	Apocynaceae	Apocynaceae_1	0.00	0.00	0.19
		Lamiales	Oleaceae	Oleaceae_1	0.00	0.04	0.06
		Laurales	Lauraceae	Lauraceae_1	0.00	0.00	0.06
		Malvales	Malvaceae	*Sida salviifolia*	0.00	0.08	0.06
		Myrtales	Myrtaceae	*Psidium guajava*	0.00	0.00	0.06
		Rosales	Rosaceae	Rosaceae_1	0.00	0.04	0.00
				Rosaceae_2	0.00	0.04	0.00
				Rosaceae_3	0.00	0.04	0.00
		Sapindales	NI	Sapindales_1	0.04	0.00	0.00
		Solanales	Convolvulaceae	*Ipomoea cairica*	0.00	0.00	0.06
			Solanaceae	*Capsicum*_1	0.00	0.08	0.00
				*Physalis*_1	0.04	0.00	0.00
		Zygophyllales ¥	Zygophyllaceae ¥	*Tetraena simplex* ¥	0.65	0.19	0.38
				Tribulus cistoides	0.04	0.08	0.06
Arthropoda	Arachnida	Ixodida	Argasidae	*Carios capensis*	0.00	0.00	0.06
	Chilopoda	Geophilomorpha	Mecistocephalidae	Mecistocephalidae_1	0.00	0.00	0.06
	Insecta	NI	NI	Insecta_2	0.00	0.04	0.00
		Blattodea ¥	Blattidae	*Periplaneta*_1	0.00	0.04	0.00
			Rhinotermitidae	Reticulitermes_1 ¥	0.00	0.58	0.38
				Reticulitermes_2	0.00	0.00	0.06
		Coleoptera	Curculionidae	Curculionidae_1	0.12	0.00	0.00
			Staphylinidae	Staphylinidae_1	0.00	0.08	0.00
				Staphylinidae_2	0.00	0.15	0.00
			Tenebrionidae	Tenebrionidae_1	0.00	0.04	0.00
				*Oxycara*_1	0.00	0.00	0.19
				*Tentyria*_1	0.00	0.04	0.00
		Diptera	NI	Diptera_1	0.00	0.04	0.00
			Chironomidae	Chironomidae_1	0.00	0.04	0.00
				*Chironomus*_1	0.00	0.00	0.06
			Culicidae	Culicidae_1	0.00	0.00	0.06
			Sarcophagidae	*Wohlfahrtia*_1	0.00	0.00	0.06
				*Wohlfahrtia*_2	0.00	0.00	0.06
		Hemiptera	Acanthosomatidae	Acanthosoma_1	0.00	0.04	0.00
			Lygaeidae	*Nysius cymoides*	0.00	0.04	0.00
			Pseudococcidae	Pseudococcidae_1	0.04	0.00	0.00
		Hymenoptera	Apidae	Apidae_1	0.00	0.00	0.06
			Formicidae	*Lepisiota capensis*	0.04	0.00	0.00
				Solenopsis geminata	0.00	0.04	0.00
			Pteromalidae	*Anisopteromalus calandrae*	0.00	0.08	0.00
			Thynninae	*Neozeleboria*_1	0.08	0.00	0.00
		Lepidoptera	Lycaenidae	Lycaenidae_1	0.00	0.04	0.00

Phylum	Class	Order	Family	MOTU	Tz_SL	Tz_ra	Tg_ra
			Noctuidae	*Agrotis ipsilon*	0.00	0.04	0.00
				Noctua pronuba	0.00	0.00	0.06
			Pieridae	*Eurema hecabe*	0.04	0.19	0.06
			Pterophoridae	Pterophoridae_2	0.00	0.04	0.00
				*Agdistis*_1	0.12	0.04	0.06
			Pyralidae	Pyralidae_1	0.08	0.04	0.00
			Tortricidae	*Lobesia*_1	0.00	0.04	0.00
		Mecoptera	NI	Mecoptera_1	0.00	0.19	0.00
		Orthoptera	Acrididae	*Acrida turrita*	0.00	0.04	0.00
				*Sphingonotus*_1	0.00	0.04	0.06
				*Sphingonotus*_2	0.00	0.00	0.06
		Plecoptera ¥	NI	Plecoptera_1 ¥	0.00	0.27	0.06
		Psocodea	Trogiidae	*Cerobasis*_1	0.15	0.00	0.00
		Psocoptera	Amphientomidae	Amphientomidae_1	0.00	0.04	0.00
		Zygentoma ¥	NI	Zygentoma_1	0.00	0.04	0.00
				Zygentoma_2	0.00	0.12	0.00
			Lepismatidae ¥	Lepismatidae_1	0.00	0.19	0.00
				Lepismatidae_2	0.00	0.12	0.06
				Lepismatidae_3	0.08	0.00	0.00
				Lepismatidae_4	0.00	0.04	0.00
				Lepismatidae_5	0.00	0.12	0.00
				Ctenolepisma_1	0.00	0.12	0.00
				*Heterolepisma*_1	0.00	0.12	0.00
				*Heterolepisma*_2	0.00	0.08	0.00
				Neoasterolepisma myrmecobia	0.00	0.00	0.06
				Thermobia domestica ¥	0.00	0.23	0.19
	Malacostraca	Decapoda	Alpheidae	Alpheidae_1	0.00	0.00	0.06
			Decapoda_1	Decapoda_1	0.00	0.00	0.06
			Grapsidae	*Grapsus adscensionis*	0.00	0.00	0.06
				*Pachygrapsus*_1	0.00	0.00	0.06
		Euphausiacea	NI	Euphausiacea_1	0.04	0.04	0.00
Chordata	Actinopterygii	Beloniformes	Exocoetidae	*Exocoetus volitans*	0.00	0.00	0.06
		Clupeiformes	Clupeidae	*Sardina pilchardus*	0.04	0.00	0.00
		Perciformes	Carangidae	*Caranx crysos*	0.00	0.00	0.06
			Coryphaenidae	*Coryphaena hippurus*	0.00	0.04	0.00
			Moronidae	*Dicentrarchus labrax*	0.00	0.04	0.00
			Scaridae	*Sparisoma cretense*	0.00	0.04	0.00
		Spariformes	Sparidae	Sparidae_1	0.00	0.04	0.00
	Aves	Galliformes	Phasianidae	*Gallus gallus*	0.08	0.00	0.00
		Passeriformes*	Alaudidae *	*Alauda razae* *	0.00	0.00	0.44
			Fringillidae	Fringillidae_1	0.00	0.04	0.00
			Passeridae	*Passer iagoensis*	0.00	0.04	0.25
		Phaethontiformes	Phaethontidae	*Phaethon aethereus*	0.00	0.00	0.06
		Procellariiformes*	Hydrobatidae	*Oceanodroma jabejabe*	0.00	0.00	0.06
			Procellariidae *	*Bulweria bulwerii* *	0.00	0.00	0.44
				Calonectris edwardsii	0.00	0.04	0.13
				Puffinus boydi	0.00	0.04	0.00

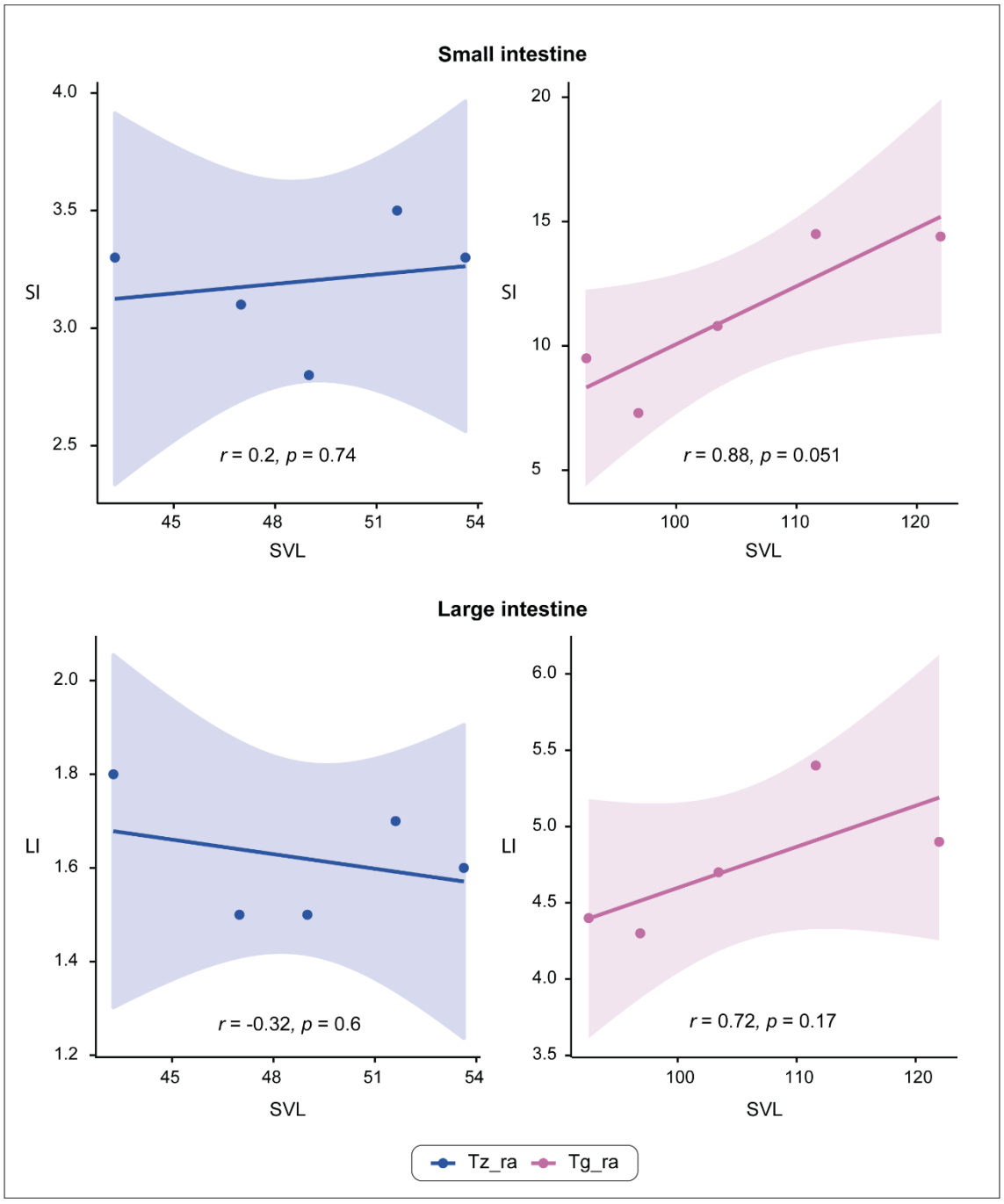

Figure S1. Plot of gastrointestinal length (in mm) versus snout-vent length (SVL in mm). The correlations for the small intestine (SI) are plotted at the top and the correlations for the large intestine (LI) are plotted at the bottom. Regression lines are shown for each *Tarentola* species with the corresponding colour (*T. raziana*, Tz_ra in blue and *T. gigas*, Tg_ra in pink). Pearson correlation coefficients (r) and corresponding significant values (p) are shown in each plot. Shaded areas represent 95% confidence intervals.

Article II

Smaller islands, bigger appetites: evolutionary strategies of insular endemic skinks

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Abstract

Competitive dietary segregation and morphological divergence among co-occurring species are fundamental aspects of ecological communities, especially in island environments where evolutionary pressures are more pronounced. The Cabo Verde Archipelago serves as a simplified natural laboratory, hosting a group of endemic reptile species descended from a single common ancestor, with sympatric species exhibiting wide morphological variation, particularly in size, and competing for limited food resources. In this context, DNA metabarcoding was used to compare the diets of the large skink *Chioninia vaillantii* and the smaller *Chioninia delalandii* in three contexts: on Fogo, where the species occur in sympatry in humid uplands with abundant vegetation, but where an allopatric population of *C. delalandii* inhabits lowland areas, and on Cima Islet, where the species are sympatric and competitive pressures are expected to be higher. The morphological variation of these populations was also studied using linear measures. Results showed significant differences in diet composition between species and populations, coupled with divergent morphological traits. In particular, the two sympatric populations of *C. delalandii* on Fogo and Cima showed similar changes in head morphology compared to the allopatric population, suggesting *in situ* evolution of head shape traits that facilitate coexistence with the larger competitor *C. vaillantii*. These observations suggest the presence of character displacement as an evolutionary strategy to facilitate resource partitioning and stable coexistence. Furthermore, increased trophic competition on Cima Islet correlated with distinct dietary composition and morphological variation, highlighting the influence of higher competitive pressures on the evolution of these species. This comprehensive study enhances our understanding of how endemic species adapt and compete in isolated environments, providing vital insights that are crucial for formulating effective conservation strategies.

Keywords: Cabo Verde; competition; resource partitioning; morphology; DNA metabarcoding; reptiles

Introduction

Competition plays a fundamental role in shaping ecological communities, primarily by driving dietary segregation between coexisting species (Arlettaz *et al.*, 1997; Arrizabalaga-Escudero *et al.*, 2018; Kartzinel *et al.*, 2015a). This is particularly expected among closely related species, as they tend to have similar morphologies, comparable feeding habits, and distribution patterns (Harvey *et al.*, 1991). Thus, in environments where resources are limited, it is expected that interspecific competition between them will be marked (Crombie, 1947; Schoener, 1977). Consistent with this, syntopic species have been found to rapidly diverge in their ecological niches by altering morphology, feeding preferences or shifting ranges to cope with competitors (Amorim *et al.*, 2017; Dufour *et al.*, 2017, 2018a, 2018b). As trophic competition intensifies, traits involved in the exploitation of food resources are expected to come under greater evolutionary pressure (Schoener, 1977), with such divergence allowing species to specialize on different prey, optimising resource use (Brown *et al.*, 1956; Schluter, 2000; Žagar *et al.*, 2017) to favour their stable coexistence (Pianka, 2011). Thus, understanding dietary niche partitioning and associated morphological divergence between evolutionarily and ecologically related co-occurring species is important for developing a general understanding of community assembly and co-evolution (Losos *et al.*, 1994; Masson *et al.*, 2023; Sottas *et al.*, 2020; Žagar *et al.*, 2017). Ultimately, this may help to predict how ecosystems might respond to increased pressures, such as prey extinction or the introduction of alien species.

Competition-driven phenotypic specialisation has been observed in several groups of vertebrates, including birds (Grant *et al.*, 2006), fishes (Wainwright, 1996) and reptiles (Verwaijen *et al.*, 2002). Particularly in the reptiles, variation in traits such as body size, head size and shape, and limb length have been shown to provide functional advantages to competing species (De Meyer *et al.*, 2019; Losos *et al.*, 2009; Maher *et al.*, 2022; Žagar *et al.*, 2017). These variations contribute to microhabitat segregation and are highly relevant in social activities such as mating and aggressive interactions (Attum *et al.*, 2007; Fabre *et al.*, 2016; Losos, 2009; McBrayer *et al.*, 2007; Pinho *et al.*, 2023; Williams *et al.*, 2016). Notably, head shapes and dimensions play a direct role in dietary specialization (De Meyer *et al.*, 2019; Herrel *et al.*, 2001a; Masson *et al.*, 2023; Verwaijen *et al.*, 2002). Larger heads have been shown to result in stronger jaws, and therefore in increased bite force (Herrel *et al.*, 2001b; Masson *et al.*, 2023; Verwaijen *et al.*, 2002). This variation allows the consumption of larger and/ or harder prey items and more plant matter (Herrel *et al.*, 2004, 2006; Verwaijen *et al.*, 2002). In contrast, species with smaller

heads tend to specialise in capturing smaller and/ or softer prey (Herrel *et al.*, 2001a, 2001b, 2004). Moreover, limb length is strongly associated with locomotor performance. Variations in limb morphology allow species to occupy different microhabitats and adopt different foraging strategies (Goodman *et al.*, 2008; McBrayer *et al.*, 2009; Tan *et al.*, 2020; Verwaijen *et al.*, 2007). Therefore, differences in locomotor ability may lead to dietary segregation due to disparities in prey capture ability (Attum *et al.*, 2007; Edwards *et al.*, 2013). For example, longer limbs are associated with improved sprinting ability, which is linked to the capture of faster prey (Vanhooydonck *et al.*, 2007).

Islands offer unique opportunities to study the impact of competition on ecological and evolutionary processes, particularly those related to morphological and dietary specialization (Lomolino *et al.*, 2012; Losos *et al.*, 2009; Sagonas *et al.*, 2015). Small islands and islets are particularly interesting, often harbouring endemic species with unique morphological and behavioural adaptations (Lomolino, 1985; Luiselli *et al.*, 2015). Comprehensive studies on dietary niche partitioning strategies among evolutionary and ecologically related and co-occurring species are greatly facilitated within systems where it is possible to characterise and compare dietary habits and morphological variation between sympatric and allopatric populations. This sampling framework allows a better understanding of the extent to which the presence/ absence of direct competition influences these species. An illustrative example of these dynamics is evident among the diverse *Anolis* lizards from the Greater Antillean Islands, which present remarkable morphological diversity associated with dietary preferences and foraging strategies according to the presence/ absence of different competitors (Losos, 2009).

Cabo Verde consists of ten oceanic islands and several islets within the Macaronesia biogeographical region (Figure 1A–B), with ages between 2–26 million years, My (Florencio *et al.*, 2021). This arid archipelago, within the Mediterranean Basin biodiversity hotspot (Mittermeier *et al.*, 2011), hosts a remarkable reptile biodiversity, including a total of 32 endemic terrestrial reptile taxa from three genera (Vasconcelos *et al.*, 2013, 2020). The genus *Chioninia* is exclusively found in the archipelago, and all species are endemic to these islands (Carranza *et al.*, 2001, 2003; Miralles *et al.*, 2011). Prior to recent phylogenetic studies, these species were thought to belong to the genus *Mabuya*, except for the giant *Chinionia coctei* Duméril & Bibron, 1839, which was thought to belong to the endemic and monophyletic genus *Macroscincus* (Carranza *et al.*, 2001; Greer, 1976, 2000). It is now known that *Chioninia* skinks originated in Cabo Verde from a single colonisation event from continental Africa 26 My ago (Pereira *et al.*, 2017), which most likely took place in the north-western islands (Brehm *et al.*, 2001; Miralles *et al.*, 2011).

There are currently six extant species and seven subspecies of *Chioninia*, and one extinct species, *C. coctei* (Miralles *et al.*, 2011). An increased rate in the evolution of body size has been demonstrated for the genus (Pereira *et al.*, 2017). Most *Chioninia* species have retained the medium size of the ancestral species, such as Delalande's skink *Chioninia delalandii* (Duméril & Bibron, 1839), but several species developed larger body sizes, such as the extinct *C. coctei* and the extant Vaillant's skink *Chioninia vaillantii* (Boulenger, 1887) (Miralles *et al.*, 2011). This latter species is Endangered and is represented by two subspecies, *C. vaillantii vaillantii* (Miralles *et al.*, 2011) on Santiago Island and *C. vaillantii xanthotis* (Miralles *et al.*, 2011), on Fogo Island and Rombo's Islets. *Chioninia vaillantii* coexists throughout its range with the smaller Least Concern *C. delalandii*, which is widespread on the Leeward islands of Santiago, Fogo, Brava Islands and Rombo's Islets and has been introduced to Maio (Miralles *et al.*, 2011; Vasconcelos *et al.*, 2013). Unlike *C. vaillantii*, Delalande's skink can also be found in allopatry (Miralles *et al.*, 2011; Vasconcelos *et al.*, 2013). These are sister species that are estimated to have diverged between 6.9 and 5.9 My ago (Miralles *et al.*, 2011). Both species are diurnal and syntopic at high altitudes on Fogo and Santiago and on Cima Islet from Rombo's (Figure 1C; Vasconcelos *et al.*, 2013), potentially competing for trophic resources. This provides a useful framework to investigate evolutionary responses to competitive pressures for this species pair. Studying these processes can help to develop sustainable conservation strategies and improve the general understanding of the ecological and evolutionary mechanisms that facilitate the coexistence of endemic species in island settings.

To this end, DNA metabarcoding of faecal pellets was used to characterise the diet composition of both species in three contexts (Figure 1C): on Fogo, where the species occur in sympatry in humid high-altitude areas with abundant vegetation, but with an allopatric population of *C. delalandii* inhabiting lowland areas, and on Cima Islet, where the species are sympatric. In the latter, competitive pressures are expected to be higher due to its restricted area and increased ecological constraints. On this islet, resources are very limited and mouse populations reach high densities, which has a major impact on biodiversity (Semedo *et al.*, 2021). In addition, linear morphological measures were compared among populations to explore how ecological and competitive pressures associated with species coexistence have shaped the evolution of body morphology. Both species are expected to exhibit a more generalist and diverse diet on Cima when compared to Fogo, presenting a less pronounced diet overlap and evidence of resource partitioning, reflecting the impact of ecological constraints on resource use. The Cima populations are expected to present more morphological divergences when compared

to sympatric and allopatric species on Fogo. This should be particularly evident in head morphology, which is strongly associated with adaptations to different resource use and may reflect the expected more generalist habits of these species.

Methods

Study area

Fogo Island and the Rombo's Islets are located in the Leeward group of the Cabo Verde archipelago (Figure 1B). Fogo is an inhabited island with a total area of 476 km^2, reaching the highest altitude of the country at 2829 m above sea level, asl (Mitchell-Thomé, 1976). The landscape is characterised by the active volcano, which last erupted in 2014 (Mata *et al.*, 2017), and its large caldera. The island also presents large agricultural and pasture areas. The climate on the island varies from dry in the lower areas to sub-humid in higher altitude areas, where vegetation is more prevalent (Rivas-Martínez *et al.*, 2017). In 2020, it was declared a biosphere reserve by UNESCO due to its unique ecosystem and biodiversity (UNESCO, 2020).

The uninhabited Rombo's Islets are composed of two main islets, Grande and Cima, surrounded by the smaller islets of Luiz Carneiro, Rei and Sapado (<0.25 km^2). These are classified as Integral Nature Reserve (Anonymous, 2003) and constitute Important Bird Areas by BirdLife International (BirdLife International, 2023). Cima, located approximately 15 km west of Fogo, is a dry flat islet with about 1.5 km^2 of area and a small hill of approximately 77 m asl. This islet is considered a key biodiversity hotspot for seabird species (Lopes *et al.*, 2022; Semedo *et al.*, 2021). However, its biodiversity is severely affected by mice, which are reaching high densities (Semedo *et al.*, 2021).

Study species

Delalande's skink *C. delalandii* is medium-sized (adults between 52 to 92 mm snout-vent length, SVL) and can be easily recognized by the presence of a black dot on the axilla and yellow eyelids (Miralles *et al.*, 2011). It is diurnal and often found in stonewalls and beneath rocks within agricultural and livestock areas (Chadwick *et al.*, 2005). On Fogo, it can also be found in modified areas, including at higher altitudes (Figure 1C; Vasconcelos *et al.*, 2013). Despite its abundance and conspicuousness (Vasconcelos, 2013a), little is known about its ecology except for anecdotal reports of an omnivorous diet (Schleich, 1987). This is also supported by the description of sharply pointed

unicuspid teeth for this species (Böhme *et al.*, 2022). It is classified as Least Concern according to the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (Vasconcelos, 2013a) and as Low Risk on the National Red List at a national and main islands level, but Data Deficient on Rombo's (Schleich, 1996).

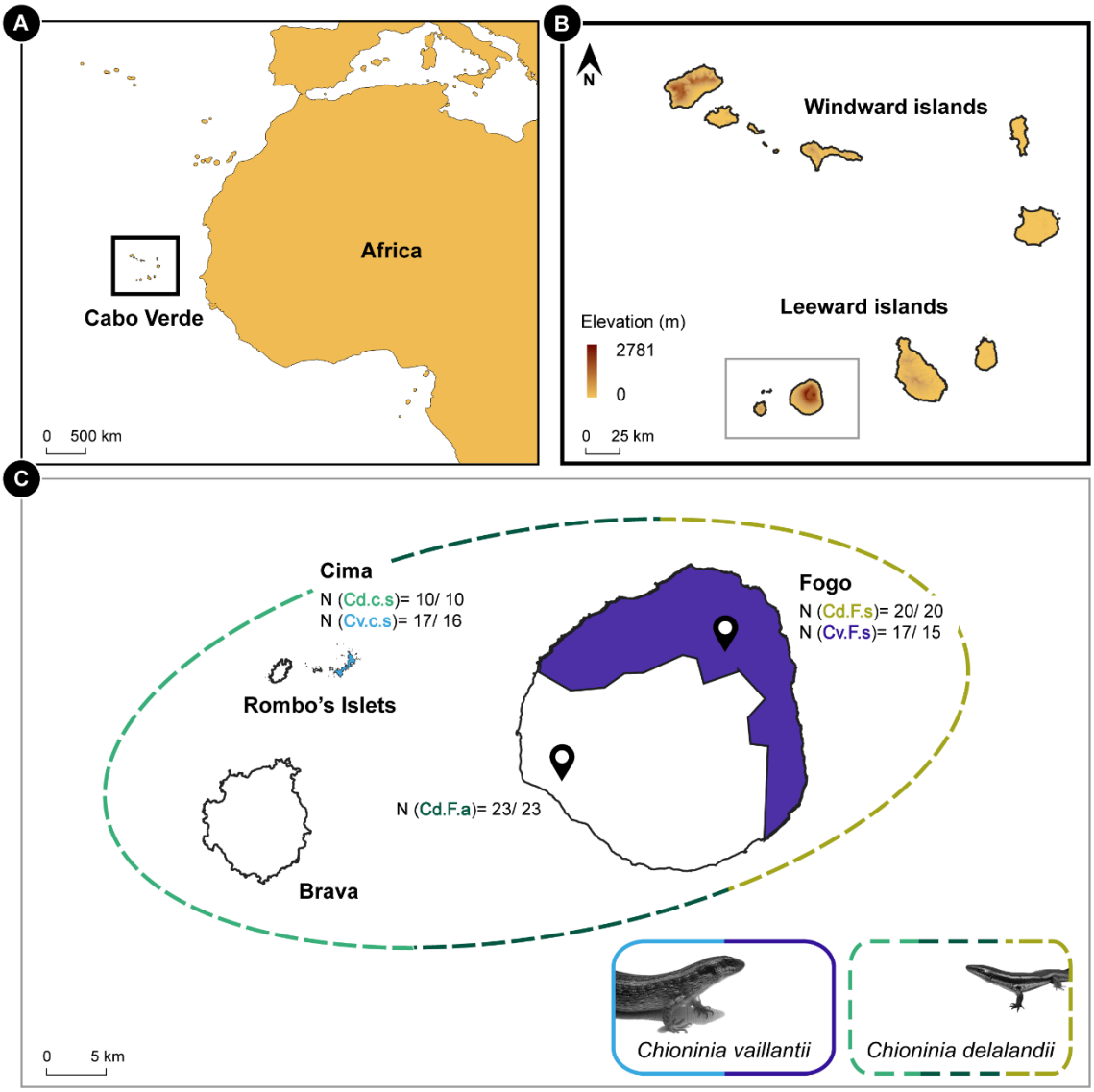

Figure 1. Study area and study taxa. A) Geographic location of the Cabo Verde Archipelago (Geographic Coordinate System, Datum WGS84). B) Islands of Cabo Verde, depicting their elevation and indicating the two island groups: Windward (Santo Antão, São Vicente, Santa Luzia, São Nicolau, Sal, and Boavista) and Leeward (Maio, Santiago, Fogo, and Brava). C) Geographical distributions of the five sampled *Chioninia* populations (Cd, *C. delalandii* in green tones; Cvx, *C. vaillantii* in blue tones). Reptile taxa are represented showing their size discrepancies. Dashed lines represent the species with the largest distribution of the pair and filled areas correspond to their sympatric areas. The number of faecal samples (N) extracted, and used in the analyses, respectively, are also shown for each of the five populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima).

The subspecies of Vaillant's skink *C. vaillantii xanthotis* is a large-sized skink with adults ranging from 87.5 to 105 mm SVL, distributed on Fogo Island and the Rombo's Islets, more precisely on Cima Islet (Vasconcelos *et al.*, 2013). It can be identified by its three well-contrasted stripes along the body and the bright yellow scales around the ear opening (Miralles *et al.*, 2011). On Fogo, it is restricted to the north-western side (Figure 1C) and less abundant than *C. delalandii*. It can typically be found in stone walls in high-altitude localities characterized by vegetated humid habitats (Vasconcelos *et al.*, 2013). It is diurnal, viviparous and its diet is thought to be composed mostly of insects and plants (Schleich, 1987). The adaptation to a more herbivorous diet is supported by the presence of tricuspid teeth in this species (Böhme *et al.*, 2022). The species is classified as Endangered according to the IUCN Red List of Threatened Species (Vasconcelos, 2013b), as Undetermined on a national level on Fogo, and as Data Deficient on the Rombo's Islets in the National Red List (Schleich, 1996).

Sampling

Sampling took place on Fogo and Cima in May 2019, with additional samples of *C. delalandii* collected on Fogo in September 2021 in the allopatric area. On Cima, a total of 10 and 17 faecal samples of *C. delalandii* and *C. vaillantii xanthotis* were collected, respectively. On Fogo, 20 samples of *C. delalandii* were collected in sympatric areas and 23 in allopatric areas, and 17 of *C. vaillantii,* present in sympatry. Specimens were captured using either hoop fishing or bucket traps with bait (banana and mango). Faecal samples were collected by abdominal massage and immediately preserved in 96% ethanol tubes. These were stored at -20ºC until further processing. Individuals were sexed, photographed and geolocated using a GPS device. Prior to release, morphological linear measures of both species were taken to the nearest 0.1 mm using digital callipers (Figure 2): snout-vent length (SVL), trunk length (TL), head length (HL), width (HW), and height (HH), eye-snout (ES) and eye-ear distance (EE), eye diameter (ED), and front (FLL) and hind limb length (HLL). Animals were placed in separate opaque tissue bags while others were processed to reduce their stress.

In addition, pitfall traps with water were placed close to the areas where skinks were captured to collect available invertebrates to build a DNA reference database of available food items. Captured specimens were preserved in falcon tubes with 96% ethanol until examination under a magnifying lens with an assembled camera. Individuals were sorted into different high-level taxonomic groups based on morphological identification and photographed for higher-resolution identification by experts.

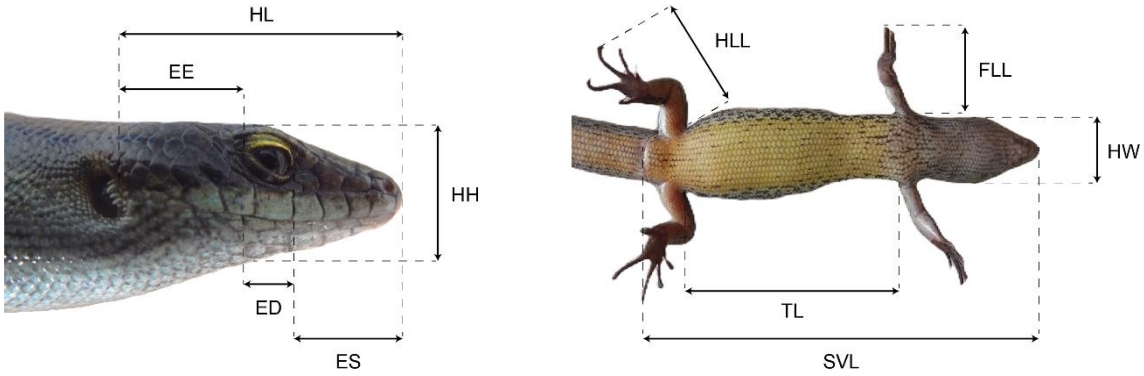

Figure 2. Linear measures taken for both *Chinonina* species. The measurements of the head included head length (HL), width (HW), and height (HH), eye-snout distance (ES), eye-ear distance (EE), and eye diameter (ED). Body measurements included snout-vent length (SVL), trunk length (TL), front (FLL), and hind limb length (HLL). The photos given as examples correspond to specimens of *Chioninia delalandii* (photographs by R. Vasconcelos).

Diet analysis

Library preparation

DNA from faecal samples was extracted using the Stool DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada), following the manufacturer's instructions. Each batch of 23 samples included an extraction negative control. Extracted DNA was amplified using previously validated markers (Pinho *et al.*, 2018) for three diet groups: invertebrates with the modified IN16STK-1F-mod/IN16STK-1R-mod primers, targeting the mitochondrial 16S gene (Kartzinel *et al.*, 2015b; Pinho *et al.*, 2018); plants with g/ h primers targeting the short P6-loop of chloroplast *trnL* (UAA) gene (Taberlet *et al.*, 2007) and vertebrates with the 12sv5F/ 12Ssv5R primer pair, targeting V5-loop within the mitochondrial 12S gene (Riaz *et al.*, 2011). To prevent the amplification of DNA from *C. delalandii* and *C. vaillantii* with the 12S primers, a blocking primer was designed (5'-TCCTCTAGGTCGGTATGGGGCACCGCCA(C3 spacer)-3'). For this, 12S sequences of both *Chioninia* species were obtained from GenBank (Brehm *et al.*, 2001; Carranza *et al.*, 2001) and aligned with sequences from birds, fish and reptiles known to occur in Cabo Verde. Its effectiveness for *C. delalandii* and *C. vaillantii* was validated by comparing PCR amplification with and without the blocking primer, using previously extracted DNA from tail tissues.

PCR reactions for the *trnL* and 16S markers were performed in 10 µL volumes, comprising 5 µL of QIAGEN Multiplex PCR Master Mix, 0.2 µL of each 10 nM primer, 3.6 µL of ultrapure water, and 1 µL of DNA extract. For the 12S marker, 3 µL of 10 nM blocking primer were added and only 1.6 µL of ultra-pure water were used. Cycling conditions used an initial denaturing at 95°C for 15 min, followed by 39 cycles of

denaturing at 95°C for 30s, annealing at 45°C (trnl), 52°C (16S) and 48°C (12S), for 90s and extension at 72°C for 30s, with a final extension at 72°C for 10 min. All PCR reactions included negative controls. After 1:4 dilutions of the PCR products, a second PCR reaction was performed to incorporate the Illumina adapters and indexes in 10 µL volumes consisting of 5 µL KAPA HiFi HotStart ReadyMix, 1 µL index mix, 2 µL ultrapure water and 2 µL PCR product. Cycling conditions were similar as the first PCR but only 10 cycles and annealing temperature of 55°C. PCR products were cleaned using Agencourt AMPure XP beads, quantified using Nanodrop, diluted to 15nM, and pooled for each marker. The tree libraries were then quantified using qPCR and pooled to obtain a final library with 4nM, that was sequenced in a MiSeq sequencer using the MiSeq Reagent Kit V2 for an expected average of 20,000 paired-end reads per PCR product.

Invertebrate DNA for the reference library was extracted from legs or whole individuals (when very small), using saline extraction methods following Carranza *et al.* (1999). Invertebrate DNA from the reference collection was amplified using two primer sets. The IN16STK primer set, allowing the match with diet items, following the previously described methods, and the LCO1490/HC02198 primer set, targeting the animal barcode region of the cytochrome oxidase I (COI). PCR conditions used for the latter are described in Folmer *et al.* (1994). Barcode sequencing was undertaken to assist with taxonomic assignments.

Bioinformatics filtering

Sequences were bioinformatically processed using tools incorporated in the software package OBITtools (http://metabarcoding.org/obitools). This process included the alignment of forward and reverse sequences (command *illuminapairedend*, --score-min=40), removal of unaligned sequences (command *obigrep*, mode!="joined"), assignment of reads to samples and removal of primers and adapters (command *ngsfilter*) and the collapsed of reads into unique sequences (command *obiuniq*). Unique sequences were discarded if the read count was less than 10 and the sequence length did not match the expected base pair size (16S: ±110; *trnL*: 10–143; 12S: 73–110). Finally, the command *obiclean* was used to remove potential PCR/sequencing errors and singletons, and to obtain the molecular operational taxonomic units (MOTUs), by removing sequences that differed by 1 base pair from the most abundant ones. The LULU R package was used to combine MOTUs with >84% similarity (Frøslev *et al.*, 2017), removing potential remaining PCR and sequencing errors. Based on PCR blank counts, PCR products with less than 2000 reads were removed and within the remaining samples, MOTUs representing less than 1% of the total reads of that PCR product were

also excluded. The remaining MOTUs were then compared against the NCBI Nucleotide Database and our reference collection, using the BLAST+ software, and classified to the lowest possible taxonomic rank. Our local database was created using *makeblastdb* and compared against obtained diet sequences using *blastn*. If a sequence matched more than one species with equal similarity values all belonging to the same genus, a genus-level assignment was considered. When a sequence matched more than one species or genus with similar probabilities, only species or genera known to occur in the archipelago were considered, otherwise a higher ranking would be attributed (e.g., family). If different MOTUs corresponded to the same taxon, a number would be attributed to each one of them (e.g., Araneae_1 and Araneae_2). MOTUs detected in extraction blanks and considered as contaminations were removed from the corresponding batch of samples (e.g., human, fungi, host DNA). Lastly, items used for bait were also excluded from the final dataset (banana and mango).

Data analysis

The frequencies of occurrence (FO) of each of the three diet item groups (plant, invertebrate, vertebrate) were estimated for the sympatric populations from Fogo (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* from hereon) and Cima Islet (Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* from hereon) and the allopatric population of *C. delalandii* from Fogo (Cd.F.a from hereon). To test for potential differences in proportions of each diet item group between populations, FOs were compared using two-proportion z-tests with the function *prop.test* in R software version 4.3.2 (R Core Team, 2023).

Family-level identification was used for the analysis due to the inability to achieve species-level taxonomic resolution for several MOTUs. The FOs of each prey family were calculated for each population. To visualize the dietary networks at the family level for the five populations, bipartite networks were generated using the function *plotWeb* from bipartite package v2.19 (Dormann *et al.*, 2008). Adjacency plots were calculated at the order level using the function *metaComputeModules* employing the ‘Beckett’ method and visualized using *plotModuleWeb*. Rarefaction curves were used to compare resource richness and niche breadth at the family level among the five populations. Richness values were estimated for the double of species with the lower sample size and differences were considered significant if the 95% confidence intervals did not overlap (Chao *et al.*, 2020). The functions *iNEXT* and *ggiNEXT* from the INEXT package (Hsieh *et al.*, 2022) were used to perform this analysis and visualized it, respectively.

Dietary niche overlap within the two sympatric populations (Cd.F.s vs. Cv.F.s and Cd.c.s vs. Cv.c.s) was calculated using Pianka's index at the family level (Pianka, 1973). This index ranges from 0 to 1, where 0 indicates no common resources and 1 indicates complete resource overlap. To test whether the dietary niche overlap differed significantly from what would be expected by chance, null models were generated based on the RA3 randomization algorithm (Lawlor, 1980), generating 10,000 null matrices that were to compare with our observed data, using the EcoSimR package (Gotelli *et al.*, 2015). Non-metric multidimensional scaling (NMDS) was used to evaluate prey-use differences among the five populations. Samples were ordinated in a two-dimensional space according to diet dissimilarity, using the function *metaMDS* from the vegan package (Oksanen *et al.*, 2022) and based on a Jaccard distance matrix.

Finally, generalised linear models (GLMs) were performed to test for significant differences in diet composition at the MOTU, family and order levels in the following contexts: within sympatric species (Cd.F.s vs. Cv.F.s and Cd.c.s vs. Cv.c.s) to investigate resource partitioning mechanisms; between species from different islands (Cd.F.s vs. Cd.c.s and Cv.F.s vs. Cv.c.s) and between *C. delalandii* allopatric populations (Cd.F.s vs. Cd.F.a) to investigate the influence of different competitive pressure scenarios. GLMs for multivariate presence/ absence data were fitted by implementing the *manyglm* function of the mvabund package (Wang *et al.*, 2022). The complementary log-log distribution provided the best fit and was used for subsequent tests. The significance of the GLMs was tested using the *anova.manyglm* function. The same function was then implemented with the argument *p.uni= 'adjusted'* to perform univariate tests to identify which prey items were responsible for differences in diet composition between species and populations.

Morphological analysis

Morphological measures were log-transformed before further analyses to ensure the normality of the data distribution. A principal component analysis (PCA) of all morphological measurements was performed, using the function *prcomp* from the package *stats* in R software version 4.3.2 (R Core Team, 2023), to obtain a comprehensive view of the morphological diversity in our dataset, taking into account the five populations and both sexes. Analyses of variance (ANOVAs) were performed, using the *adonis2* function of the *vegan* package (Oksanen *et al.*, 2022), to test for the possibility of sexual dimorphism within the study species, as well as to examine potential differences in total body size between the different populations, represented by SVL.

Before further significance testing, the effect of size was removed for each variable, given the much larger size of *C. vaillantii* when compared with *C. delalandii*. Size correction was performed using the *allom* function from the package GroupStruct (Onn *et al.*, 2022), which follows the formula from Thorpe (1975): $X_{adj} = \log(X) - \beta[\log(SVL) - \log(SVL_{mean})]$, where X_{adj}= adjusted value; X= measured value; β= unstandardized regression coefficient for each population; SVL= measured SVL; SVL_{mean}= grand mean of all SVLs. In this way all the individuals are scaled to the same size and their shape is adjusted to the new size according to the allometry principle (Lleonart *et al.*, 2000; Thorpe, 1975). A new PCA was performed on the adjusted values and a plot of the principal components (PC) was generated, showing the contribution of each variable to the observed variances. ANOVAs were performed using each of the adjusted linear measures as response variables, and the populations as independent variables. Additionally, post-hoc Tukey's Honest Significant Difference (HSD) tests (Abdi *et al.*, 2010) were performed on each of the ANOVAs, using the function *TukeyHSD* from the package *stats* (R Core Team, 2023), to show how populations differed from each other.

Results

Diet composition

The final dataset, after filtering, was comprised of a total of 84 samples (Cd.F.s= 20, Cv.F.s=Cvx_F_sym= 15, Cd.F.a= 23, Cd.c.s= 10, Cv.c.s= 16) with an average of 16,347 reads per sample and approximately four prey items per sample. A total of 144 MOTUs were identified (Cd.F.s= 19, Cv.F.s= 21, Cd.F.a= 62, Cd.c.s= 42, Cv.c.s= 39) of nine taxonomic classes (Cd.F.s= 3, Cv.F.s= 5, Cd.F.a= 7, Cd.c.s= 7, Cv.c.s= 5), 40 orders (Cd.F.s= 12, Cv.F.s= 13, Cd.F.a= 21, Cd.c.s= 21, Cv.c.s= 23) and 88 families (Cd.F.s= 13, Cv.F.s= 14, Cd.F.a= 41, Cd.c.s= 32, Cv.c.s= 29; Figure 3). For Cd.F.s, the most frequent families identified were Malvaceae and Fabaceae (Figure 3), namely the MOTUs Malvaceae_1 (FO= 60%) and *Sida acuta* Burm.fil. (FO= 40.00%). For the diet of Cv.F.s, the most frequent families were Fabaceae and Convolvulaceae (Figure 3), namely the MOTUs *Ipomoea*_1 (FO= 40.00%) and *Phaseolus*_1 (FO= 33.33%). The most frequent families in the diet of Cd.F.a were Fabaceae and Blattidae (Figure 3), namely the MOTUs *Periplaneta americana* (Linnaeus, 1758) (FO= 30.43%) and *Cardiocondyla obscurior* Wheeler, 1929 (FO= 21.74%). On Cima islet, the most frequent families in the diet of Cd.c.s population were Formicidae and Tenebrionidae (Figure 3), namely *Oxycara*_1 (FO= 40.00%) and *Lepisiota capensis* (Mayr, 1862) MOTUs and for

Cv.c.s were Zygophyllaceae and Caryophyllaceae (Figure 3), namely the MOTUs *Zygophyllum simplex* L. (FO= 68.75%) and *Paronychia illecebroides* Webb (FO= 43.75%).

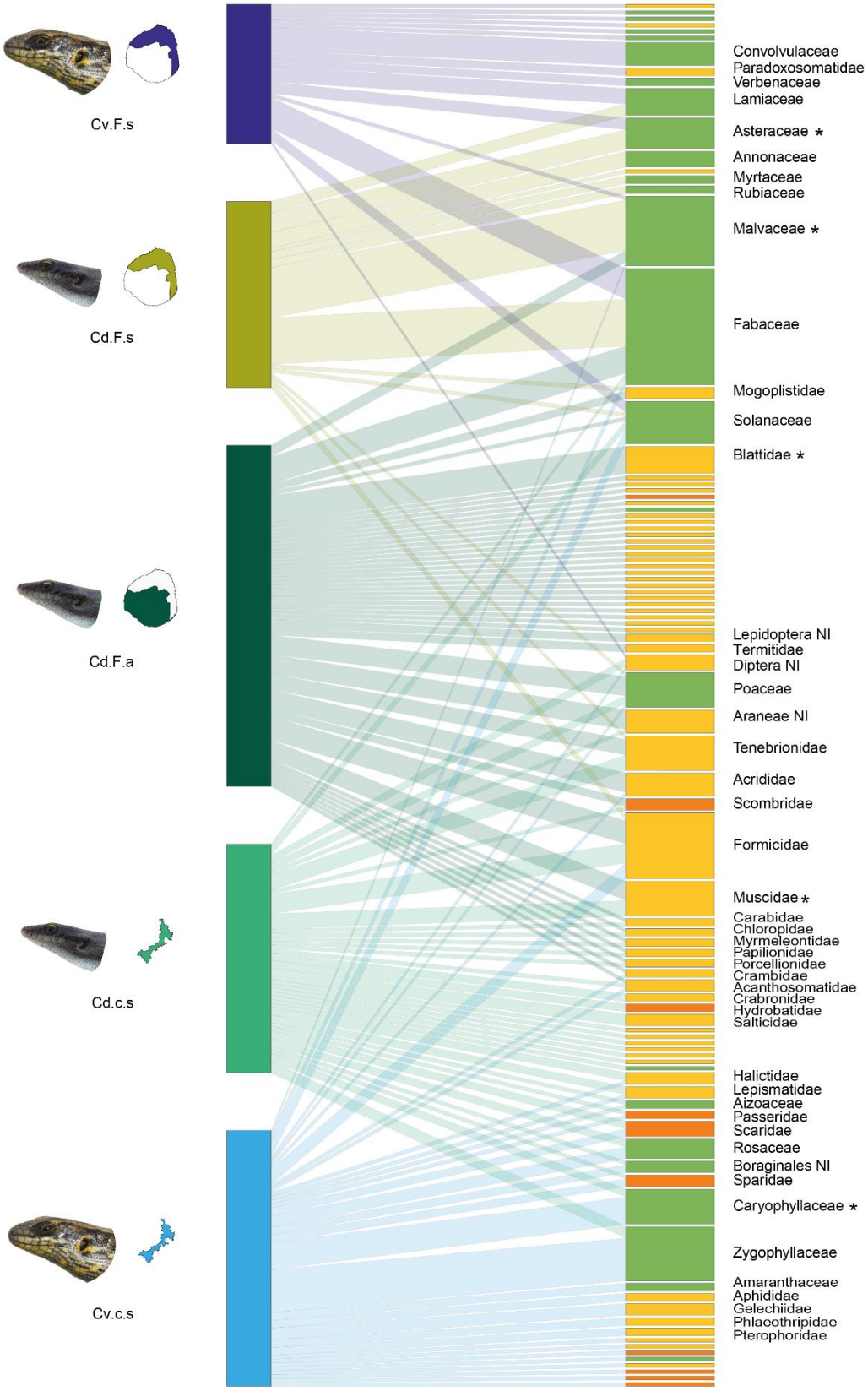

Figure 3 (on top). Bipartite network representing associations among the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima) and their diet items. The populations are on the left and arthropod (yellow), plant (green) and vertebrate (orange) prey families are on the right. The width of the connecting bars represents the frequency of occurrence of each family in the pellets of each population. The symbol (*) indicates the families that contributed significantly to differences among populations. Only family names with a frequency of occurrence above two percent are mentioned. NI stands for non-identified families.

Frequencies of plants were similar across all populations, except for Fogo allopatric populations Cd.F.a (FO= 52.00%), which presented significantly lower values (χ^2 $_{Cd.F.s\ vs.\ Cd.F.a}$= 10.460, p= 0.001). Invertebrate frequencies on Fogo populations (FO $_{Cd.F.s}$= 1; FO $_{Cv.F.s}$= 20.00%) were lower than on Cima (FO $_{Cd.c.s}$= 100%; FO $_{Cv.c.s}$= 69.00%), showing significant differences between islands (χ^2 $_{Cd.F.s\ vs.\ Cd.c.s}$= 16.30, p= 5.38e^{-05}; χ^2 $_{Cv.F.s\ vs.\ Cv.c.s}$= 15.50, p= 0.020). Additionally, there were significant differences in frequencies of invertebrates between Cd.F.s and Cd.F.a (χ^2= 28.87, p= 7.72e^{-08}). Vertebrates were only present in the diet of the sympatric Cima populations (FO $_{Cd.c.s}$= 30%; FO $_{Cv.c.s}$= 25.00%) and the allopatric population of Fogo (FO $_{Cd.F.a}$= 9.00%).

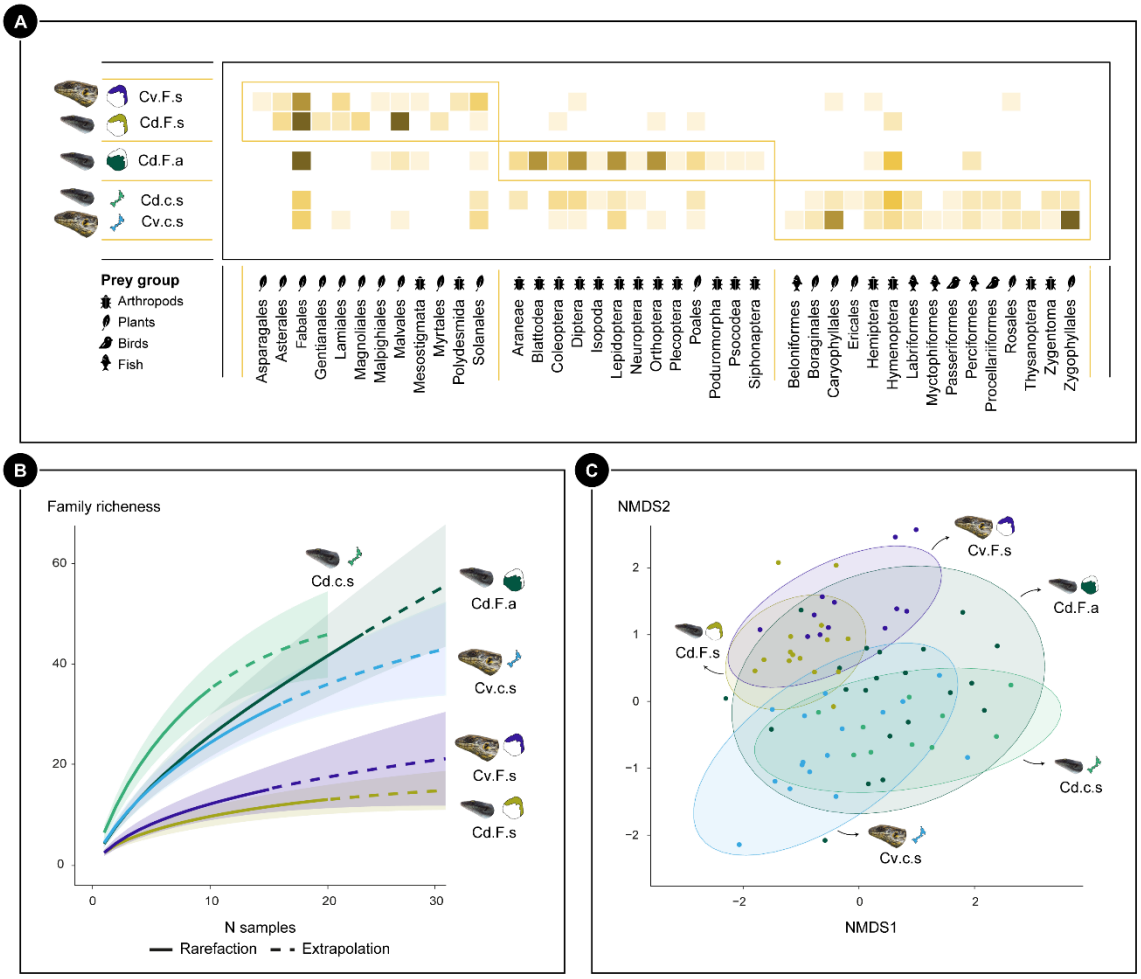

Figure 4 (on top). Diet composition results for the *five Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima). A) Adjacency matrix summarizing the population subgroups according to their identified diet item orders. The species are represented proportionally according to their sizes along the vertical axis, and plant, arthropod and vertebrate orders are shown along the horizontal axis. Subgroups are delineated by yellow boxes. The darker the yellow squares the more frequent the association between a diet item and its predator. B) Accumulation curves depicting observed (full line) and extrapolated (dashed line) family richness with their 95% confidence intervals. C) Non-metric multidimensional scaling ordination (NMDS) of the diet composition at the family level. Points closer together correspond to samples with a more similar dietary composition. Ellipses represent the standard error of the dietary composition centroids at 95% confidence intervals.

The adjacency matrix defined a total of three modules at the order level (Figure 4A), grouped by location. The sympatric populations of Fogo (Cd.F.s and Cv.F.s) are grouped according to the majority of plants consumed, Cd.F.a according to the majority of invertebrate orders consumed, and the sympatric populations of Cima (Cd.c.s and Cv.c.s) according to the diversity of orders of plants, invertebrates and vertebrates (Figure 4A). The two species living in sympatry on Cima (Cd.c.s and Cv.c.s) as well as the allopatric Cd.F.a presented no differences in family-level richness, both observed or extrapolated (Figure 4B). The sympatric species on Fogo (Cd.F.s and Cv.F.s) also showed no differences in family-level richness between them, but had significantly lower richness values compared to the other three populations (Figure 4B). Diet niche overlap (O) at the family level was similar for both sympatric comparisons and not significantly different than expected by chance ($O_{Cd.F.s\ vs.\ Cv.F.s}$ = 0.580; $O_{Cd.c.s\ vs.\ Cv.c.s}$= 0.530). The NMDS showed a generally moderated diet overlap within sympatric populations (Figure 4C), which is consistent with the results of Pianka's dietary niche overlap.

Multivariate analyses revealed significant differences in the diet composition of all the populations at the MOTU, family, and order levels (p= 0.001). Pairwise comparisons were also significant, except for the comparison Cd.c.s vs. Cv.c.s at the order level (Table 1). Univariate tests showed that differences in diet composition were due to seven MOTUs: *Paronychia illecebroides* Webb (p= 0.001) exclusively consumed by the sympatric populations of Cima Islet (Cd.c.s and Cv.c.s; Table S1); *Sida acuta* Burm.f. (p= 0.002), *Cajanus cajan* (L.) Millsp. (p= 0.004), *Phaseolus*_1 (p= 0.006) and *Desmodium scorpiurus* (Sw.) Poir. (p= 0.041) exclusively consumed by the sympatric populations of Fogo (Cd.F.s and Cv.F.s; Table S1) and *Periplaneta americana* (Linnaeus, 1758) (p= 0.004) exclusively consumed by Cd.F.a (Table S1). The families that contributed significantly to these differences were Caryophyllaceae (p= 0.001), Malvaceae (p= 0.001), Blattidae (p= 0.002), Muscidae (p= 0.004) and Asteraceae (p= 0.046; Figure 3). The orders Blattodea (p= 0.001), Caryophyllales (p= 0.001), Malvales

(*p*= 0.001), Hymenoptera (*p*= 0.003), Diptera (*p*= 0.004), Lepidoptera (*p*= 0.008), Araneae (*p*= 0.021), Orthoptera (*p*= 0.029), Coleoptera (*p*= 0.039), Asterales (*p*= 0.042) and Fabales (*p*= 0.044) contributed significantly to these differences. The results for the univariate tests for specific population pairwise comparisons are presented in Table S2.

Table 1. Results of pairwise comparisons. Multivariate comparisons in diet composition between the five Chioninia populations (Cd.F.s, C. delalandii and Cv.F.s, C. vaillantii sympatric on Fogo; Cd.F.a, C. delalandii allopatric on Fogo; Cd.c.s, C. delalandii and Cv.c.s, C. vaillantii sympatric on Cima), considering three taxonomic levels (Order, Family and MOTUs). Significant p-values are in bold and Dev stands for deviance values.

	MOTU		Family		Order	
Pairwise comparisons	Dev	*p*	Dev	*p*	Dev	*p*
Cd.F.s vs. Cv.F.s	88.42	**0.001**	64.32	**0.001**	53.62	**0.002**
Cd.c.s vs. Cv.c.s	112.00	**0.014**	94.58	**0.014**	47.13	0.061
Cd.F.s vs. Cd.c.s	189.80	**0.001**	158.20	**0.001**	142.2	**0.001**
Cv.F.s vs. Cv.c.s	163.60	**0.001**	129.90	**0.001**	92.33	**0.001**
Cd.F.s vs. Cd.F.a	217.50	**0.001**	130.20	**0.001**	114.9	**0.001**
Cd.c.s vs. Cd.F.a	189.30	**0.001**	130.50	**0.001**	80.01	**0.001**

Morphological analysis

There were no significant differences between the sexes (*p*= 0.144), therefore all subsequent analyses were performed combining both. The PCA of unadjusted measures showed a clear division between both species, *C. delalandii* and *C. vaillantii*, due to the expected significant differences in SVL (*p*= 0.001; Figure S1A). There were significant differences in SVL between the two sympatric populations of *C. delalandii* from the different islands (Cd.F.s vs. Cd.c.s: *p*= 0.023; Figure S1A; Table S3).

After size adjustment, the PCA showed a clear separation along the first PC axis between the populations of the two islands (Figure 5A). This axis explained about 31.00% of the variance in the dataset and was associated with variation in EE, ES and HL (Figure 5A). The second PC axis explained about 27.00% of the variation and was mostly associated with variation in the limbs (HLL and FLL) and in HH (Figure 5A). Finally, the third PC explained about 18.00% of the variation and was associated with HW, HH, TL and ED (Figure 5A).

The Tukey HSD tests showed several significant differences between the pairs of populations across the linear measures (Figure 5B). Regarding head variables, HL, showed significant differences in the pairs Cd.F.s vs. Cd.F.a (*p*= 0.002), Cd.c.s vs. Cd.F.a (*p*= $3.85e^{-10}$), Cd.c.s vs. Cd.F.s (*p*= 0.000) and Cv.c.s vs. Cv.F.s (*p*= $1.33e^{-10}$),

with both species showing lower values for Cima populations (Figure 5B and Figure S1B). HW showed significant differences in the pairs Cd.F.s vs. Cd.F.a (p= 0.002) and Cv.c.s vs. Cv.F.s (p= 0.01), with Cd.F.a and Cv.c.s showing lower values (Figure 5B and Figure S1B). Considering ocular and ear variables, EE showed significant differences between the pairs Cd.c.s vs. Cd.F.s (p= 0.02) and Cv.c.s vs. Cv.F.s (p= $3.43e^{-05}$) and ES and ED showed significant differences between Cd.c.s vs. Cd.F.a (p_{ES}= $1.21e^{-07}$; p_{ED}= 0.012), Cd.c.s vs. Cd.F.s (p_{ES}= $4.59e^{-08}$; p_{ED}= 0.008) and Cv.c.s vs. Cv.F.s (p_{ES}= $6.29e^{-09}$; p_{ED}= $2.85e^{-05}$; Figure 5B). These results are explained by the lower values of both Cima populations for these variables (Figure S1B).

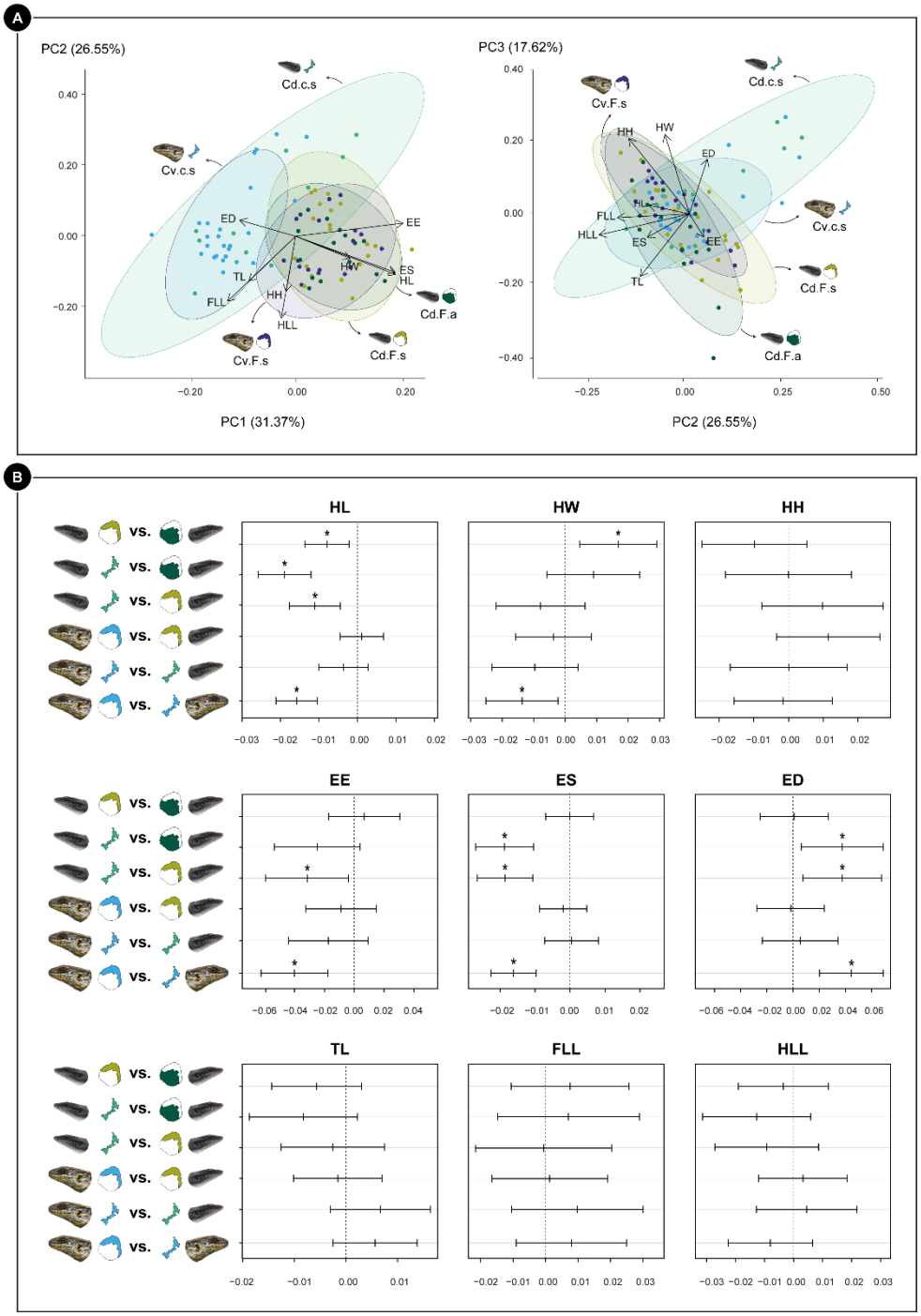

Figure 5 (on top). Morphological analysis results considering the snout-vent length (SVL) adjusted values for the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima). A) Principal component analysis (PCA) of the SVL-adjusted linear measures. Each individual is represented by a circle in the PCA space, and ellipses represent the 95% confidence intervals for each population. The contribution of the variables for the PCs is also represented. B) Results from Tukey's Honest Significant Difference (HSD) tests in SVL-adjusted linear measures (SVL, snout-vent length; HL, head length, HW, width and HH, height; ES, eye-snout distance; EE, eye-ear distance; ED, eye diameter; TL, trunk length; FLL front, and HLL, hind limb length) among populations. The horizontal bars represent the 95% confidence intervals for each comparison and the symbol (*) indicates the significant differences in mean levels among populations.

Discussion

The observed differences in dietary composition and morphological traits between sympatric and allopatric populations of *C. delalandii* provide strong evidence for character displacement as a possible evolutionary strategy that has enabled the stable coexistence with *C. vaillantii.* Furthermore, increased competitive pressure on Cima Islet is not only associated with a significantly different diet composition, but is also associated with morphological variation between the two species, as compared to their counterparts on Fogo. As expected, with increased trophic competition, the traits that showed significant differences were those involved in the exploitation of food resources, such as head size. This supports the existence of greater evolutionary pressure on these traits underpinning stable coexistence.

Diet composition comparisons

As initially predicted, both small and large species from Cima Islet present more generalist diets, consuming all food groups (plants, invertebrates and vertebrates), while the diet of Fogo sympatric populations is mainly composed of plants. A striking difference between islands is the presence of vertebrates in Cima population diets. This is the first time that vertebrates have been described in the diet of these species. This dietary shift may indicate an adaptation to the limited resources on the islet, leading to the inclusion of more nutritionally valuable prey in their diet, as previously seen in geckos of Cabo Verde (Pinho *et al.*, submitted) and lacertids in the Azores (Neves *et al.*, 2022). The presence of fish items in the diet is most likely a result of the ingestion of seabird regurgitations and/ or faeces, as evidenced by the presence of *Pelagodroma marina* (Latham, 1790) and *Bulweria bulwerii* (Jardine & Selby, 1828) in the diet. Additionally, fish may have originated from leftovers from fishing activity, reflecting opportunistic feeding on vertebrates rather than active foraging. The *C. delalandii* allopatric population

on Fogo also presented vertebrates in its diet, particularly fish, albeit at low frequencies. This is certainly related to anthropogenic waste, as exemplified by the presence of *Selar crumenophthalmus* (Bloch, 1793), a widely consumed fish in Cabo Verdean cuisine. Furthermore, in general, species of Cima consumed more native plant species than Fogo sympatric populations, which consumed more crops. This is reflected in the MOTUs that contributed to the significant differences in the diet composition between species from different islands. For instance, *Sida acuta* Burm. f. was exclusively present in the diet of *C. delalandii* sympatric population on Fogo while *Zygophyllum simplex* L. was present in the diet of *C. vaillantii* from Cima. Finally, the significant differences between the two *C. delalandii* populations on Fogo are the result of more plants being present in the diet of the sympatric and more invertebrates in the diet of the allopatric. This reflects a possible niche shift in the presence/ absence of a direct competitor, similarly to what was observed with another endemic Cabo Verdean reptile *T. raziana* (Pinho *et al.*, submitted).

Both sympatric skinks on Cima, showed significantly greater dietary richness, along with the *C. delalandii* allopatric population, when compared to both species in sympatry on Fogo. These findings align with the optimal foraging theory, which indicates that a decrease in the availability of preferred resources is associated with an expansion of the dietary niche breadth (Perry *et al.*, 1997). On Cima, given the low resource availability, the preferred food items of both species likely are very restricted. This, coupled with interspecific competition, may have compelled individuals to broaden their dietary niche, incorporating previously overlooked prey items (Bonesi *et al.*, 2004). This is consistent with an adaptive response to cope with competitive pressures in the face of scarcity. These populations have more opportunity to prey on a smaller set of high-value resources, making foraging decisions to maximize their net energy gain (Parent *et al.*, 2014; Wilson *et al.*, 1994). In contrast, Fogo sympatric populations are expected to have easier access to preferred resources, resulting in a more restricted diet. On the other hand, the richer diet observed in the allopatric population of *C. delalandii* on Fogo may indicate that these individuals have the opportunity to diversify their diet in the absence of direct competition (Herrmann *et al.*, 2021; Pianka, 2011). Indeed, the rarefaction curve obtained for this population did not reach a plateau, suggesting that additional sampling effort would likely reveal greater dietary diversity, further supporting the ecological release hypothesis.

Contrary to the initial hypothesis, diet niche overlap values at the family level were similar for both sympatric pairs on Fogo and Cima. Both pairs showed moderate dietary overlap, however, these results were not significantly different from what would be expected by chance and should be further explored. In spite of this, Fogo and Cima sympatric species

exhibit a shared presence of around a quarter of the MOTUs and families. This, coupled with the significantly different diet compositions within sympatric species, highlights the distinct exploitation of the resources by the two species despite the similar dietary richness values. In particular, on Fogo, small and large species exhibit distinct feeding patterns on invertebrates, with no shared MOTUs identified in this group. Moreover, they differ in their utilization of two plant families, although the majority of plant MOTUs were shared. On Cima, the observed significant differences were mainly due to consumption of Tenebrionidae, such as the small darkling beetles *Oxycara*, exclusively consumed by *C. delalandii* and in high frequency. This suggests that a stable coexistence is already established with dietary niche segregation as a mechanism through which competition is avoided between *C. delalandii* and *C. vaillantii* in both areas (du Preez *et al.*, 2017; Razgour *et al.*, 2011; Westeen *et al.*, 2023).

Morphological comparisons

The first striking outcome from the morphological analysis highlights a divergence in overall body morphology between the two sympatric scenarios on Fogo and Cima. While there were no significant differences in all traits analysed within sympatric species in Fogo and Cima, differences emerge when comparing the same species from the two islands.

The significantly smaller body size of *C. delalandii* from Cima compared to the sympatric population from Fogo may indicate phenotypic plasticity in response to harsh environmental conditions (Chevin *et al.*, 2017) or may already reflect selective forces acting on this population. Given the low resource availability on Cima Islet, *C. delalandii* may need to adopt more active foraging and predation techniques and reduce food intake needs. Insectivorous and omnivorous species tend to be smaller than herbivorous species because they need to be more agile in order to hunt active prey, such as insects or small vertebrates (Masson *et al.*, 2023; Van Damme, 1999). This is consistent with the diet results, showing a more omnivorous diet on Cima and a more herbivorous diet on Fogo. Additionally, variation in body size can affect other characteristics such as reproduction and thermoregulation (Georges, 1985; Sagonas *et al.*, 2013). Species with smaller body sizes usually exhibit shorter gestation periods and times, reducing reproduction energy costs (Georges, 1985). Smaller reptiles can absorb and dissipate heat more rapidly than larger ones, enhancing their ability to regulate body temperature faster (Bogert, 1949; Sagonas *et al.*, 2013). Both of these characteristics are very advantageous for island populations facing suboptimal conditions. This is exemplified by

the case of the Kotschy's gecko *Mediodactylus kotschyi* (Steindachner, 1870), which has the smallest body size of all its populations on Plakida Island, most likely due to increased competition or predation by the larger *Podarcis erhardii* (Bedriaga, 1882) (Itescu *et al.*, 2021).

Regarding SVL-adjusted head dimensions, both sympatric *C. delalandii* populations showed significantly shorter and wider heads in comparison with the *C. delalandii* allopatric population. Wider and shorter heads are usually associated with more plant-eating patterns or more diverse diets in reptiles (Herrel *et al.*, 2001a; Masson *et al.*, 2023; Sagonas *et al.*, 2014). A wider head creates additional space for jaw adductors, potentially contributing to an enhanced bite force (Herrel *et al.*, 2005; Masson *et al.*, 2023). In contrast, longer heads provide faster yet relatively weaker bites (Masson *et al.*, 2023), typical of active predators for capturing highly mobile prey. Despite not presenting significant differences in head width, the two sympatric *C. delalandii* populations significantly differed in head length, with the population from Cima showing shorter heads. This latter also presented significantly shorter snouts relative to the other two *C. delalandii* populations, suggesting an adaptation to further increase the mechanical advantage of the jaw without increasing muscle forces (Herrel *et al.*, 2007). These results are consistent with the presented dietary results showing the diet of the *C. delalandii* allopatric population is more dominated by invertebrates, presenting longer and narrow heads; a more plant-based diet of the *C. delalandii* sympatric population from Fogo, thus shorter and wider heads and a more generalist diet, of *C. delalandii* sympatric population from Cima, reflected in even shorter heads. Similar patterns of head size variation are observed between the two *C. vaillantii* populations. The Cima population shows significantly shorter heads and snouts, while the Fogo population shows wider heads. This is likely associated with the observed broader diet of the Cima population and a more plant-focused diet on Fogo. Wider heads may allow the effective reduction of fibrous plant material before swallowing, facilitating digestion (Herrel *et al.*, 1999a, 1999b). The head morphology of the *C. vaillantii* Cima population likely allows a more omnivorous and diverse diet (Herrel *et al.*, 2004; Masson *et al.*, 2023) in response to the low resource availability in the islet, supported by the dietary results.

Significant differences were also observed in the eye diameter of the populations with *C. delalandii* and *C. vaillantii* from Cima presenting larger eyes. This divergence in eye size suggests a potential adaptation associated with the activity period of these populations, with larger eyes often being associated with a more nocturnal activity (Garamszegi *et al.*, 2002; Werner *et al.*, 2006). These are typically diurnal species (Chadwick *et al.*, 2005; Schleich, 1987), but they may have evolved larger eyes, enhancing low light sensitivity,

as an adaptive strategy to increase their temporal window for prey capture (Thomas *et al.*, 2020). This would allow them to hunt prey during twilight when nocturnal invertebrates are more active. In fact, several MOTUs of nocturnal moths were exclusively found in the diet of these two species (e.g., Noctuidae), further supporting this hypothesis. Additionally, larger eyes improve focal resolution which may improve their ability to detect prey (Thomas *et al.*, 2020; Werner *et al.*, 2006). This adaptation may help increase their overall foraging efficiency and meet nutritional requirements in this resource-constrained islet, in contrast with the Fogo populations which do not face these challenges. Larger eyes occupy more space within the head, which might explain the significant reduction in the eye-ear distance in the Cima species. This can translate into smaller jaw adductor muscles usually correlated with less powerful bite (Herrel *et al.*, 2007). However, these populations most likely are compensating for this through the observed reduction of snout length. This might reduce the distance between the input and output force of the jaw lever and maximise bite force (Fabre *et al.*, 2014). Alternatively, the observed variations in head morphology may be associated with aggressive interactions. Mice are present in high densities on Cima Islet (Semedo *et al.*, 2021), which could have fostered morphological variation that allows greater bite forces in these populations. This enhanced biting capability may confer a competitive advantage in direct encounters or conflicts (Huyghe *et al.*, 2005; Lailvaux *et al.*, 2007). Furthermore, the Cima species showed behavioural differences compared to the Fogo populations, and are clearly more aggressive when handled (Vasconcelos, pers. comm.). This hypothesis can be explored in detail using geometric morphometrics or exploring the cranial morphology of this species if museum vouchers are available.

Evolutionary and conservation perspectives

The discussed morphological variations associated with differential resource use are strong indicators of character displacement. This evolutionary process is defined by an initial interspecific competition for resources, which generates natural selection favouring those individuals best adapted to resource partitioning, which is then reflected in the adaptive divergence of morphological traits (Brown *et al.*, 1956; Dufour *et al.*, 2017; Herrmann *et al.*, 2021; Stuart *et al.*, 2013). The case presented here represents a manifestation of historical competitive interactions and fulfils several criteria to consider character displacement (Stuart *et al.*, 2013). The *C. delalandii* and *C. vaillantii* are sister species that evolved *in situ* from a medium-sized ancestral form, displaying striking phenotypic variation associated with resource use (Böhme *et al.*, 2022; Miralles *et al.*,

2011). Apart from the marked difference in body size, the previously reported variation in tooth morphology reflects a clear functional link with different resource exploitation (Böhme *et al.*, 2022). While *C. delalandii* has sharp unicuspid teeth, often associated with insectivorous and omnivorous diets, *C. vaillantii* has tricuspid teeth, associated with adaptations to greater plant consumption (Böhme *et al.*, 2022; Melstrom, 2017). In the dietary results presented, the frequencies of plants and invertebrates were similar in the diet of both species, but they may consume plants in different quantities. Unfortunately, it is still not possible to accurately quantify ingested biomass with metabarcoding (Deagle *et al.*, 2018) and this should be further investigated. Additionally, *C. delalandii* allopatric population presented a diet mostly based on invertebrates, indicating a resource use shift when in the presence of the larger species. Furthermore, both sympatric *C. delalandii* populations on Fogo and Cima presented similar modifications in head morphology when compared with the *C. delalandii* allopatric population. Presenting additional evidence that the sympatric populations of *C. delalandii* have evolved head shape characteristics that allow coexistence with the larger competitor *C. vaillantii*. A similar case likely occurred in the northern islands of the archipelago with the sympatric pair of the extinct giant *C. coctei* and the smaller *Chioninia stangeri* (Gray, 1845) (Carranza *et al.*, 2001).

Apart from this already established dietary and morphological divergence between *C. delalandii* and *C. vaillantii*, additional ecological and evolutionary forces are acting on the Cima Islet populations. These include a more generalist diet in the Cima populations and the significant morphological differences observed between the two species on different islands. This is consistent with an adaptive response to ecological pressures, with both species experiencing similar selective challenges posed by the constrained conditions on Cima Islet. Plastic responses to harsh environmental conditions (Chevin *et al.*, 2017) can be hypothesised to justify these differences. However, particularly for *C. delalandii* genetic differences have already been described through the identification of haplotypes unique to these islets (Miralles *et al.*, 2011). Therefore, there is a possibility that a new subspecies of *C. delalandii* exists on the Rombo's Islets, supported by genetic evidence and the morphological variations presented here. This emphasizes the need for further genetic, ecological and behavioural studies to unravel the complexity of evolutionary processes in these and other reptile populations of the archipelago.

Finally, all these data provide valuable information for conservation plans. As already reported for birds (Semedo *et al.*, 2021; Lopes *et al.*, 2022), mice are a major threat to reptiles on Cima Islet and eradication plans need to be implemented urgently. It is important to note that *C. vaillantii* is already considered Endangered due to its limited

distribution, and habitat loss (Vasconcelos, 2013b) and that *C. delalandii* population on the islet is very reduced (authors pers. obs.) and classified as Data Deficient at national level (Schleich, 1996). Furthermore, despite the wide distribution of *C. delalandii*, there is a need to conserve the diversity of this species, and Cima population seems to hold unique genetic and morphological traits. In small remote populations, the synergy of genetic and demographic factors significantly increases the probability of extinction (Frankham, 1997). Therefore, given the proven interrelationship between these two species and their fragile/ unknown conservation status on the islet, it is crucial to maintain the balance between the two, implementing holistic conservation strategies.

Conclusions

In conclusion, through the use of DNA metabarcoding and linear measures, this study highlights the complex interplay between diet, morphology and competitive pressures in shaping the ecological dynamics of sympatric reptile populations, particularly on small islands. The observed variation between sympatric and allopatric populations provides strong evidence for character displacement as a possible evolutionary strategy allowing resource partitioning and stable coexistence of these two species. In addition, increased trophic competition on the small islet further emphasised dietary and morphological variation in sympatric species. The present findings contribute to the understanding of how these species adapt to resource constraints and compete in isolated environments, providing valuable insights for conservation strategies.

Acknowledgements

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Supplementary material Article II

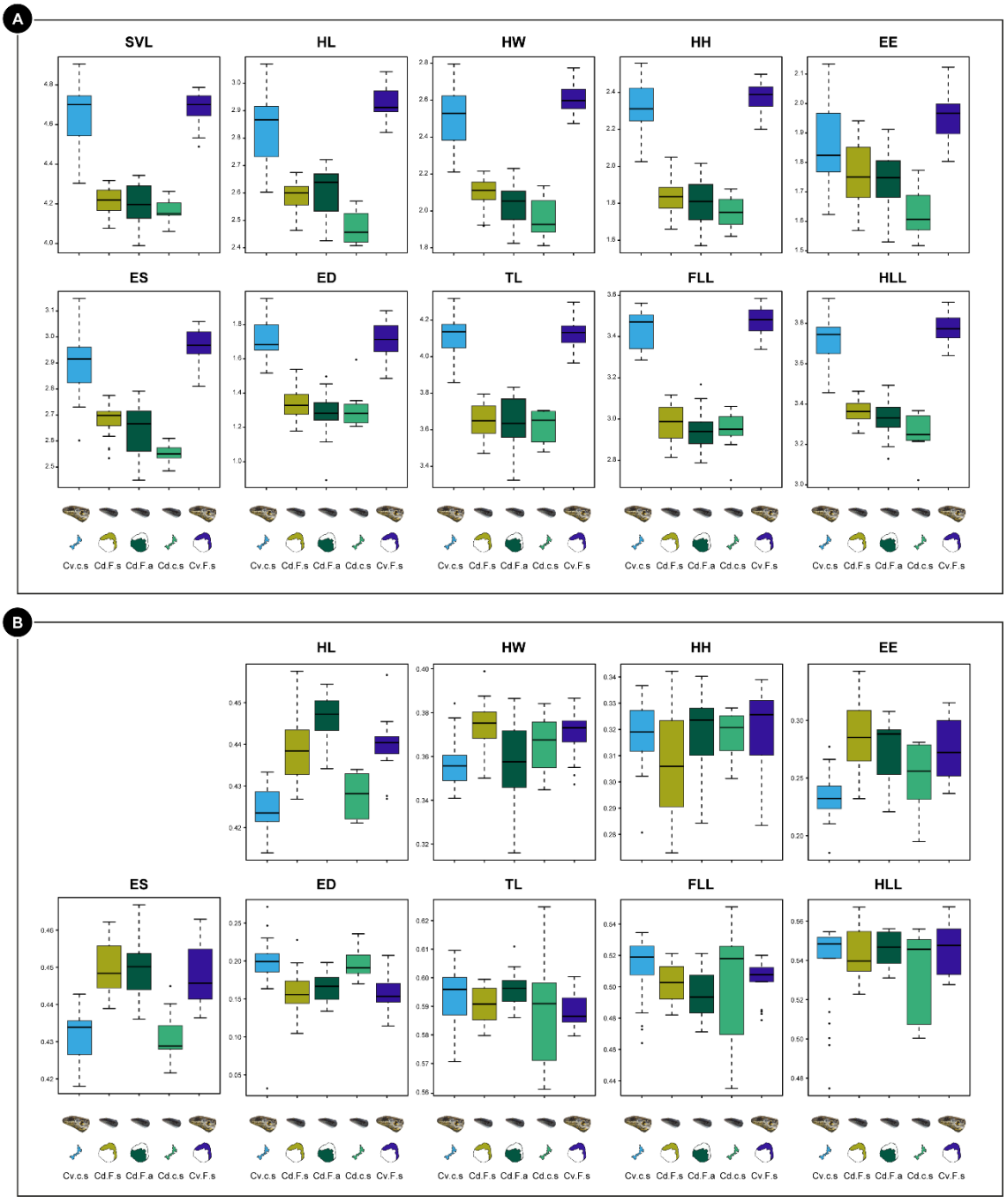

Figure S1. Boxplots of the linear measures (SVL, snout-vent length; HL, head length, HW, width and HH, height; ES, eye-snout distance; EE, eye-ear distance; ED, eye diameter; TL, trunk length; FLL front, and HLL, hind limb length) obtained for the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima). A) Log-transformed unadjusted values. B) SVL-corrected values.

Table S1. List of the taxonomic identified MOTUs to the maximum resolution obtained. MOTU's frequency of occurrence is provided for each *Chioninia* population (Cd, *C. delalandii*; Cv, *C. vaillantii*; s, sympatric; a, allopatric). The symbol (*) indicates the orders, families and MOTUs that contributed significantly to the differences among the five populations. NI stands for non-identified orders or families.

					Fogo		Cima		
Phyllum	**Class**	**Order**	**Family**	**MOTU**	**Cd.s**	**Cv.s**	**Cd.a**	**Cd.s**	**Cv.s**
Tracheophyta	Liliopsida	Asparagales	Asparagaceae	*Furcraea foetida*	0.00	0.07	0.00	0.00	0.00
		Poales	Poaceae	Poaceae_1	0.05	0.00	0.00	0.20	0.06
				Aristida adscensionis	0.00	0.00	0.04	0.00	0.00
				Dactylis smithii	0.00	0.00	0.04	0.00	0.00
				Oryza 1	0.00	0.00	0.13	0.00	0.00
	Magnoliopsida	Asterales*	Asteraceae*	Asteraceae_1	0.10	0.13	0.00	0.00	0.00
				Asteraceae_2	0.15	0.00	0.00	0.00	0.00
				Asteraceae_3	0.05	0.00	0.00	0.00	0.00
				Bidens bipinnata	0.00	0.07	0.00	0.00	0.00
		Boraginales	Boraginales NI	Boraginales_1	0.00	0.00	0.00	0.10	0.13
		Caryophyllales*	Aizoaceae	*Trianthema portulacastrum*	0.00	0.00	0.00	0.10	0.06
			Amaranthaceae	*Atriplex halimus*	0.00	0.00	0.00	0.00	0.13
			Caryophyllaceae*	*Paronychia illecebroides**	0.00	0.00	0.00	0.20	0.44
			Nyctaginaceae	*Mirabilis jalapa*	0.00	0.07	0.00	0.00	0.00
		Ericales	Actinidiaceae	*Actinidia chinensis*	0.00	0.00	0.00	0.10	0.00
		Fabales*	Fabaceae	Fabaceae_1	0.00	0.00	0.04	0.00	0.00
				*Cajanus cajan**	0.35	0.20	0.00	0.00	0.00
				*Desmodium scorpiurus**	0.25	0.20	0.00	0.00	0.00
				Leucaena leucocephala	0.00	0.07	0.04	0.00	0.00
				*Lotus*_1	0.00	0.00	0.00	0.10	0.13
				Phaseolus vulgaris	0.00	0.00	0.13	0.00	0.00
				*Phaseolus*_1 *	0.20	0.33	0.00	0.00	0.00
				Vigna unguiculata	0.00	0.00	0.09	0.00	0.00
		Gentianales	Rubiaceae	Rubiaceae_1	0.05	0.00	0.00	0.00	0.00
				Coffea arabica	0.05	0.00	0.00	0.00	0.00
		Lamiales	Gesneriaceae	Gesneriaceae_1	0.00	0.00	0.00	0.00	0.06
			Lamiaceae	Lamiaceae_1	0.15	0.27	0.00	0.00	0.00
			Verbenaceae	Verbenaceae_1	0.00	0.07	0.00	0.00	0.00
				*Stachytarpheta*_1	0.00	0.07	0.00	0.00	0.00
		Magnoliales	Annonaceae	*Annona*_1	0.20	0.00	0.00	0.00	0.00
		Malpighiales	Euphorbiaceae	*Ricinus communis*	0.00	0.00	0.04	0.00	0.00
			Passifloraceae	*Passiflora*_1	0.00	0.07	0.00	0.00	0.00
		Malvales*	Malvaceae*	Malvaceae_1	0.60	0.00	0.00	0.00	0.00
				Corchorus tridens	0.00	0.00	0.13	0.00	0.00
				*Sida acuta**	0.40	0.07	0.00	0.00	0.00
				Sida cordifolia	0.00	0.00	0.00	0.00	0.06
		Myrtales	Myrtaceae	*Psidium guajava*	0.10	0.00	0.00	0.00	0.00
		Rosales	Rosaceae	Rosaceae_1	0.00	0.00	0.00	0.10	0.19
				Malus domestica	0.00	0.00	0.00	0.00	0.06
				*Prunus*_1	0.00	0.00	0.00	0.10	0.06
			Urticaceae	*Laportea aestuans*	0.00	0.07	0.00	0.00	0.00
		Solanales	Convolvulaceae	*Ipomoea*_1	0.00	0.40	0.00	0.00	0.00
			Solanaceae	Solanaceae_1	0.05	0.00	0.00	0.00	0.00
				Capsicum_1	0.00	0.00	0.00	0.10	0.19
				Solanum lycopersicum	0.00	0.13	0.00	0.10	0.06
				Solanum_1	0.00	0.07	0.04	0.00	0.00
		Zygophyllales	Zygophyllaceae	*Zygophyllum simplex*	0.00	0.00	0.00	0.30	0.69
				Tribulus terrestris	0.00	0.00	0.00	0.00	0.13
Arthropoda	Arachnida	Araneae*	Araneae NI	Araneae_1	0.00	0.00	0.22	0.00	0.00
				Araneae_2	0.00	0.00	0.00	0.10	0.00
			Gnaphosidae	Gnaphosidae_1	0.00	0.00	0.04	0.00	0.00
			Philodromidae	*Thanatus vulgaris*	0.00	0.00	0.00	0.10	0.00
			Salticidae	Salticidae_1	0.00	0.00	0.00	0.30	0.00
			Theridiidae	*Theridion*_1	0.00	0.00	0.04	0.00	0.00
		Mesostigmata	Laelapidae	Laelapidae_1	0.00	0.00	0.04	0.00	0.00
			Parasitidae	Parasitidae_1	0.00	0.07	0.00	0.00	0.00
	Collembola	Poduromorpha	Hypogastruridae	Seira 1	0.00	0.00	0.04	0.00	0.00
	Diplopoda	Polydesmida	Paradoxosomatidae	Paradoxosomatidae_1	0.00	0.13	0.00	0.00	0.00
	Insecta	Blattodea*	Blattidae*	*Periplaneta americana**	0.00	0.00	0.30	0.00	0.00
			Termitidae	Termitidae_1	0.00	0.00	0.09	0.00	0.00
		Coleoptera*	Anthicidae	Anthicidae_1	0.00	0.00	0.04	0.00	0.00
			Carabidae	Carabidae_1	0.00	0.00	0.04	0.00	0.00
				*Microlestes*_1	0.00	0.00	0.00	0.10	0.00
			Dytiscidae	Dytiscidae_1	0.00	0.00	0.00	0.00	0.06
			Hybosoridae	*Hybosorus illigeri*	0.00	0.00	0.04	0.00	0.00
			Leiodidae	*Bathysciola*_1	0.05	0.00	0.00	0.00	0.00
			Staphylinidae	Staphylinidae_1	0.00	0.00	0.00	0.10	0.00
			Tenebrionidae	Tenebrionidae_1	0.00	0.00	0.04	0.10	0.00
				Tenebrionidae_2	0.00	0.00	0.04	0.00	0.00
				Tenebrionidae_3	0.00	0.00	0.04	0.00	0.00

					Fogo		Cima		
Phyllum	**Class**	**Order**	**Family**	**MOTU**	**Cd.s**	**Cv.s**	**Cd.a**	**Cd.s**	**Cv.s**
				*Gonocephalum*_1	0.00	0.00	0.09	0.00	0.00
				*Oxycara*_1	0.05	0.00	0.09	0.40	0.00
		Diptera*	Diptera NI	Diptera_2	0.00	0.00	0.00	0.20	0.06
				Diptera_4	0.00	0.07	0.00	0.00	0.00
			Bombyliidae	*Exoprosopa*_1	0.00	0.00	0.04	0.00	0.00
			Chloropidae	Chloropidae_1	0.00	0.00	0.04	0.10	0.00
			Muscidae*	Muscidae_1	0.00	0.00	0.00	0.10	0.00
				Musca domestica	0.00	0.00	0.17	0.30	0.00
				Stomoxys calcitrans	0.00	0.00	0.04	0.00	0.00
			Psychodidae	*Psychoda*_1	0.00	0.00	0.04	0.00	0.00
			Scenopinidae	*Scenopinus*_1	0.00	0.00	0.00	0.10	0.00
			Syrphidae	Syrphidae_1	0.00	0.00	0.04	0.00	0.00
		Hemiptera	Hemiptera NI	Hemiptera_1	0.00	0.00	0.00	0.00	0.06
			Acanthosomatidae	Acanthosomatidae_1	0.00	0.00	0.04	0.10	0.06
			Aleyrodidae	*Bemisia tabaci*	0.00	0.07	0.00	0.00	0.00
			Aphididae	*Aphis craccivora*	0.00	0.00	0.00	0.00	0.13
			Cicadellidae	*Anaceratagallia*_1	0.00	0.00	0.00	0.10	0.00
			Pentatomidae	Pentatomidae_1	0.00	0.00	0.04	0.00	0.00
			Reduviidae	*Coranus aegyptius*	0.00	0.00	0.00	0.10	0.00
		Hymenoptera*	Bethylidae	Bethylidae_1	0.00	0.00	0.04	0.00	0.00
			Crabronidae	Crabronidae_1	0.00	0.00	0.00	0.20	0.00
			Formicidae	Formicidae_1	0.00	0.00	0.00	0.10	0.06
				Cardiocondyla obscurior	0.00	0.00	0.22	0.00	0.00
				Lepisiota capensis	0.00	0.00	0.00	0.30	0.06
				Monomorium subopacum	0.00	0.00	0.00	0.10	0.13
				*Pachycondyla*_1	0.00	0.00	0.04	0.10	0.00
				Pheidole megacephala	0.10	0.00	0.00	0.00	0.00
			Halictidae	*Lasioglossum*_1	0.00	0.00	0.00	0.00	0.06
				*Nomioides*_1	0.00	0.00	0.00	0.20	0.00
		Lepidoptera*	Lepidoptera NI	Lepidoptera_1	0.00	0.00	0.09	0.00	0.00
			Autostichidae	Autostichidae_1	0.00	0.00	0.00	0.00	0.06
			Crambidae	Crambidae_1	0.00	0.00	0.04	0.00	0.06
			Erebidae	Erebidae_1	0.00	0.00	0.04	0.00	0.00
			Gelechiidae	Gelechiidae_1	0.00	0.00	0.00	0.00	0.19
			Hesperiidae	Hesperiidae_1	0.00	0.00	0.04	0.00	0.00
			Noctuidae	Noctuidae_1	0.00	0.00	0.00	0.10	0.00
			Nymphalidae	Nymphalidae_1	0.00	0.00	0.04	0.00	0.00
			Papilionidae	Papilionidae_1	0.00	0.00	0.00	0.10	0.00
				Papilionidae_2	0.00	0.00	0.04	0.00	0.00
			Pieridae	*Colias*_1	0.00	0.00	0.04	0.00	0.00
			Pterophoridae	Pterophoridae_1	0.00	0.00	0.00	0.00	0.13
			Sphingidae	Sphingidae_1	0.00	0.00	0.04	0.00	0.00
		Neuroptera	Myrmeleontidae	Myrmeleontidae_1	0.00	0.00	0.04	0.10	0.00
		Orthoptera*	Acrididae	Acrididae_1	0.00	0.00	0.04	0.00	0.00
				Acrididae_2	0.00	0.00	0.04	0.00	0.00
				Anacridium incisum	0.00	0.00	0.04	0.00	0.00
				Diabolocatantops axillaris	0.00	0.00	0.00	0.00	0.06
				*Oedaleus*_1	0.00	0.00	0.13	0.00	0.00
				Schistocerca gregaria	0.00	0.00	0.04	0.00	0.00
			Gryllidae	*Acheta domesticus*	0.00	0.00	0.04	0.00	0.00
			Mogoplistidae	Mogoplistidae_1	0.05	0.00	0.09	0.00	0.00
			Orthoptera NI	Orthoptera_1	0.00	0.00	0.04	0.00	0.00
			Pyrgomorphidae	*Pyrgomorpha conica*	0.00	0.00	0.04	0.00	0.00
		Plecoptera	Plecoptera NI	Plecoptera_1	0.00	0.00	0.04	0.00	0.00
		Psocodea	Lachesillidae	*Lachesilla*_1	0.00	0.00	0.04	0.00	0.00
		SiphoNIptera	Pulicidae	*Ctenocephalides felis*	0.00	0.00	0.04	0.00	0.00
		Thysanoptera	Phlaeothripidae	Phlaeothripidae_1	0.00	0.00	0.00	0.00	0.13
		Zygentoma	Lepismatidae	Lepismatidae_1	0.00	0.00	0.00	0.20	0.00
				Thermobia domestica	0.00	0.00	0.00	0.00	0.06
	Malacostraca	Isopoda	Isopoda NI	Isopoda_1	0.00	0.00	0.04	0.00	0.00
			Porcellionidae	*Porcellionides pruinosus*	0.00	0.00	0.04	0.10	0.00
Chordata	Actinopterygii	Beloniformes	Exocoetidae	*Exocoetus volitans*	0.00	0.00	0.00	0.00	0.06
			Hemiramphidae	*Oxyporhamphus similis*	0.00	0.00	0.00	0.00	0.06
		Labriformes	Scaridae	*Sparisoma choati*	0.00	0.00	0.00	0.20	0.13
				Sparisoma cretense	0.00	0.00	0.00	0.00	0.06
		Myctophiformes	Myctophidae	Myctophidae_1	0.00	0.00	0.00	0.00	0.06
		Perciformes	Carangidae	*Selar crumenophthalmus*	0.00	0.00	0.04	0.00	0.00
			Scombridae	*Acanthocybium solandri*	0.00	0.00	0.04	0.00	0.00
				*Auxis*_1	0.00	0.00	0.00	0.10	0.00
				Euthynnus alletteratus	0.00	0.00	0.04	0.00	0.00
			Sparidae	*Diplodus*_1	0.00	0.00	0.00	0.10	0.13
	Aves	Passeriformes	Passeridae	*Passer iagoensis*	0.00	0.00	0.00	0.10	0.06
		Procellariiformes	Hydrobatidae	*Pelagodroma marina*	0.00	0.00	0.00	0.20	0.00
			Procellariidae	*Bulweria bulwerii*	0.00	0.00	0.00	0.00	0.06

Table S2. Results of univariate tests of the pairwise comparisons. The prey items that significantly ($p < 0.05$) contributed to the differences in diet composition between Chioninia populations (Cd.F.s, C. delalandii and Cv.F.s, C. vaillantii sympatric on Fogo; Cd.F.a, C. delalandii allopatric on Fogo; Cd.c.s, C. delalandii and Cv.c.s, C. vaillantii sympatric on Cima), considering three taxonomic levels (order, family and MOTUs) are given.

Comparison	**Order**	***p***	**Family**	***p***	**MOTUs**	***p***
Cd.F.s vs. Cv.F.s	Malvales	0.004	Malvaceae	0.002	Malvaceae_1	0.001
			Convolvulaceae	0.005	*Ipomoea*_1	0.010
Cd.c.s vs. Cv.c.s			Tenebrionidae	0.050	*Oxycara*_1	0.050
Cd.F.s vs. Cd.c.s	Diptera	0.002	Malvaceae	0.002	Malvaceae_1	0.004
	Malvales	0.002	Muscidae	0.009	*Sida acuta*	0.050
	Hymenoptera	0.005	Fabaceae	0.041		
	Coleoptera	0.027				
	Fabales	0.031				
Cv.F.s vs. Cv.c.s	Zygophyllales	0.001	Zygophyllaceae	0.001	*Zygophyllum simplex*	0.001
	Caryophyllales	0.020	Caryophyllaceae	0.008	*Ipomoea*_1	0.004
			Convolvulaceae	0.010	*Paronychia illecebroides*	0.004
					*Phaseolus*_1	0.014
Cd.F.s vs. Cd.F.a	Malvales	0.007	Malvaceae	0.006	Malvaceae_1	0.001
	Blattodea	0.010	Blattidae	0.028	*Sida acuta*	0.001
	Diptera	0.010	Asteraceae	0.050	*Cajanus cajan*	0.002
	Lepidoptera	0.010			*Desmodium scorpiurus*	0.023
	Asterales	0.050			*Periplaneta americana*	0.023

Table S3. Linear measures used for morphological analyses. The codes of the sampled individuals of the five *Chioninia* populations (Cd.F.s, *C. delalandii* and Cv.F.s, *C. vaillantii* sympatric on Fogo; Cd.F.a, *C. delalandii* allopatric on Fogo; Cd.c.s, *C. delalandii* and Cv.c.s, *C. vaillantii* sympatric on Cima) and the respective measurements of the 10 variables (SVL, snout-vent length; HL, head length, HW, width and HH, height; ES, eye-snout and EE, eye-ear distances; ED, eye diameter; TL, trunk length; FLL front, and HLL, hind limb length) are given.

Code	Population	SVL	HL	HW	HH	EE	ES	ED	TL	FLL	HLL
06.19.069	Cd.F.s	71	13.50	8.76	5.96	6.30	14.08	4.17	43.03	19.21	26.76
06.19.070	Cd.F.s	66	13.02	7.83	6.33	5.31	14.58	4.27	38.05	18.12	29.37
06.19.071	Cd.F.s	72	13.74	8.38	6.24	5.74	14.98	3.35	44.50	21.30	28.50
06.19.072	Cd.F.s	63	13.16	7.74	5.98	5.75	14.76	3.76	36.64	20.70	29.49
06.19.073	Cd.F.s	65	13.81	8.44	6.63	6.66	15.07	3.73	38.80	17.01	28.73
06.19.076	Cd.F.s	68	14.08	8.12	6.93	5.71	15.34	3.97	39.28	21.51	31.85
06.19.077	Cd.F.s	68	13.48	8.21	5.94	5.92	14.94	3.44	38.82	20.36	30.93
06.19.078	Cd.F.s	73	14.51	9.01	6.60	6.63	16.04	3.76	42.65	22.57	30.67
06.19.079	Cd.F.s	70	14.22	8.92	6.55	6.36	15.65	4.00	37.21	21.96	31.48
06.19.080	Cd.F.s	59	11.75	6.81	5.37	5.16	12.60	3.45	34.29	16.68	27.21
06.19.081	Cd.F.s	75	13.77	8.41	6.35	6.38	15.07	4.45	41.80	21.24	28.44
06.19.082	Cd.F.s	63	11.97	6.84	5.26	5.23	13.03	3.36	34.96	17.87	27.96
06.19.083	Cd.F.s	66	12.72	7.88	5.82	5.39	14.47	3.72	36.59	18.96	25.93
06.19.086	Cd.F.s	64	12.76	8.06	5.84	4.80	13.71	4.66	35.23	18.27	29.04
06.19.100	Cd.F.s	72	14.13	9.17	7.76	6.57	15.80	4.05	41.59	21.27	31.92
06.19.101	Cd.F.s	63	12.02	6.99	5.79	5.44	13.09	3.25	32.18	19.29	26.47
06.19.102	Cd.F.s	71	13.11	8.51	6.27	5.35	14.66	3.82	39.80	18.38	29.18
06.19.103	Cd.F.s	75	13.47	9.01	6.27	6.97	15.03	3.75	44.24	19.34	29.18
06.19.105	Cd.F.s	67	13.30	8.15	6.58	5.76	14.75	3.79	35.39	20.47	27.76
06.19.106	Cd.F.s	68	13.47	8.30	6.95	5.93	15.10	3.97	36.42	21.25	28.28
06.19.074	Cv.F.s	109	18.32	13.42	11.35	8.23	18.86	5.76	61.88	34.12	43.44
06.19.075	Cv.F.s	117	19.53	14.59	11.60	7.20	20.20	6.24	71.04	33.91	42.28
06.19.085	Cv.F.s	110	18.27	13.24	10.22	7.11	19.45	5.54	64.65	32.49	43.77
06.19.089	Cv.F.s	102	17.91	12.45	10.65	7.61	17.81	5.11	63.73	30.80	45.91
06.19.090	Cv.F.s	106	18.38	13.87	10.89	7.15	18.83	5.23	59.37	31.11	43.54
06.19.091	Cv.F.s	100	17.70	12.87	10.22	6.99	20.49	5.06	55.61	28.87	40.46
06.19.092	Cv.F.s	110	18.95	13.10	10.81	7.36	19.93	5.55	62.34	30.56	46.78
06.19.093	Cv.F.s	120	20.11	14.45	11.36	7.38	21.04	5.70	73.16	34.65	47.42
06.19.094	Cv.F.s	115	20.21	16.03	12.16	8.36	21.32	6.01	62.95	36.01	49.64
06.19.096	Cv.F.s	104	18.10	12.82	9.95	6.46	19.42	5.42	57.85	31.11	42.94
06.19.097	Cv.F.s	110	20.95	13.53	11.30	7.09	20.99	6.56	58.95	32.23	44.14
06.19.098	Cv.F.s	116	18.68	14.02	11.37	7.22	19.40	6.10	65.22	35.54	44.42
06.19.099	Cv.F.s	107	18.15	13.01	10.10	6.67	18.66	5.17	59.65	33.35	41.58
06.19.104	Cv.F.s	116	18.89	14.50	12.09	8.18	20.48	6.11	73.64	35.20	46.69
08.21.126	Cv.F.s	110	20.08	14.29	11.34	6.41	19.64	5.83	64.22	32.61	40.95
08.21.127	Cv.F.s	89	16.79	12.01	9.31	6.07	17.12	4.42	54.13	29.19	38.13
08.21.128	Cv.F.s	93	17.00	11.86	9.03	6.20	16.62	4.55	52.76	28.16	39.04
08.21.103	Cd.F.a	63	11.94	6.20	5.02	4.78	12.39	2.44	36.24	18.22	27.04
08.21.104	Cd.F.a	54	11.31	6.42	4.81	4.95	11.58	3.05	27.77	17.44	22.85
08.21.105	Cd.F.a	67	14.12	8.21	6.75	6.29	15.37	3.62	39.03	19.07	30.81
08.21.106	Cd.F.a	74	14.25	7.46	6.04	5.94	14.62	3.52	44.13	20.92	28.63
08.21.108	Cd.F.a	62	12.42	6.71	5.60	5.32	12.85	3.45	34.42	16.25	25.64
08.21.109	Cd.F.a	73	14.60	8.64	6.30	6.77	15.28	3.71	44.02	18.94	32.90
08.21.110	Cd.F.a	65	13.35	7.33	5.71	5.74	14.18	3.48	34.61	19.41	29.10
08.21.111	Cd.F.a	71	14.00	7.81	6.75	6.23	14.60	3.54	42.62	18.89	26.66
08.21.114	Cd.F.a	73	15.20	9.30	7.17	6.30	16.31	3.82	41.50	22.19	31.12
08.21.116	Cd.F.a	77	14.96	8.70	7.51	5.70	14.96	4.47	46.20	20.19	29.89
08.21.120	Cd.F.a	66	14.00	8.07	6.18	5.75	13.69	3.85	42.58	23.76	28.13
08.21.121	Cd.F.a	62	12.79	7.07	5.47	5.43	13.04	3.43	35.29	18.85	26.77
08.21.122	Cd.F.a	62	12.87	7.76	5.80	5.51	13.24	3.59	36.79	17.30	28.71
08.21.123	Cd.F.a	74	14.48	8.25	6.64	5.89	15.20	4.28	44.71	19.44	27.81

Code	Population	SVL	HL	HW	HH	EE	ES	ED	TL	FLL	HLL
08.21.124	Cd.F.a	61	12.04	7.03	5.35	4.62	12.57	3.69	34.99	16.89	24.26
08.21.125	Cd.F.a	67	14.38	8.02	6.43	5.94	15.00	3.86	35.39	18.65	27.64
06.19.017	Cd.c.s	68	12.50	8.04	6.38	4.75	13.12	4.93	40.65	20.69	20.55
06.19.018	Cd.c.s	65	12.07	6.54	5.98	4.81	12.87	3.80	40.47	21.35	29.00
06.19.028	Cd.c.s	64	11.27	6.65	5.06	5.18	12.62	3.34	39.02	19.28	25.02
06.19.056	Cd.c.s	71	13.07	8.47	6.54	5.89	13.60	3.88	40.53	20.32	28.94
06.19.057	Cd.c.s	58	11.11	6.12	5.09	5.02	12.00	3.44	33.27	17.75	24.89
06.19.058	Cd.c.s	63	11.30	6.58	5.40	4.85	12.06	3.49	39.99	18.97	26.54
06.19.062	Cd.c.s	58	11.24	6.66	5.47	4.56	12.70	3.41	38.06	20.22	28.27
06.19.066	Cd.c.s	63	11.25	7.08	5.61	4.95	12.78	3.78	35.09	18.55	26.46
06.19.067	Cd.c.s	63	12.05	7.45	5.92	5.41	12.86	3.38	32.41	18.56	25.10
06.19.068	Cd.c.s	67	12.64	7.81	6.18	5.68	13.48	3.72	34.23	14.94	25.02
06.19.002	Cv.c.s	112	17.26	13.75	11.28	7.17	19.19	5.78	62.25	32.15	39.72
06.19.003	Cv.c.s	113	16.88	12.69	10.01	5.54	17.85	6.61	64.34	33.51	43.85
06.19.004	Cv.c.s	135	21.54	16.37	12.85	8.45	23.28	6.55	75.05	33.05	50.54
06.19.005	Cv.c.s	131	20.21	15.39	11.96	7.17	20.65	6.29	72.05	34.37	46.99
06.19.019	Cv.c.s	120	18.67	14.53	11.35	7.55	18.38	7.04	65.94	35.21	43.78
06.19.020	Cv.c.s	74	13.50	9.12	7.58	5.07	13.49	4.56	62.37	33.26	31.69
06.19.021	Cv.c.s	112	18.47	12.48	10.90	7.00	19.89	5.77	64.13	32.37	44.55
06.19.022	Cv.c.s	90	14.88	10.71	8.57	5.91	16.28	4.71	48.15	27.31	36.91
06.19.023	Cv.c.s	115	18.19	13.16	11.03	7.15	19.68	6.08	70.67	33.23	42.29
06.19.024	Cv.c.s	93	15.33	9.96	8.32	5.63	16.49	5.18	57.11	28.24	38.48
06.19.025	Cv.c.s	93	14.67	10.31	8.57	6.13	16.50	4.88	54.59	28.12	37.09
06.19.026	Cv.c.s	88	14.81	10.20	8.13	5.80	15.94	6.04	54.64	26.84	36.84
06.19.027	Cv.c.s	112	18.41	13.86	11.50	6.20	18.80	5.21	62.46	34.67	43.81
06.19.030	Cv.c.s	108	17.96	11.98	10.33	6.50	18.46	5.78	65.21	33.22	41.33
06.19.034	Cv.c.s	112	18.30	13.08	10.13	6.70	18.97	5.56	60.41	34.38	43.17
06.19.052	Cv.c.s	84	14.05	9.69	8.46	5.52	15.34	4.72	47.36	26.74	33.44
06.19.053	Cv.c.s	99	16.28	11.54	9.84	5.86	17.82	5.38	57.26	29.26	40.84
06.19.054	Cv.c.s	125	19.43	15.80	12.09	7.13	21.28	6.73	70.38	33.62	46.70
06.19.055	Cv.c.s	120	18.00	14.21	11.82	6.75	19.31	5.22	74.36	32.34	42.68
06.19.059	Cv.c.s	106	16.49	11.42	9.57	5.89	17.66	5.30	62.59	27.41	39.06
06.19.060	Cv.c.s	108	17.38	12.12	9.48	6.13	17.26	5.25	60.47	30.13	38.96
06.19.061	Cv.c.s	94	15.36	10.83	9.45	5.79	16.84	5.11	55.43	27.75	37.32
06.19.063	Cv.c.s	120	18.78	13.19	10.09	5.98	18.97	5.35	65.14	31.73	45.29
06.19.064	Cv.c.s	110	18.74	13.78	10.52	7.41	20.18	5.91	62.90	30.50	44.60
06.19.065	Cv.c.s	108	17.58	12.53	9.50	7.22	19.26	5.32	64.02	31.83	43.48

Article III

Diet study of geckos reveals the first records of pseudoscorpions on Desertas Islands (Cabo Verde)

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SHORT COMMUNICATION

Diet study of geckos reveals the first records of pseudoscorpions on Desertas Islands (Cabo Verde)

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Abstract. Pseudoscorpions are known worldwide and yet are poorly studied mainly due to the difficulty of detecting them. Among their predators are ground-dwelling taxa, such as arthropods, amphibians, birds, and reptiles. Only four pseudoscorpion species are known to occur in the Cabo Verde Archipelago, and none in the Desertas Islands, located in the northwest of the country. In this study, we record the first two species for the Desertas Islands. We used molecular and morphological methods to taxonomically identify the specimens retrieved from reptile faecal pellets and pitfall traps. We identified the presence of *Garypus* cf. *saxicola* on Raso Islet, *Olpium pallipes* (Lucas, 1849) on Raso and Santa Luzia Island, and a putative new species of *Olpium* L. Koch, 1873 on Branco Islet. This study emphasizes how an indirect measure of biodiversity and ecological interactions via potential predators, using non-invasive sampling combined with metabarcoding and morphological studies, can be used to uncover unknown biodiversity, particularly of cryptic groups from highly inaccessible locations. Likewise, this study highlights the lack of 16S genetic resources for pseudoscorpions in online reference databases.

Keywords: Arachnida, genetics, morphology, Pseudoscorpiones, Macaronesia

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Pseudoscorpions are small invertebrates (frequently 0.5–5 mm length) that usually occur in low numbers in cryptic habitats, which makes them hard to detect. The ca. 3,700 described species occur worldwide, except in Antarctica, but are more diverse in the tropics and subtropics (Dunlop & Penney 2012; Harvey 2013; Zaragoza 2017). While found in nearly all terrestrial habitats, they are more common in cracks, leaf litter, soil, moss, under stones, in tree hollows, in caves or in nests of birds and mammals (Harvey 1988). Among their predators are mainly ground-dwelling taxa, such as arthropods, amphibians, reptiles, and birds. Vertebrates can have 0.2–6% of their diet composed of pseudoscorpions (Zaragoza & Seva Román 1990). Small reptiles seem to prey on them in higher proportions, even positively selecting them in their diet (Zaragoza & Seva Román 1990).

The Cabo Verde Archipelago (Republic of Cabo Verde), located in the Atlantic Ocean about 500 km off the African coast, comprises ten main oceanic islands and several islets of volcanic origin (Mitchell-Thomé 1976). The Desertas Islands (Santa Luzia, Branco and Raso) are located on the northwest alignment of the archipelago (Fig. 1A). In Cabo Verde, only four pseudoscorpion species of three families, all considered native, have been recorded, in five islands (Table 1, Fig. 1A): *Paratemnoides feai* (Ellingsen, 1906), *Withius simoni* (Balzan, 1892), *Olpium pallipes* (Lucas, 1849), and *Parolpium minor* (Ellingsen, 1910) (*P. minor* not shown in Fig. 1A; see Table 1) (Dippenaar-Schoeman & Harvey 2000; Arechavaleta et al. 2005; Harvey 2013). An unidentified specimen was recently found on São Vicente Island (Vasconcelos et al. 2009), but until this study there were no records of pseudoscorpions on the uninhabited Desertas Islands.

Here, we present the first record of pseudoscorpions for the Desertas Islands. These specimens were obtained during a study of the diet of the Cabo Verde giant wall gecko *Tarentola gigas* (Bocage, 1875). One pseudoscorpion was found within a fresh faecal pellet from a gecko captured on Raso on 29 July 2016, out of the 62 pellets collected by performing belly massages. A previous study, carried out at São Vicente Island and focused on the diet of the São Vicente wall gecko *Tarentola substituta* (Joger, 1984), also found remains of unidentified pseudoscorpions in several samples (Vasconcelos et al. 2009), indicating they might be common in the diet of Cabo Verde endemic geckos. The remaining six pseudoscorpions were caught in pitfall traps in July 2016 (Raso) and September 2017 (Branco) that were set to build a reference collection of invertebrates from those islands (Table 1, Fig. 1). All specimens were preserved in 70% ethanol until morphological and genetic identification. Several images of the seven specimens were taken through a magnifying glass and deposited in Morphobank (M681498–M681526). The following measurements of the pedipalps were taken from the photographs, for further morphological identification after Beier (1963), using ImageJ 1.47 software (online at http://imagej.nih.gov/ij/): femur length (FL) and width (FW), patella length (PL) and width (PW), chela length with pedicel (CL), chela width (CW), and fixed finger length (FiL). DNA was extracted from the whole faecal pellet using a Stool DNA Isolation Kit, following the manufacturer's instructions, and from a portion of the individuals from the pitfalls using saline extraction. A short 16S (~110 base pairs, bp) and COI (~710 bp) mitochondrial fragments were amplified and sequenced [see details in Pinho et al. (2018)] using an Illumina MiSeq (16S) and Sanger (COI) sequencing, for the faecal pellet and pitfall individuals, respectively. A Maximum likelihood (ML) tree was built using our COI sequences and sequences from Harvey et al. (2020) available in GenBank (Fig. 1B), following Vasconcelos et al. (2016).

The barcoded 16S sequence from the pellet returned only one hit with megablast search of the GenBank database (online at https://www.ncbi.nlm.nih.gov/) with 96.97% identity match with the scorpion *Diplocentrus sagittipalpus* Santibanez-Lopez, Francke & Prendini, 2013 (Scorpiones: Diplocentrinae), but with only 30% of query cover. Blastn results were overall inconclusive, with all matches showing $< 85\%$ similarity to different arthropods classes. This could be explained by contamination or the paucity of pseudoscorpion 16S

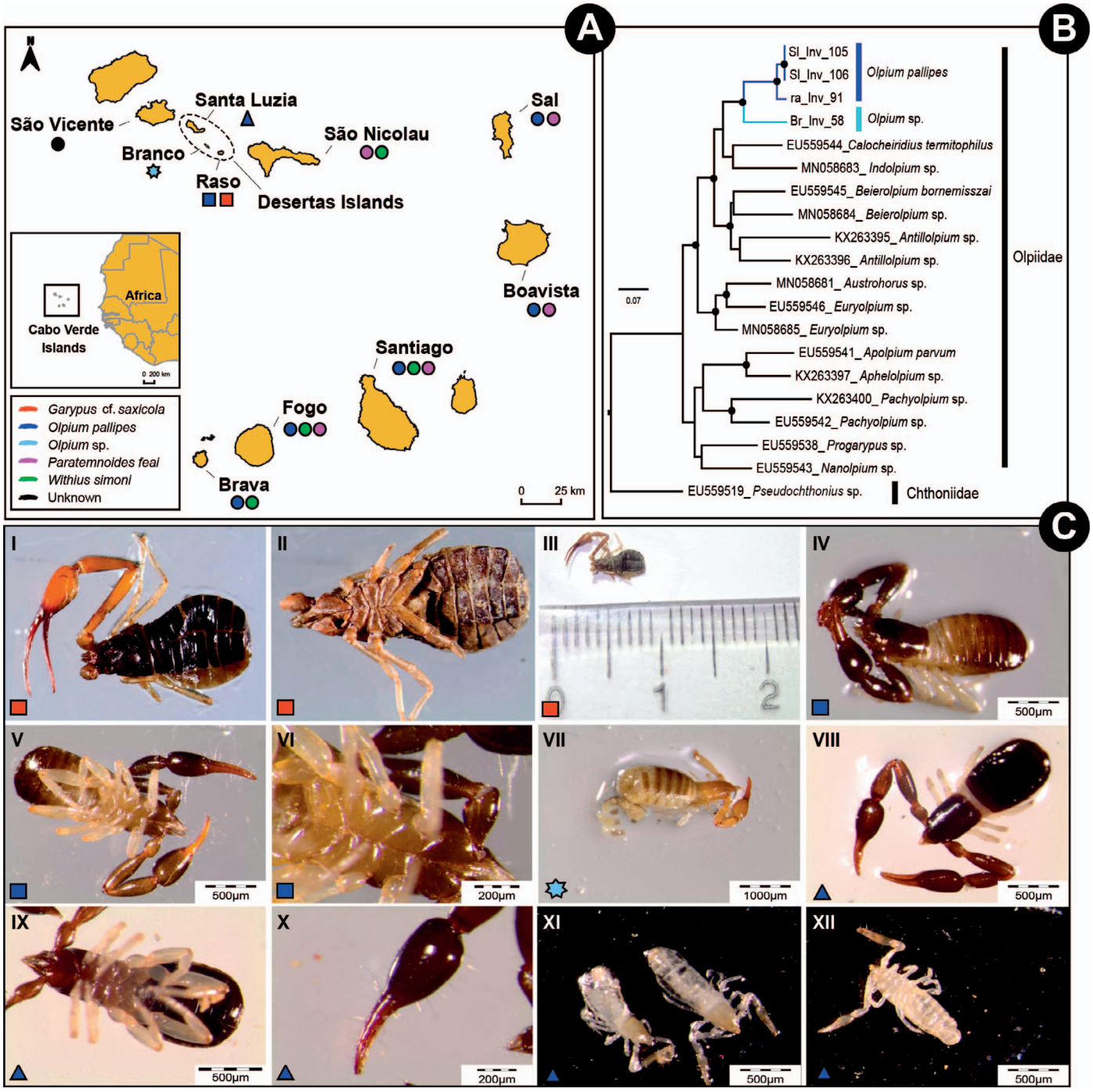

Figure 1.—Pseudoscorpion diversity of Cabo Verde. (A) Map of the archipelago showing the previously recorded (circles) and new occurrences (other symbols) of pseudoscorpions across the islands; dashed ellipse highlights our study site, the Desertas Islands; occurrences of *Parolpium minor* not shown (see Table 1); (B) Maximum likelihood phylogenetic tree (GTR+G model) of available Olpiidae sequences; black dots indicate bootstrap values > 70%; (C) Photos of the pseudoscorpion specimens found on Desertas, Cabo Verde in 2016 and 2017 (colours and shapes on the left corner correspond to taxa and islands in the map): (I) *Garypus* cf. *saxicola* (found within a faecal pellet) from Raso Islet, Chã Branca (16.617723°N, 24.602239°W; specimen Tg_ra_07.80), dorsal view, (II) ventral view and (III) size detail of same specimen; (IV) *Olpium pallipes* from Raso Islet, Chã do Castelo (16.617110°N, 24.586627°W; specimen ra_Inv_91) dorsal view, (V) ventral view and (VI) pedipalp detail; (VII) *Olpium* sp. from Branco Islet, Ponta Parede – Tope Berta (16.65071°N, 24,66607°W; specimen Br_Inv_58), dorsal view; (VIII) *Olpium pallipes* from Santa Luzia Island (16.764°N, 24.743°W; SL_Inv_105), dorsal view, (IX) ventral view and (X) pedipalp detail; (XI) nymphs of *Olpium pallipes* from Santa Luzia Island (specimens SL_Inv_106 I and II), dorsal and (XII) ventral view.

Table 1.—Pseudoscorpion diversity of Cabo Verde Archipelago. Taxonomic details, distribution, values of pedipalpal femur length (FL) and width (FW), patella length (PL) and width (PW), chela length with pedicel (CL), chela width (CW), and fixed finger length (FiL) in mm, location – São Vicente (SV), Raso (Ra), Branco (Br), Santa Luzia (SL), São Nicolau (SN), Sal (S), Boavista (BV), Santiago (ST), Fogo (F), Brava (B), the mitochondrial marker sequenced and Morphobank and GenBank codes are also given. Samples marked with an (*) correspond to juveniles. Old records were retrieved from Arechavaleta et al. (2005), Vasconcelos et al. (2009), and Harvey (2013).

Superfamily	Family	Species	Sample Code	Location	FL	FW	PL	PW	CL	CW	FiL	Marker	Morphobank	GenBank
Garypoidea	Garypidae	*Garypus* cf. *saxicola*	Tg_ra_07.80	Ra	2.75	0.44	1.62	0.44	3.80	0.87	2.35	16S	M681498–M681501	-
Garypoidea	Olpiidae	*Olpium pallipes*	ra_Inv_91	Ra	0.59	0.17	0.55	0.19	0.93	0.30	0.49	COI	M681503–M681506	MW760701
Garypoidea	Olpiidae	*Olpium* sp.	Br_Inv_58	Br	0.67	0.18	0.54	0.21	1.01	0.32	0.55	COI	M681526; M693785	MW760700
Garypoidea	Olpiidae	*Olpium pallipes*	SL_Inv_105_I	SL	0.63	0.19	0.59	0.22	1.11	0.34	0.54	COI	M681507–M681511; M693786	MW760702
Garypoidea	Olpiidae	*Olpium pallipes*	SL_Inv_105_II	SL	0.63	0.17	0.57	0.20	1.01	0.30	0.53	-	M681512–M681514	-
Garypoidea	Olpiidae	*Olpium pallipes*	SL_Inv_106_I*	SL	-	-	-	-	-	-	-	COI	M681523–M681524	MW760703
Garypoidea	Olpiidae	*Olpium pallipes*	SL_Inv_106_II*	SL	-	-	-	-	-	-	-	-	M681525	-
Garypoidea	Olpiidae	*Olpium pallipes*,	-	S, BV, ST, F, B	-	-	-	-	-	-	-	-	-	-
Garypoidea	Olpiidae	*Parolpium minor*	-	Cabo Verde	-	-	-	-	-	-	-	-	-	-
Cheliferoidea	Atemnidae	*Paratemnoides feai*	-	SN, S, BV, ST, F	-	-	-	-	-	-	-	-	-	-
Cheliferoidea	Withiidae	*Withius simoni*	-	SN, ST, F, B	-	-	-	-	-	-	-	-	-	-
-	-	-	-	SV	-	-	-	-	-	-	-	-	-	-

sequences in GenBank. The exoskeleton of the specimen from the pellet was mostly intact but very dry and brittle, missing one pedipalp and some legs. Interior body content was missing, presumably due to digestion by the gecko. The success of amplification and sequencing of digested diet items depends mostly on the frequency of occurrence of each item. Therefore, the likelihood of detecting low frequency items such as pseudoscorpions in the pellets of geckos with genetic methods is rather low. However, even though its morphological identification was problematic due to its deterioration, it was tentatively identified as *Garypus* cf. *saxicola* Waterhouse, 1878 (Garypidae) (Fig. 1C: I–III), based on characteristic traits such as triangular carapace, eyes on ocular tubercles situated away from anterior margin of carapace, fingers of pedipalps without accessory teeth, femora of legs I and II longer than the patellae, short arolium, and measurements of the body and the pedipalps (Table 1). This is the first time this family is recorded in the Cabo Verde Islands. Species of Garypidae occur in a wide variety of habitats, but the genus *Garypus* L. Koch, 1873 is more commonly found close to seashores and associated with halophilic vegetation (Harvey et al. 2020; Zaragoza 2007). Due to the small size of the Desertas Islands, the seashore is a predominant habitat where halophilic vegetation thrives (Freitas et al. 2015; Lobin 2015). Altogether, 31 species of the genus *Garypus* are recognized worldwide, but only two occur on the east Atlantic coast (Harvey 2013; Harvey et al. 2020). Hence, considering that natural colonisations of the Cabo Verde Islands by pseudoscorpions would have to be through marine dispersal, using the Canary current, it is logical to assume continental Iberia and the Atlantic islands as probable source areas. *Garypus saxicola* was previously known only from Iberia (Helversen 1965; Zaragoza 2007) with two subspecies: *G. saxicola saxicola* Waterhouse, 1878 from continental Iberia and the Canary Islands, and *G. saxicola salvagensis* Helversen, 1965 from the Selvagens Islands, both North Atlantic archipelagos. It should be noted that the measurements of the pedipalps of our sample (Table 1) slightly differed from those two taxa (FL = 2.22–2.42 mm, FW = 0.50–0.53 mm, PL = 1.80–1.88 mm, PW = 0.50–0.54 mm (Beier 1963; Mahnert 2011), and FL = 2.33–2.40 mm, FW = 0.47–0.50 mm, PL = 1.76–2.00 mm, PW = 0.51–0.62 mm (Helversen 1965), respectively) and we cannot exclude the possibility of an introduction. Additionally, two other *Garypus* species are known from nearby regions: *Garypus beauvoisii* (Audouin, 1826) in the Canary Islands, and *Garypus titanius* Beier, 1961 in Ascension Island in the South Atlantic (Harvey 2013; Harvey et al. 2020). Both species have a distinctly different size of the pedipalps compared to our specimen (FL = 1.73 mm, FW = 0.40 mm, PL = 1.40 mm, PW = 0.42 mm (Beier 1963); FL = 3.20–3.35 mm, FW = 0.63–0.71 mm, PL = 2.52–2.65 mm, PW = 0.68–0.72 mm (Beier 1960), respectively; Table 1). Some pseudoscorpions exhibit phoretic behaviour, where individuals attach to other organisms such as other arachnids, insects, mammals or birds, which facilitates their fast transport to different areas (e.g., Opatova & Šťáhlavský 2018). However, within the family Garypidae, there is no evidence of phoresy (Poinar et al. 1998). Our specimen could thus represent a putative cryptic new species, since the majority of known *Garypus* species are endemic to small areas and only a few have wide distribution ranges (Harvey et al. 2020). Therefore, without a genetic comparison with the other Macaronesian specimens, it is difficult to say whether this new record represents an addition to the occurrence range of known *Garypus* species or a new taxon.

The COI sequences of specimens collected from the pitfall traps were all assigned to the family Olpiidae (superfamily Garypoidea), with a 79.0–81.9% identity match. The ML tree supported the four specimens as a monophyletic clade within Olpiidae (Fig. 1B). Even though this family has been found previously in Cabo Verde (Arechavaleta et al. 2005), this is the first record for Desertas. The morphological identification confirmed that all specimens belonged to the genus *Olpium* L. Koch, 1873, which contains 34 described species distributed across Africa, southern Europe, Asia and Australia

(Harvey 2013). The specimens from Raso (Fig. 1C: IV–VI) and Santa Luzia (Fig. 1C: VIII–X) were identified as *O. pallipes*, as they possess the smooth and bicolored pedipalps that are characteristic of this species. Moreover, measurements of the sample SL_Inv_105 (Table 1) fully corresponded to this species (Beier 1963). *Olpium pallipes* has a wide distribution range, especially in the Mediterranean region, including North Africa, and it was previously recorded in Cabo Verde (Arechavaleta et al. 2005; Harvey 2013). It should be noted that cytogenetic analysis of this species demonstrated great differences between the karyotypes of individuals from Portugal and Greece (Šťáhlavský et al. 2006). Hence, a detailed taxonomical revision of this species is necessary, as cryptic species might be present.

The sample from Branco was not possible to identify to species level (Fig. 1C: VII). This *Olpium* sp. possessed a unicolored pedipalp (yellowish-brown), with only little granulation on the small, distal part of the pedipalp femur. The measurements of the female from Branco are similar to those for specimen SL_Inv_105_I (see Table 1), however, the position of the trichobothrium *st* on the movable finger of pedipalp was distinctly nearer to *t* than to *sb*, which differs from *O. pallipes* (M693785; M693786). Thus, more individuals both from Cabo Verde and other unsampled areas, such as the West African coast, are necessary to test if that is due to intraspecific variability of *O. pallipes* or if it is a diagnostic feature of a new species.

In conclusion, since arachnid research is often focused on scorpions and spiders, it is important to bridge the gap in our knowledge on pseudoscorpions, especially on understudied islands. Our study is, to our best knowledge, the first to use faeces to indirectly sample pseudoscorpions. Whether their presence in the pellets results from active predation—as pseudoscorpions of the superfamily Garypoidea also live under the same rocks as the gecko—or from indirect consumption, is still to be clarified. Additionally, our findings demonstrated the capacity of trans-oceanic dispersal of some pseudoscorpion families that deserves further research. Finally, it emphasizes how the combination of traditional surveys with metabarcoding and morphometrics can be used to gain more insights into known taxa and to uncover unknown biodiversity, particularly of cryptic groups and from infrequently accessed locations.

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Chapter 3

Metabarcoding as a tool to explore vertebrate extinction drivers

Article IV

Digging in a 120 years-old lunch: What can we learn from collection specimens of extinct species?

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RESEARCH ARTICLE

Digging in a 120 years-old lunch: What can we learn from collection specimens of extinct species?

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Abstract

Studying collection specimens is often the only way to unravel information about recent extinctions. These can reveal knowledge on threats and life traits related to extinction, and contribute, by extrapolation, to the conservation of extant species. However, high-throughput sequencing methods have rarely been applied to extinct species to reveal information on their ecology. Insular species are especially prone to extinction. We studied the gut contents of three specimens of the extinct giant skink *Chioninia coctei* of the Cabo Verde Islands using microscopy and DNA-metabarcoding. The presence of *Tachygonetria* adult nematodes suggests plants as important diet items. Our metabarcoding approach also identified plants and, additionally, invertebrates, supporting the hypothesis of *C. coctei*'s generalist diet. The absence of vertebrates in the digestive contents may reflect the decline of seabirds on the Desertas Islands that could have contributed to the debilitation of the giant skink, already depleted by persecution and severe droughts. Even with a small sample size, this study contributes to shedding light on the trophic roles of this enigmatic extinct species and emphasizes the need to develop holistic conservation plans for island threatened taxa. Additionally, it illustrates the potential of integrating up-to-date molecular methods with traditional approaches to studying collection specimens to help to solve ecological puzzles in other ecosystems.

Introduction

Anthropogenic threats and climate change are driving more and more species to extinction faster than the discovery rate, leading to a global concern on how to halt the current biodiversity loss [1,2]. Island ecosystems present the highest rates of both unique biodiversity and

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species extinctions [3]. Even though the number of species is typically low compared to the mainland, the number of endemics is usually high [3]. Low gene flow among islands, together with lower predation pressures and more limited food resources, promote the occurrence of unusual biological characteristics, such as diet specialization [4–6], and gigantism [7–10]. However, these traits have been shown to make species more vulnerable to extinction [11–13].

Looking into the past and learning more about extinct species can unravel important information about the threats and traits related to their extinction and help to develop adequate conservation actions for closely related extant species [14–17]. One of the approaches to achieve this consists of taking advantage of the recent development in molecular biology in order to shed new light on ecology by studying historical collection specimens [18–20]. Several studies have used DNA from historic museum specimens to obtain important temporal evolutionary perspectives [21,22]. However, few have focused on extinct island species of reptiles [23]. Most recently extinct lizard species were island endemics, and frequently larger than extant ones [7,24]. Extinction processes have been associated with anthropogenic pressure, affecting even more habitat specialists inhabiting reduced areas [25]. Insular reptiles often exhibit peculiar feeding habits [4,26,27] such as herbivory [8,27,28], omnivory or cannibalism, thus, playing valuable roles in the ecosystem as seed dispersers, pollinators and even top predators in some cases [29]. Therefore, studying their diet, ecology, and behaviour can help to raise awareness of humans about the importance of reptiles to enhance the survival of other island threatened species.

The Cabo Verde Archipelago is nearly 500 km off the Atlantic African coast and comprises ten main islands and several islets (Fig 1). This biodiversity hotspot has a remarkable reptile diversity and all native taxa are endemics [30,31]. The *Chioninia* genus holds all the endemic skinks found in Cabo Verde, namely six extant species and the extinct Cabo Verde giant skink, also known as Cocteau's skink, *Chioninia coctei* (Duméril & Bibron, 1839) [32], which present remarkable adaptive characteristics [33]. This emblematic species, which became extinct more than one century ago, was one of the largest skinks in the world. Its large dimensions (snout-vent length reaching up to 380 mm [34]), along with the five-cuspid teeth, unique dorsal and cephalic scales [32], and the diversified colouration pattern [35], made this species not only remarkably divergent from the six other species of *Chioninia* (Fig 1) but also from the ca. 1700 known species of Scincidae [32,34–37].

The Cabo Verde giant skink is classified as Extinct since the second half of the 20th century by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [38] and in the First Red List of Cabo Verde Reptiles [39]. It was last seen in its natural habitat in 1912 [40], despite repeated subsequent searches [31], plus a last unsuccessful mission performed by the authors on Branco in 2017. During the 19th century, its presence was unambiguously demonstrated on the two islets of Branco and Raso [30,34], and subfossil reports and museum specimens confirm the original distribution range of *C. coctei* on the islands of Santa Luzia and São Vicente [41,42]. Even though in 2005 a mandible of a juvenile was recovered in cat scats on Santa Luzia [43], a 2006 extensive survey on the island failed to find indications of the living presence of this species [31,32]. It might have also been present in the island of São Nicolau as stated by local fishermen, though so far, no solid evidence was found [40,44]. The probable causes of Cocteau's skink extinction seem to rely on a combination of human and natural causes. The introduction of mammals in the archipelago may have had a significant impact on the number of specimens, as well as repeated long drought periods [45]. Due to its uncommonly large size, this species was actively hunted by fishermen and prisoners deported in 1833 to the uninhabited Branco Islet, either as a food source [40], for their supposed medical properties, or to use their skins [34,46]. Furthermore, this 'giant' skink attracted the attention of natural history dealers, which led to the collection of at least 86 specimens (but very likely

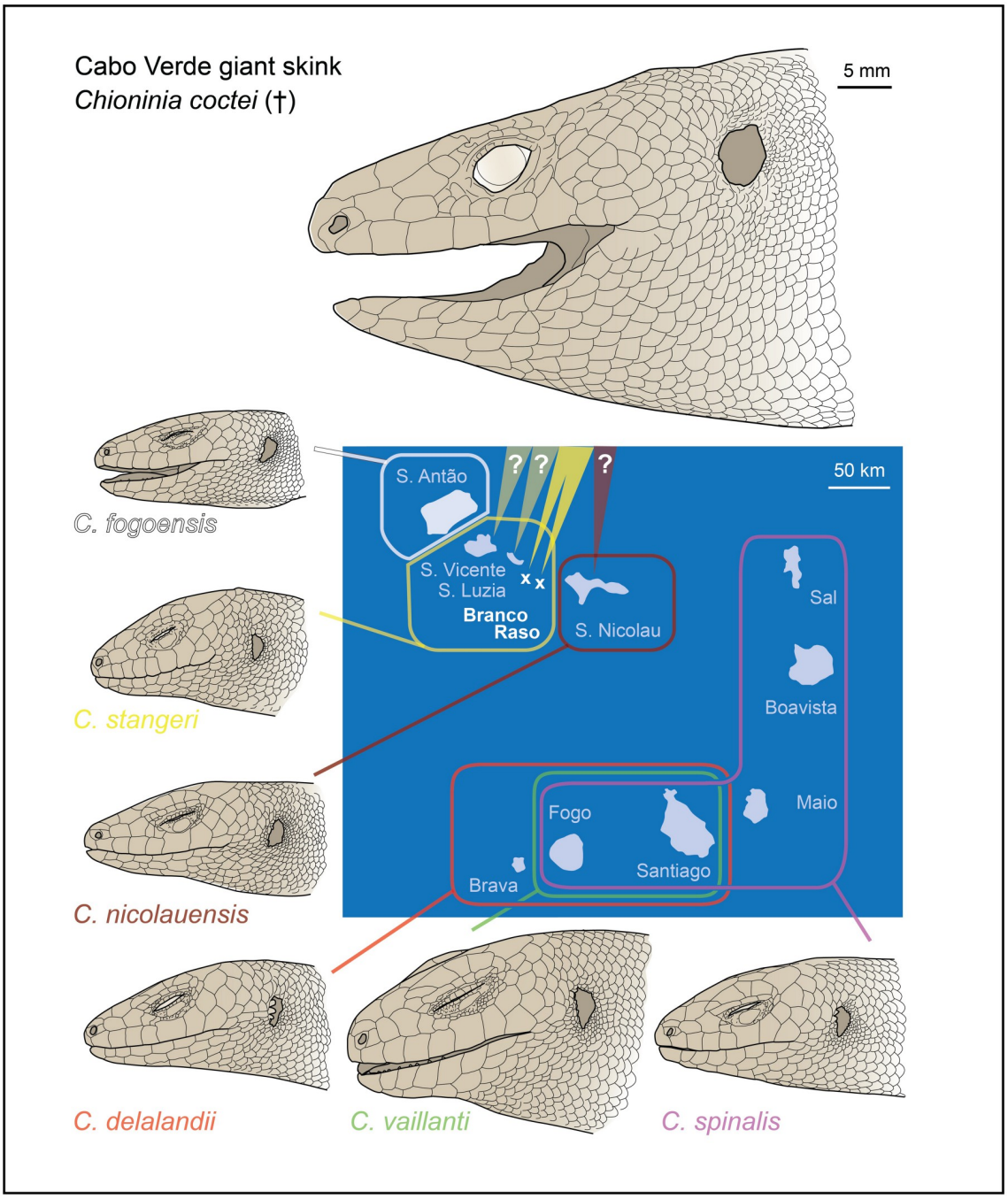

Fig 1. Extant and extinct (†) *Chioninia* species of Cabo Verde Islands. Distribution map of the seven different endemic *Chioninia* species, with illustrations showing their remarkable size variation.

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more) for museums and private collections (see S1 Table and S1 Fig) [41,45]. Based on the literature and museum data at least 63 specimens were catalogued during 1880–1930 (S1 Table and S1 Fig).

The knowledge on the ecology of *C. coctei* is scarce and mainly relies on empirical and scattered historical observations. Most authors, based on direct observations and interviews of

locals, referred to its commensalism with seabirds, such as the Cabo Verde shearwater *Calonectris edwardsii* (Oustalet, 1883), where lizards occupied rocky burrows of nesting sites and consumed the leftovers of regurgitations that adult birds spill over while feeding their chicks [40,47,48]. Also, Alexander [47] reported that skinks had been pulled out from the holes of white-faced storm petrels *Pelagodroma marina* (Latham, 1790). Nocturnal or crepuscular according to Vaillant [49], this species would prey on seabirds' eggs and insects. Its particular teeth morphology has been regarded by several authors as evidence of an adaptive shift toward a herbivorous or omnivorous diet [50,51]. It was also reported that animals kept in captivity survived feeding exclusively on plants [50,52]. Despite these invaluable past reports representing the few direct testimonies about the species, they are contradictory, and more data would be necessary to unravel the diet and other ecological features of this species.

Even though no more living individuals can be found, this charismatic species can still be studied through natural history collection vouchers. Their digestive tract contents represent the only remaining source of evidence to determine the species diet. DNA based technologies such as next-generation sequencing (NGS) metabarcoding have demonstrated their potential to recover valuable information from degraded collection specimens [53–55]. In this study, we take advantage of those technologies to shed light on the enigmatic ecology of this extinct species and to explore possible reasons that may have contributed to its extinction. Particularly, we aimed to learn more about the health status and diet of the last three known specimens of giant skink collected in 1901 on Branco Islet by the Princess Alice II expedition and which, according to our knowledge, have never been examined by scientists so far.

Methods

Type locality

Branco Islet, located between Santa Luzia Island and Raso Islet, is part of the Desertas Islands in the Cabo Verde Archipelago. This central island group is characterized by a dry tropical Sahelian climate, with very arid plane lowlands and medium elevation areas [56]. Branco, which emerged around 6 million years ago, is only 3 km^2 [57], and it is dominated by mountainous and medium-elevation arid areas, reaching its highest point in Tope Berta (353 m) [58]. This islet is of difficult access due to the roughness of the sea, lack of safe natural ports, steepness (there is just a small flat area of about 400 × 200 m), and high exposure to the trade winds in most of the north coast. The Desertas Islands present an annual rainfall ranging between 122–186 mm (mean = 148.5 ± 15.9 mm) [58].

Examination of specimens

In 2017, during a visit to the 'Musée océanographique de Monaco' (MOM), at the 'Institut Océanographique', Fondation Albert I^{er}, Prince de Monaco, five vouchers of *Chioninia coctei* (VS0000067_A–E), never referred to in the scientific literature before were discovered and studied. The specimens were collected on July 22nd and 23rd of 1901 by Prince Albert I on Branco Islet during the Expeditions of Princess Alice II [59]. The five specimens were photographed dorsally and ventrally, measured from the tip of the snout to the posterior edge of the cloaca (SVL), and sexed by examining the presence/ absence of hemipenes and in the case of dissected individuals, their internal reproductive structures. The photos of the specimens were deposited in Morphobank (Table 1). We were allowed to dissect three of them. The digestive tracts of these specimens (hereafter individuals A, B and C) were opened with a scalpel and examined. A considerable number of parasites were observed while collecting the tracts. The digestive contents were collected and preserved in 70% pure ethanol and stored at -20ºC for further analyses. At the end of this study, voucher A (UCV2017/0001) was donated by MOM

Table 1. Study specimens.

Code	SVL (mm)	Sex	Diet	Nr of helminths	Morphobank code
VS0000067_A*	240	F	Yes	236	M687941; M687942
VS0000067_B	267	M	Yes	12500 aprox.	M687944; M687945
VS0000067_C	228	M	Yes	0	M687946; M687947
VS0000067_D	237	?	No	-	M687948; M687949
VS0000067_E	188	M	No	-	M687950; M687951

Details of the *Chioninia coctei* vouchers rediscovered in 'Institut Océanographique' Fondation Albert Ier, Prince de Monaco. All specimens were collected on Branco Islet in 1901.

* This voucher was recently donated to the Natural History Museum of Cabo Verde (UCV2017/0001).

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to the collections of the Technical University of the Atlantic, São Vicente, the future Natural History Museum of Cabo Verde.

Parasite analysis

Adult helminths were removed from the digestive contents and morphologically examined with a binocular magnifying glass. The helminths were photographed, counted, and taxonomically identified based on the following variables: oesophagus length, the structure of the apical end, the structure of the caudal end of the males—including length and shape of the spicule, existence and shape of the gubernaculum, and arrangement of the caudal papillae [60–62]. Then, helminths were preserved in 70% ethanol.

In addition, the DNA of the helminths was extracted using QIAamp DNA Micro Kit (Qiagen, Crawley, UK), following the manufacturer's instructions. The DNA amplification was carried out for the nuclear 28S rDNA, including the D2 region, using the primers 28SF0001 and 28SR0990 (~850 bps) [63]. The PCR was performed in a total of 10 μL reaction volumes containing: 2 μL of QIAGEN Multiplex PCR Master Mix (Qiagen, Crawley, UK), 0.5 μL of each 10 μM primer, 3 μL of ultra-pure water, and 4 μL of DNA extract. Cycling conditions used an initial denaturing at 95ºC for 12 min, followed by 35 cycles of denaturing at 95ºC for 15s, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, with a final extension at 72ºC for 7 min. Post-PCR steps (PCR clean-up, cycle sequencing, cycle sequencing clean-up) followed standard procedures for degraded DNA [64]. PCR products were in a first attempt Sanger sequenced and in a second attempt sequenced on Illumina's MiSeq platform [64].

Metabarcoding diet analysis

The stomach contents were air-dried completely to reduce the volume and concentrate the DNA. DNA extraction was performed using the whole homogenised stomach contents, in several total volumes of 50 μL, using the Stool DNA Isolation Kit (Norgen Biotek Corp.Canada) and following the manufacturer's instructions.

Three different DNA markers were selected to identify the diet items present in the digestive contents. For plants, the g/h primers that target the short P6-loop of chloroplast trnL (UAA) intron (~10–143 bps) were used [65]. The primers IN16STK-1F/ IN16STK-1R, targeting the mitochondrial 16S rRNA gene (~110 bps) [66] and 12sv5F/ 12Ssv5R targeting the V5-loop fragment of the mitochondrial 12S gene (~73–110 bps) [67] were used to amplify invertebrates and vertebrates [67], respectively. All primers were modified to contain Illumina adaptors and individual barcodes to allow individual identification for the three genetic loci. The regions were amplified by PCR cycles in a total volume of 25 μL, following specific

conditions according to the taxonomic group (following [68]). Library preparation was carried out following Illumina MiSeq protocol 16S Metagenomic Sequencing Library Preparation [69]. The final library was run on a MiSeq sequencer (Illumina, San Diego, CA, USA) using a 2 × 150 bp MiSeq Reagent Kit (Illumina, San Diego, CA, USA) for a projected average of 12,000 paired-end reads per sample.

The software package OBITools (http://metabarcoding.org/obitools) was used for sequence processing. After filtering, the final haplotypes were blasted against known reference sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and our Molecular Operational Taxonomic Units (MOTU) reference database [68]. Sequences with less than 90% of similarity were classified only to the class level, whereas the ones with values between 90% to 95% were classified to the family level. In addition, sequences with similarity values beyond 95% were classified to the genus or species level. If a haplotype was similar to more than one species or genus, only species or genera recognized to occur on Branco Islet, or the surrounding islands of the archipelago were considered [70]. Haplotypes identified as contaminations (e.g., human DNA) were removed.

Results

Examination of specimens

Vouchers had an average SVL of 232 mm (n = 5, Table 1). Voucher A was identified as a female; voucher D was not possible to sex, and the remaining specimens were identified as males (Table 1). Individuals A, B and E appeared to be unusually skinny and/or dehydrated (see photos on Morphobank).

Parasite analysis

The analysis of the intestine contents revealed the presence of nematode parasites in two of the three specimens analysed, all of them belonging to the family Pharyngodonidae (Table 1). All sampled nematodes were identified morphologically to species-level based on the above-mentioned morphological variables (Fig 2A–2D). Five different taxa were identified, all belonging to the same genus *Tachygonetria* Wedl, 1862: *T*. *longicollis longicollis* (Schneider, 1866), *T*. *longicollis setosa* (Petter, 1966), *T*. *macrolaimus* (Linstow, 1899), *T*. *numidica* Seurat, 1918, and *T*. *conica* (Drasche, 1884). In specimen A, we isolated a total of 236 nematodes, all females of the first three taxa. In voucher B, it was possible to observe a compact mass of plant matter to which a large number of nematodes were attached. In this voucher, thousands of nematodes were found, both males and females, with an estimated amount of approximately 12.500 individuals belonging to the five species mentioned above (Table 1). On the intestinal contents of voucher C, no nematodes were observed. Despite several attempts, sequencing failed for the DNA samples of the nematodes, probably due to the highly degraded state of the samples.

Metabarcoding diet analysis

The overall diet composition of the three studied specimens, obtained with metabarcoding, is depicted in Fig 3. After bioinformatic filtering, the average coverage obtained was about 11200 sequence reads per sample (see S2 Table). We identified a total of 29 diet items, 12 corresponding to plants—comprising six orders and seven families—and 17 to arthropods—comprising nine orders and 12 families. The haplotype sequences of the 29 MOTUs are available in the S2 Table. The analysis identified plant items in two of the three samples and invertebrates in all three samples. Sample A presented four MOTUs of plants and 13 of invertebrates, sample B 10 MOTUs of invertebrates, and sample C 10 of plants and 16 of invertebrates. No vertebrate

Fig 2. Ventral views of *Tachigonetria* nematodes identified from *Chioninia coctei*'s digestive contents (photos by V. Roca). A) *T. longicollis*, male caudal end (only *T. l. longicollis* is represented as differences between subspecies are difficult to depict); B) *T. numidica*, female; C) *T. macrolaimus*, male caudal end; D) *T. conica*, female.

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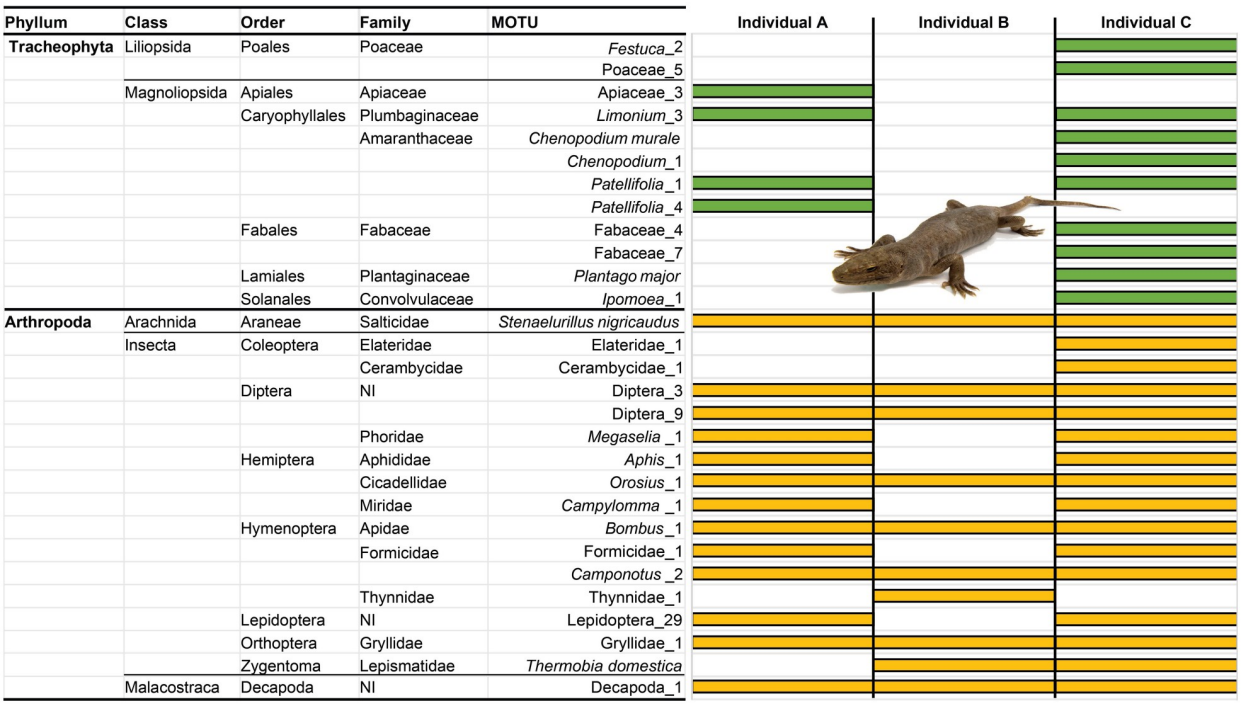
DNA sequences were detected. Plumbaginaceae (e.g.: *Limonium*) and Amaranthaceae (e.g.: *Patellifolia*) plant families had the highest frequency of occurrence for plants. Five arthropod families were present in all samples (Salticidae, Cicadellidae, Apidae, Formicidae, Gryllidae), having the highest overall frequency of occurrence, along with non-identified Diptera and Decapoda (Fig 3).

Discussion

Even though our results only represent a very small part of the last extant population of the Cabo Verde giant skink and a snapshot of its diet, as we studied only the last meal ingested by three specimens, we provide valuable data regarding the conditions and dietary habits of this emblematic extinct species. Considering this is an extinct and rare species, with roughly 80 specimens in worldwide museums, of which only about 30 specimens are putatively available, getting information from three vouchers, corresponding to 10% of all vouchers (see S1 Table



Phyllum	Class	Order	Family	MOTU	Individual A	Individual B	Individual C
Tracheophyta	Liliopsida	Poales	Poaceae	*Festuca*_2			
				Poaceae_5			
	Magnoliopsida	Apiales	Apiaceae	Apiaceae_3			
		Caryophyllales	Plumbaginaceae	*Limonium*_3			
			Amaranthaceae	*Chenopodium murale*			
				*Chenopodium*_1			
				Patellifolia _1			
				Patellifolia _4			
		Fabales	Fabaceae	Fabaceae_4			
				Fabaceae_7			
		Lamiales	Plantaginaceae	*Plantago major*			
		Solanales	Convolvulaceae	*Ipomoea*_1			
Arthropoda	Arachnida	Araneae	Salticidae	*Stenaelurillus nigricaudus*			
	Insecta	Coleoptera	Elateridae	Elateridae_1			
			Cerambycidae	Cerambycidae_1			
		Diptera	NI	Diptera_3			
				Diptera_9			
			Phoridae	*Megaselia* _1			
		Hemiptera	Aphididae	*Aphis*_1			
			Cicadellidae	*Orosius*_1			
			Miridae	*Campylomma* _1			
		Hymenoptera	Apidae	*Bombus*_1			
			Formicidae	Formicidae_1			
				Camponotus _2			
			Thynnidae	Thynnidae_1			
		Lepidoptera	NI	Lepidoptera_29			
		Orthoptera	Gryllidae	Gryllidae_1			
		Zygentoma	Lepismatidae	*Thermobia domestica*			
	Malacostraca	Decapoda	NI	Decapoda_1			

Fig 3. Diet items detected in the stomach contents of the *Chioninia coctei* vouchers (photo by M. Dagnino). Items are represented until the higher taxonomic identification possible to obtain. Plant and invertebrate MOTUs are represented in green and yellow, respectively. NI describes non-identified families.

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and S1 Fig), represents the first opportunity to describe the diet of this species and provide unique clues on the ecology of this enigmatic species.

Parasites and herbivory hypothesis

The report from the Princess Alice II expeditions described the captured individuals as 'miserable' looking [52]. This gives us clues on the health status of the last *C. coctei* individuals of a population on the brink of extinction. This observation may be related to the extremely high parasitic load of one of the vouchers that we found. Naturally, a sense of balance between parasites and reptile hosts prevents the progress of diseases, however, if exposed to environmental stressors this equilibrium can be broken [71,72]. In this latter case, the health of the host can be directly or indirectly affected by helminths [73]. Host social behaviour can be modified by parasites promoting aggressive actions, reducing mobility which can reduce the possibility of finding a partner or food, reducing reproduction investment by females [74] and ultimately survival rates [75,76]. However, the other two vouchers presented parasite loads similar to the ones observed in other large insular lizards in the nearby Canary Islands [77,78], positing to the existence of other main drivers affecting the health of the individuals.

Several studies showed an association between the nematode fauna and the type of diet of the reptile hosts [77,79,80]. The helminths from the Pharyngodonidae family have two evolutionary lineages, comprising different genera, commonly associated with different diets, carnivorous saurians or herbivorous iguanids/ tortoises [81]. The genus *Tachygonetria* identified in our samples corresponds to the lineage that usually infects herbivorous reptiles. Their higher incidences are typically found in tortoises and lizards with omnivorous or herbivorous diets [82–84]. The finding of *Tachygonetria* in our study suggests an, at least partial,

herbivorous diet of *C. coctei*. This lineage could have infected this Cabo Verde reptile through the endemic and extinct Cabo Verdean tortoise *Geochelone atlantica* López-Jurado *et al.* 1998, as they could easily disperse between islands carrying nematodes with them, or by host switching with another infected sympatric herbivorous host [85], such as *Chioninia stangeri* (Gray, 1845) lizards. Unfortunately, our unsuccessful attempts to sequence these nematodes prevented us from disentangling the evolutionary history of the host-parasite interactions and the origin of these nematodes. Since Pharyngodonidae has a typical monoxenous life cycle, i.e., infecting a single host, it has been proposed [80] that the infection of herbivorous reptiles is favoured as they may have greater chances of inadvertently ingesting eggs dropped in plants through faecal pellets of infected hosts [80]. Besides, an intensification of plant matter consumption offers an ideal environment for the development of more rich and diverse helminth communities [80,81,83].

Insular reptile populations can reach higher densities as terrestrial predators are generally scarce [86]. In the drier periods, where food resources are scarcer, plant items may play an important role in their diets to balance the lower availability of arthropod prey [87], exactly when our vouchers were collected. At the same time, very small distribution ranges favour the transmission of nematodes among individuals [88,89]. Large body and gut sizes favour the recruitment of nematodes and are usually associated with higher consumption of plant matter [90]. Both characteristics were recorded in the studied *C. coctei* specimens. However, the low caloric intake provided by plants and the need for longer digestions makes the individuals more susceptible to introduced predators and humans. Indeed, several old records describe how easily these animals were caught by hand [34,40,52,91].

Omnivory hypothesis

Our molecular results also support the adaptation towards the consumption of plant matter by *C. coctei*. Plant consumption was identified in two out of three analysed samples, and even though we could not amplify plants in sample B, it was possible to observe traces of plants (e.g., green, and fibrous mass) when we examined the digestive content under the binocular magnifying glass. So, all studied individuals consumed plants. The morphology of the conic teeth with five cuspids [34,50], typical of herbivores [44], and empirical studies describing that some captured specimens survived several years feeding only on plants, gave origin to the assumption that the species was exclusively herbivorous [51]. However, the diet of *C. coctei* was mostly referred to in the literature as a generalist [44,48,49]. Our results support this hypothesis since we did find arthropods in all the three digestive contents analysed. And, in fact, some invertebrates were present in all samples (e.g., Decapoda) and had a higher diversity of MOTUs than plants. Skinks could be directly feeding on Decapoda, as observed in other island reptile species, such as *Cryptoblepharus cognatus* (Boettger, 1881) in Madagascar [92]. Alternatively, the secondary consumption of marine organisms is expected considering the commensal link of the giant skink with seabirds [93]. In fact, several reptiles adapted to oceanic islands feed on seabird eggs or their regurgitations [44,68,94,95].

No traces of vertebrates were detected in the gut content of the three sampled specimens, precluding to confirm several historical reports mentioning predation of this skink on seabirds (e.g. [48]). These reports mentioned a commensal and predatory link with endemic seabirds such as the Cabo Verde shearwater *Calonectris edwardsii* [40,47,48], especially after bird nesting season [44]. The lack of bird sequences in digestive contents could have been explained if the samples were collected outside the birds breeding season, but this was not the case. Although the breeding season of Boyd's shearwaters *Puffinus boydi* Mathews, 1912, white-faced storm petrels *Pelagodroma marina* and Cabo Verde storm petrels *Oceanodroma jabejabe*

(Bocage, 1875) extend only until June, the more abundant Cabo Verde shearwaters usually breed from June to November [96]. The latter one, in particular, was severely persecuted for food by fishermen for centuries [97]. Besides, in 1833 prisoners were deported to the deserted Branco Islet [40], and without any available resources, along with the skinks themselves, the chicks and adult seabirds could have been a valuable food source. That led to a severe decline of the seabird populations on Branco, and their almost disappearance from Santa Luzia, where the skink first disappeared [96]. Even though the islet populations were safe from introduced mammals, the seabirds decline through persecution may have reduced the access by skinks to this highly energetic food resource, while contributing to their fitness debilitation, especially in drought periods [87]. Our study specimens were collected after several decades of overexploitation of these resources, which could explain our results. An alternative explanation for the absence of vertebrates could be due to the small sample size and technical limitations, such as the lack of power of the 12S primers to amplify the highly degraded DNA of our samples. However, these markers have been effectively used to amplify museum samples [98] and were used in a variety of dietary studies with successful results [68,99]. Moreover, the other markers worked very well on these samples despite being more than 100 years old and possibly poorly preserved for DNA amplification.

To disentangle the several hypotheses related to the lack of seabird sequences we could try to get permission to check the digestive contents of more specimens of the beginning and mid-19th century, even though the chances of getting quality DNA are very low [53]. Several specimens of *C. coctei* were recently rediscovered in other institutions, such as the ones in the Natural History Museum of the University of Porto and Passos Manuel Lyceum in Lisbon [100], and are waiting to be studied using other techniques (e.g. osteology, isotopic analysis) that could shed some light on this [45]. We could also check the diet of the co-occurring reptile species and indeed the geckos feed on seabirds and their regurgitations [68], which suggests that herbivory is a common strategy to survive in such harsh and limited resourced islet [44]. Unfortunately, no diet studies were conducted for the extant smaller *Chioninia* species, even though anecdotal observations reported they prey on insects and plants confirming an omnivorous diet [30].

Alternative hypotheses for extinction drivers

In addition to the severe decline of seabirds, other potential factors may have contributed to *C. coctei* extinction. Droughts are a centuries-old problem in the archipelago and the 18th and 19th centuries were noticeable by long-term droughts that lead to severe famines and epidemics across the islands [101]. These also represented serious threats to biodiversity on islands, for instance, populations of *Alauda razae* (Alexander, 1898) fell to extremely low levels during these periods [102]. Consequently, long periods of droughts might have reduced drastically vegetation and arthropod availability on the islands. This skink presumably had a very low fecundity rate [45]. It is hypothesized that the average age at which sexual maturity was reached was five years for males and six years for females [45]. And, as an oviparous species [35,103], females normally laid two eggs per clutch [45]. All these factors that lead to the intensification of hunting of the skinks and seabirds [45], namely the introduction of mammal predators, such as cats and rodents, and the over-exploitation by natural history dealers, may have led to the extinction of the Cabo Verde giant skink [41,45]. All these extinction drivers were especially severe at the end of the 19th century, to the point that researchers feared its extinction at that time [87]. The example of this remarkable extinct species proves that species are vastly interdependent, especially on islands, as they co-evolved in disharmonic and isolated systems. Since the number of insular species is reduced, the trophic links among species are

fewer and stronger, leading to a more fragile balance [104]. This is especially evident in the smaller, most isolated, low-elevation islands [104,105] like the Desertas. Consequently, the local disappearance or reduction of even one element of that network, such as shearwater, might have led to the collapse of this delicate equilibrium, especially at the top of the ecological chain [106], such was probably the case of *C. coctei*. For this reason, it is crucial to design conservation plans that focus on protecting the ecological processes and not individual species [107]. In this way, it will be possible to ensure that processes such as pollination, seed dispersal and predation continue their viability in island ecosystems that are more vulnerable to disruptions [29]. However, the development of holistic conservation plans relies on studies of the diet and trophic roles of endemic species that are generally lacking [94]. This is striking for reptiles and with our work, we hope to contribute to efficiently diminishing this shortfall by integrating classical morphological with cutting-edge genetic technologies (e.g. [99]). This study can foster conservation measures for the six extant *Chioninia* species, two of which are classified as Endangered in the IUCN Red List of Threatened Species [31].

Genetics leveraging museum collections

Previous studies using DNA sequencing of ancient coprolites, rumen and gut microbiomes already provided valuable information on the diet of New Zealand's Upland Moa *Megalapteryx didinus* (Owen, 1883), Yakutian bison *Bison priscus* (Bojanus 1827), woolly mammoth *Mammuthus primigenius* (Blumenbach 1799) and woolly rhinoceros *Coelodonta antiquitatis* (Blumenbach, 1799), highlighting the informativeness of molecular approaches to unravel species extinction causes [108,109]. Likewise, molecular studies of sub-fossil remains and collection vouchers have been important to reconstruct the phylogeography and demographic history of the Tasmanian tiger *Thylacinus cynocephalus* (Harris, 1808) presently extinct [110]. However, genetic methods of high-throughput sequencing, such as metabarcoding, have rarely been applied to extinct species to reveal information on habitat preferences, behaviour, and ecology [19,109].

Our study illustrates once more the potential of new molecular tools, even when applied to old and degraded digestive contents. It also emphasizes the value of collection specimens to obtain reliable data on the ecology of extinct species and to provide some clues about their extinction drivers. Disclosing the existence of these overlooked vouchers will allow further studies to be performed and to solve other pieces of the ecological puzzle of Cabo Verde and other understudied ecosystems.

Supporting information

S1 Fig. Number of *C. coctei* specimens catalogued in museums per 50-year period. Unknown clusters group all vouchers with unavailable cataloguing dates (check S1 Table for details).
(TIF)

S1 Table. Voucher data published in the literature or available on candidate museum websites. Information regarding the voucher code, number (Nr), cataloguing date (Year), origin, museum name and location, the name of the collector/donor, and some notes are given. Listing according to ascending cataloguing date.
(PDF)

S2 Table. Details on the diet items identified in the stomach contents of the *Chioninia coctei* vouchers. Taxonomic identifications, frequency of occurrence (FO), haplotype sequences and the respective number of reads of the diet items. The final ID of MOTUs corresponds to

the highest taxonomical classification possible.
(PDF)

S1 Appendix. Abstract in Portuguese.
(PDF)

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Supplementary Material Article IV

Appendix I. Abstract in Portuguese

Estudar espécimes de colecções é frequentemente a única maneira de desvendar informações sobre extinções recentes. Estes podem revelar conhecimento sobre ameaças e características das espécies relacionados com a extinção e contribuir, por extrapolação, para a conservação das espécies existentes. No entanto, métodos moleculares de sequenciação em massa de ADN, raramente foram usados para desvendar informação sobre a ecologia das mesmas. As espécies insulares são especialmente propensas à extinção. Estudámos os conteúdos do intestino do lagarto gigante das ilhas de Cabo Verde extinto usando microscopia e *metabarcoding* de ADN. A presença de nemátodes *Tachygonetria* sugerem que as plantas eram importantes itens alimentares. A nossa abordagem de *metabarcoding* também identificou itens de plantas e ainda de invertebrados, apoiando a hipótese de uma dieta generalista do *Chioninia coctei*. A ausência de vertebrados nos conteúdos digestivos é um indício da possível extirpação das aves marinhas que pode ter contribuído para a debilitação do lagarto gigante, já debilitado pela perseguição e secas intensas. Este estudo contribui para lançar luz sobre os papéis tróficos desta espécie extinta enigmática e enfatiza a necessidade de desenvolver planos de conservação holísticos para espécies insulares ameaçadas. Adicionalmente, ilustra o potencial de integrar métodos moleculares recentes com abordagens tradicionais para estudar espécimes de museus para desvendar peças de outros enigmáticos puzzles ecológicos que aguardam serem montados.

Figure S1. Number of *C. coctei* specimens catalogued in museums per 50-year period. Unknown clusters group all vouchers with unavailable cataloguing dates (check Table S1 for details).

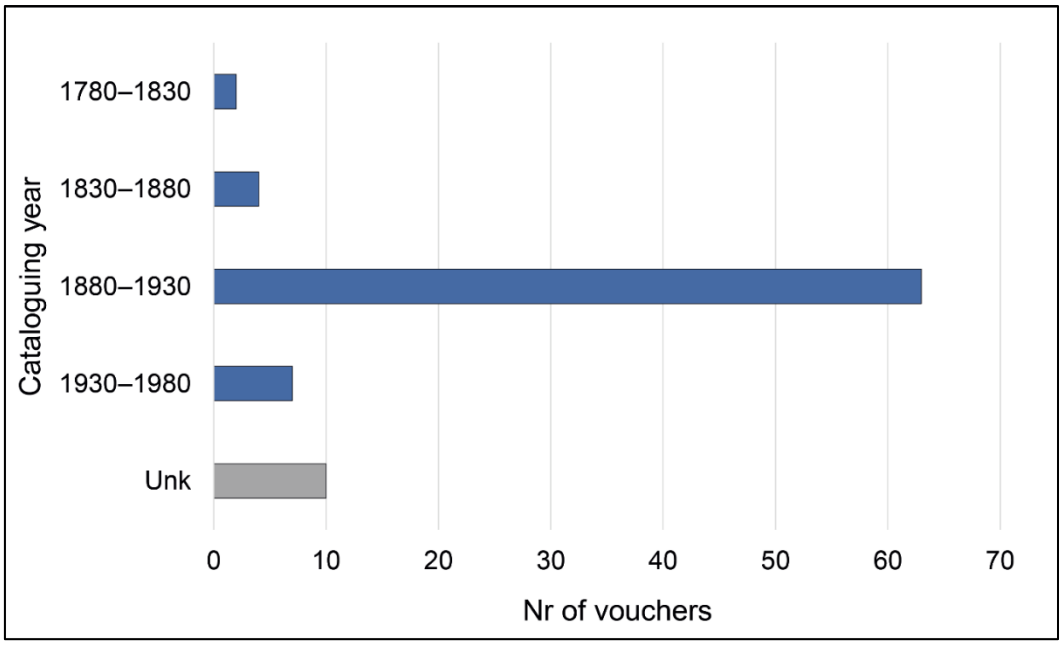

Table S1. Voucher data published in the literature or available in candidate museum websites. Information regarding the **voucher code, number (Nr), cataloguing date, origin, museum name and location, the name of the collector/donor, and some notes** are given.

Voucher code	Nr	Year	Origin	Museum name	Location	Collector/donor	Notes
1891.9.24.1-2	2	1809	Cabo Verde	Natural History Museum	London, UK	-	Part taken from Lisbon in 1809
MNHN-RA-0.8299	1	1839	Branco	Muséum national d'Histoire Naturelle	Paris, France	João da Silva Feijó	Skin; part taken from Lisbon in 1809
RMNH.RENA.17057	1	1839	Branco	Naturalis Biodiversity Center	Leiden, Netherlands	João da Silva Feijó	Part taken from Lisbon in 1809
UP-MHNFCP - 017374	1	1874	Raso	Natural History Museum and Sciene	Porto, Portugal	D. Hopffer	
1877.9.26.1	1	1877	Cabo Verde	Natural History Museum	London, UK	-	
MNHN-ZA-AC-1891-1134	1	1881	Cabo Verde	Muséum national d'Histoire Naturelle	Paris, France	-	Skeletons
MNHN-RA-1884.148; MNHN-RA-1884.227, 228, 229, 231, 233, 236, 237; MNHN-RA-1987.941	9	1883	Branco	Muséum national d'Histoire Naturelle	Paris, France	Expédition scientifique du Navire 'Talisman'	
MSNG 8769.1-2	2	1891	Branco	Musei di Storia Naturale di Genova	Genova, Italy	D. Schiavetti	
-	2	1891	Branco	Museo delle Scienze	Trento, Italy	Mario G.Peracca	
MZUT R1981.1-26	26	1891	Branco	Musei di Zoologia e di Anatomia Comparata	Turim, Italy	Mario G.Peracca	26 adults, 6 eggs (MZUT R1981.27-32)
MZST RI-6	6	1891	Branco	Museo Zoologico "G. Scarpa" di Treviso	Treviso, Italy	Mario G.Peracca	
NMW 10721 3/112/7	1	1892	Santa Luzia	Naturhistorisches Museum Wien	Vienna, Austria	Dr. Brillant	
MSNG 28891.1-2; 34516	3	1898	Raso	Musei di Storia Naturale di Genova	Genova, Italy	Leonardo Fea	
MZUF 176	1	1898	São Vicente	Museo Zoologico La Specola	Florence, Italy	G. Cecconi	
VS0000067_A	1	1901	Branco	Universidade Técnica do Atlântico	Cabo Verde	Prince Albert I	
VS0000067_B-E	4	1901	Branco	Musée Océanographique de Monaco	Monaco	Prince Albert I	
MNHN-RA-1906.295	1	1906	-	Muséum national d'Histoire Naturelle	Paris, France	Mario G.Peracca	
MNHN-ZA-AC-1906-458	1	1906	-	Muséum national d'Histoire Naturelle	Paris, France	Mario G.Peracca	Skeletons
NMW 885	1	1906	Cabo Verde	Naturhistorisches Museum wien	Vienna, Austria	Steindachner	
MNHN-ZA-AC-1907-255; MNHN-ZA-AC-1907-344	2	1907	Cabo Verde	Muséum national d'Histoire Naturelle	Paris, France	-	Skeletons
USNM 58495	1	1910	Cabo Verde	National Museum of Natural History	Washington, USA	-	https://www.gbif.org/pt/species/8412194
NMP-P6V 32744	1	1925	Cabo Verde	National Museum of Prague	Prague, Czech	R. M. Boušek	Partially damaged adult
MNHN-ZA-AC-1941-227	1	1941	Cabo Verde	Muséum national d'Histoire Naturelle	Paris, France	-	Skeletons
MNHN-ZA-AC-1943-133	1	1943	Cabo Verde	Muséum national d'Histoire Naturelle	Paris, France	-	Skeletons
1960.1.8.3-4	2	1960	Cabo Verde	Natural History Museum	London, UK	-	
AMNH 7116	1	-	Cabo Verde	American Museum of Natural History	New York, USA	-	Hutchinson (1989) - skeleton
R.10491	1	-	Cabo Verde	Cambridge University Museum of Zoology	Cambridge, USA	-	https://www.gbif.org/pt/species/8412193
MSNG 43132	1	-	Branco	Musei di Genova	Genova, Italy	-	
-	1	-	Cabo Verde	Museum für Naturkunde	Berlin, Germany	-	Ceriaco 2015 - anonymus sine anno
-	1	-	Cabo Verde	Passos Manuel Secondary School	Lisbon, Portugal	-	
ZMS 1/1978; ZMS 1/1979; ZMS 1/1987	3	-	Cabo Verde	Zoologische Staatssammlung München	Munich, Germany	-	Schleich 1979/1982
R-21886	1	-	Cabo Verde	Museum of Comparative Zoology	Cambridge, USA	-	https://www.gbif.org/pt/species/8412194
RBINS 2325	1	1978-	Cabo Verde	Royal Belgian Institute of natural Sciences	Brussels, Belgium	-	https://www.gbif.org/pt/species/8412194

Table S2. Taxonomic identifications, frequency of occurrence (FO), haplotype sequences and the respective number of reads of the diet items. The final ID of MOTUs corresponds to the highest taxonomical classification as possibl

Phylum	Class	Order	Family	Final ID	FO	Haplotype sequence	Reads
Tracheophyta	Liliopsida	Poales	Poaceae	Festuca_2	0.3	atccgtgttttgagaaaacaaggaggttctcgaactagaatacaaaggaaaag	1659
				Poaceae_5	0.3	atccgtgttttgagaaaacaaggggttctcgaactagaatacaaaggaaaag	4831
	Magnoliopsida	Apiales	Apiaceae	Apiaceae_3	0.3	atcctattttccaaaaacaaacaaaggcccagaaggtgaaaaaag	437
		Caryophyllales	Plumbaginacea	*Limonium*_3	0.6	ctcccgctttccaaatcaaaaaag	53211
			Amaranthacea	*Chenopodium murale*	0.3	ctccttttgtcaaaagcaaaaaatactcaaaagaaaaaaataataaaaaagcaagaaaaaaaaag	7190
				Chenopodium_1	0.3	ctccttttttcaaaagcaagaataaaaaaaaag	3
				Patellifolia_1	0.6	ctccttttttttcaaaagcaaaaaaagaaggattcagaaaacaagaataaaaaaag	12224
				Patellifolia_4	0.3	ctccttttttttcaaaagcaaaaaaagaaggattcagaaaacaagaataaaaaag	89
		Fabales	Fabaceae	Fabaceae_4	0.3	atcctgttttccgaaaacaaagaaaagttcataaagtgataataaaaaagg	1076
				Fabaceae_7	0.3	atcctgttttctgaaaacaaagaaaaattaagaaagttataataaaaaagg	104
		Lamiales	Plantaginaceae	*Plantago major*	0.3	atcctgtcttctcaaaataaagttcagaaagcgaaaaggg	184
		Solanales	Convolvulacea	Ipomoea_1	0.3	atcctgttttccgaaaacaaacaaaagttcagaaaaaaag	545
Arthropoda	Arachnida	Araneae	Salticidae	*Stenaelurillus nigricaudus*	1.0	ttttttacaagtcgaacagacttcctttattaaatcttgctttaattaggttattaattcaacatcgaggtcacaatctatttttaatataaggactttga	24045
	Insecta	Coleoptera	Tenebrionidae	Tenebrionidae_1	0.3	ggtttcaaaggtcgaacagactcaataataaagctactacacctaaaattaaccttaatccaacatcgaggtcgcaaacttcactttcgattt	140
			Elateridae	Elateridae_1	0.3	ggtttcaaaagtcgaacagactcaataataaagctaccatacctaaaattaaccttaatccaacatcgaggtcgcaaacttcactttcgattt	132
		Diptera	NI	Diptera_3	1.0	gaaataaaagtcgaacagacttaaagcttaaacgactacacctaagctttatcttaatccaacatcgaggtcgcaatccttttatcgatatg	54060
				Diptera_9	1.0	gaatttaaaagtcgaacagactttaaatttaagcttctgcacctaaaattatatcttaatccaacatcgaggtcgcaatcatttttatcaataaga	4248
			Phoridae	*Megaselia*_1	0.6	gaatttaaaagtcgaacagacttaacctttaagcggctacacctaaaaatatctcttaatccaacatcgaggtcgcaatctttttatcgatatg	1182
		Hemiptera	Aphididae	*Aphis*_1	0.6	gaatttaaaggtcgaacagacctaatatttaaaattttgcacctaaaattaatcttaattcaacatcgaggtcgcaaactaatttttaaatttgaa	19654
			Cicadellidae	*Orosius*_1	1.0	gaattcaagagtcgaacagactcaccttataaggggctgccccttaaagttttcttaattcaacatcgaggtcgcaaaagaaagtatagat	26810
			Miridae	*Campylomma*_1	0.6	aaatttaaaggtcgaacagacctagtattcaatcttatactcctgaaacttattttaatccaacatcgaggtcgcaaactattttatcgataaga	12266
		Hymenoptera	Apidae	*Bombus*_1	1.0	gatttcaaaagtcgaacagacttaatatttaaactgctgcatttaatatttatcttaattcaacatcgaggtcgcaatcttctttgttaatatggtcttt	21960
			Bethylidae	Bethylidae_2	0.6	gattttaaaagtcgaacagacttaaattttaatagtaattaaattttatcttaattcaacatcgaggtcgcaaacattttattaaataaatctttgata	38061
			Formicidae	*Camponotus*_2	1.0	aattttaatagtcgaacagactaaataattaaacttctccatttaatcttaattttaattcaacatcgaggtcgcaatcatctttataaataagatct	37380
			Thynnidae	Thynnidae_1	0.3	gaatttaatagtcgaacagactaaatctattagactcctgcatctaatttttatcttaattcaacatcgaggtcacaatcatttttatagatttggtct	8116
		Lepidoptera	NI	Lepidoptera_29	0.6	gattttaatgatcgaacagatcaaaattttaaactttgcatttaaattttatcttaatccaacatcgaggtcgcaaacttttttttttatttgaactaaa	18286
		Orthoptera	Gryllidae	Gryllidae_1	1.0	gaatttaatggtcgaacagaccaattaattaaacttctgcacctaatcatattcttaatccaacatcgaggtcgcaatcttcattatcaataaga	15492
		Zygentoma	Lepismatidae	*Thermobia domestica*	0.6	gaatttaaaggtcgaacagacctttttcctaagcttctgcacctaagaaacatcttaattcaacatcgaggtcgcaatccttcttattgataagg	12968
	Malacostraca	Decapoda	NI	Decapoda_1	1.0	gattttaatagtcgaacagactatccccttaaaaaactgcccctaaaagaaatcttaattcaacatcgaggtcgcaaccattaatgtagatat	23100

Chapter 4

Metabarcoding diet analysis of terrestrial vertebrates for conservation

Article V

Trust your guts? The effect of gut section on diet composition and impact of *Mus musculus* on islands using metabarcoding

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RESEARCH ARTICLE

Trust your guts? The effect of gut section on diet composition and impact of *Mus musculus* on islands using metabarcoding

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Abstract

1. DNA metabarcoding is widely used to characterize the diet of species, and it becomes very relevant for biodiversity conservation, allowing the understanding of trophic chains and the impact of invasive species. The need for cost-effective biodiversity monitoring methods fostered advances in this technique. One question that arises is which sample type provides a better diet representation.
2. Therefore, with this study, we intended to evaluate if there were differences in diet estimates according to the section of the gastrointestinal tract analysed and which section(s) provided the best diet representation. Additionally, we intended to infer the ecological/economic impacts of an invader as a model of the potential effects in an originally mammal-free ecosystem.
3. We examined the gut contents of the house mouse *Mus musculus* introduced to Cabo Verde, considering three sections: stomach, small intestine, and large intestine. We applied a DNA-metabarcoding approach using two genetic markers, one specific for plants and another for invertebrates.
4. We showed that this invader consumed 131 taxa (73 plants and 58 invertebrates). We obtained significant differences in the composition of two of the three sections, with a higher incidence of invertebrates in the stomach and plants in the intestines. This may be due to stomach inhibitors acting on plants and/or to faster absorption of soft-body invertebrates compared to the plant fibers in the intestines. We verified that the impact of this invader in the ecosystem is predominantly negative, as at least 50% of the ingested items were native, endemic, or economically important taxa, and only 19% of the diet items were exotics.

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5. Overall, results showed the need to analyse only two gastrointestinal tract sections to obtain robust diet data, increasing the cost-effectiveness of the method. Furthermore, by uncovering the native taxa most frequently preyed on by mice, this DNA-metabarcoding approach allowed us to evaluate efficiently which are at the highest risk.

KEYWORDS
Cabo Verde Islands, diet, gastrointestinal tract, house mouse, invasive species, invertebrates, next-generation sequencing, plants

TAXONOMY CLASSIFICATION
Agroecology; Applied ecology; Biodiversity ecology; Botany; Conservation ecology; Entomology; Genetics; Invasion ecology; Zoology

1 | INTRODUCTION

Dietary studies can reveal valuable information on how species exploit the available food resources and intervene in ecological processes (Siegenthaler et al., 2019). Several methods can be implemented to characterize diet, such as direct observation, morphological identification, and stable isotopes analysis (Margalida et al., 2005; Martín et al., 2017; Symondson, 2002). However, with the recent generalization of high-throughput sequencing methodologies, the identification of prey items was further improved (Pompanon et al., 2012). DNA-metabarcoding, combined with the power of next-generation sequencing (NGS) technologies (Shendure & Ji, 2008), became widely used to characterize species diets (Taberlet et al., 2012). DNA-based methodologies allow the identification of prey material even when hard parts cannot be recovered after digestion, contrary to other methods. These provide comprehensive taxonomic identification of diet items within highly diverse diets, relying less on taxonomic expertise, and can be applied to noninvasive or degraded samples (Pompanon et al., 2012). The need for cost-effective biodiversity monitoring methods fostered the advances of this technique, becoming highly relevant for biodiversity conservation, by allowing the understanding of trophic chains, and ultimately the ecological impact of invasive species (Westfall et al., 2020). Assessment of predation by invaders using DNA-based approaches is rapidly growing, permitting wildlife managers to identify the most threatened taxa (Harms-Tuohy et al., 2016; Robeson et al., 2018).

Samples used to characterize diets using DNA-metabarcoding are generally fecal, or gut contents (Jakubavičiūtė et al., 2017; Thuo et al., 2019). Fecal samples are advantageous if applied to threatened species since they can be obtained with minimum or no impact on individual fitness (Ferreira et al., 2018). However, in comparison with faeces, DNA from gut contents is usually of superior quality and thus commonly used in diet assessments of invaders (Robeson et al., 2018; Siegenthaler et al., 2019). Yet, most studies disregard the contents of the intestines. The detectability of prey DNA in mammalian stomachs versus faeces was already explored, indicating that sample type influenced its duration but not its quality (Egeter et al., 2015). However, to our knowledge, no study explored how analyzing different gastrointestinal tract sections (stomach, large and small intestines), through DNA-metabarcoding, influences the detectability of diet items. This is particularly important to omnivorous species that consume items with different tissue cell density, digestion rate, and DNA quality decay after digestion (Pompanon et al., 2012).

Invasive species are one of the main causes of biodiversity loss (Butchart et al., 2010), particularly on islands. On geologically young oceanic islands, typically no native terrestrial mammals occur and, in most cases, non-volant land mammals have been anthropogenically introduced (Whittaker et al., 2017). These introductions already led to the loss of several island endemics worldwide (Doherty et al., 2016). The most widely introduced mammal species to islands are cats *Felis catus* L. and commensal rodents (Doherty et al., 2016). Norway rats *Rattus norvegicus* (Berkenhout 1769), black rats *Rattus rattus* L., Pacific rats *Rattus exulans* (Peale 1848), and house mouse *Mus musculus* L. were unintentional transported on boats to most islands (Jones et al., 2013), causing devastating impacts on fauna and flora. These invaders compromise the stability of native populations by competition for resources, predation, and transmission of diseases (Courchamp et al., 2003; Gaiotto et al., 2020). Furthermore, rodents are the ones causing more damage to the natural patrimony of islands (Russell et al., 2018), leading to great economic losses (Doherty et al., 2017). These omnivorous invaders can have broad impacts on health, culture, and agriculture (Russell et al., 2017). For instance, in Asia, the damage rodents cause to rice crops prevented the feeding of 200 million people (Stenseth et al., 2003). In particular, the house mouse is among the 15 species most prevalent on islands worldwide (Russell et al., 2017). In Australia, mice cause losses of up to 4% of the national agricultural production, equivalent to 40 million dollars in the worst seasons (Singleton, 1997). However, the impacts of these rodents on native and domestic species are rarely quantified, due to the lack of taxonomic knowledge of predated items and slowness in obtaining data using traditional methods of morphological identification.

In Cabo Verde, as in several other geologically young oceanic islands, no indigenous terrestrial mammals occur except for bats (Borloti et al., 2020; Hazevoet & Masseti, 2011). Therefore, all non-volant terrestrial mammals were introduced during the past 550 years, when

the first Europeans arrived in the archipelago. These invaders already caused a great impact contributing to the extinction of endemics, such as the Cabo Verde giant skink *Chioninia coctei* (Duméril & Bibron, 1839), and the extirpation of the giant wall gecko *Tarentola gigas* (Bocage, 1875) on Santa Luzia Island (Mateo et al., 2005; Medina et al., 2021). From the rodent invaders, mice are the most widely distributed occurring across all inhabited and even some uninhabited islands of the archipelago (Hazevoet & Masseti, 2011). Similar to other island ecosystems (Bunce et al., 2009), it is likely that mice have highly negative impacts on Cabo Verdean native flora and agricultural crops. This impact is expected to be higher on more vegetated islands, with higher coverage of both endemic plants and agricultural production, which is the case of Santiago and Santo Antão islands (Brilhante et al., 2021; MAA, 2021). Nevertheless, the damage caused by these invaders in Cabo Verde has never been quantified. With this study, we intend to evaluate if there are differences in diet estimates according to the section of the gastrointestinal tract analysed and which section(s) provide a better representation of the diet and the impacts of an invader using a DNA-metabarcoding approach. For that, we examined the gut contents of the house mouse introduced to Cabo Verde, considering three sections: stomach, small intestine, and large intestine. Additionally, we aimed to provide a framework to infer the ecological/economic impact of invaders on an island ecosystem, especially in agricultural areas.

2 | MATERIALS AND METHODS

2.1 | Study area

The Cabo Verde Islands are located in the Atlantic Ocean, approximately 500 km off the African coast. This volcanic archipelago belongs to the biogeographical region of Macaronesia and is composed of ten main islands and several islets (Figure 1). It is politically divided into Windward and Leeward Islands. We sampled two islands of the Windward group (Santo Antão and São Vicente) and one of the Leeward group (Santiago; Figure 1) to have a wider representation of the available diversity of diet items (Arechavaleta et al., 2005).

Santo Antão is the northernmost and second-largest island of Cabo Verde (Figure 1). With a total area of 779 km², and the second-largest agricultural area of the archipelago, corresponding to 16% of the national total (Monteiro et al., 2020), mice are expected to be abundant (Hazevoet & Masseti, 2011). The southwestern region of this mountainous island is almost completely arid, while the northeast, where we sampled, receives relatively regular rainfall and thus has more vascular plants and invertebrate endemic richness (Romeiras et al., 2015). São Vicente's total area is 227 km^2 (Figure 1), and its agricultural areas correspond to only 0.3% of the whole archipelago (Monteiro et al., 2020). Its landscape is composed of stony plains, sandy dunes, and barren hills, as it is very dry with scarce vegetation (Duarte et al., 2008). However, as it harbours one of the largest human settlements of the country (Romeiras et al., 2020), mice are expected to be abundant (Groh, 1982). It is also one of the most important islands considering terrestrial endemic arthropod richness (Lobo & Borges, 2010). Santiago, with 991 km^2 of total area, is the largest island (Figure 1) and the most important agricultural center of the archipelago (Monteiro et al., 2020). Mice were previously reported on this island in the mountains around São Jorge dos Órgãos and São Domingos (Rabaça & Mendes, 1997). The landscape of Santiago is mostly characterized by steep, tall mountains, although the southeast area is flatter (Neto et al., 2020). Encompassing a wide range of habitats, this island presents the highest number of species and endemism of the archipelago (Duarte et al., 2008; Lobo & Borges, 2010).

2.2 | Sampling

Sampling took place between the 11th and 27th of November 2019 in the municipalities of Paúl, on Santo Antão, Mindelo, on São Vicente, and São Lourenço dos Órgãos, on Santiago (Figure 1). We placed 40–70 Sherman traps per site in cultivation fields, depending on its size and crop diversity, in groups of 10, distanced by approximately 5 m. We baited each trap with oats, tuna and peanut butter to attract *Mus musculus* individuals. We euthanized the captured mice by cervical dislocation and dissected them for the collection of the gastrointestinal tracts with digestive contents for further genetic analysis. We deposited all animals in the collections of the Technical University of the Atlantic, São Vicente, the future Natural History Museum of Cabo Verde.

2.3 | DNA extraction and sequencing

We divided the digestive tracts into three sections (stomach—S, small intestine—SI, and large intestine—LI). We performed the DNA extraction of the digestive contents of 30 individuals per island, in a total of 90 specimens, for each section separately, including blanks, using the Stool DNA Isolation Kit (Norgen Biotek Corp., Canada), following the manufacturer's instructions.

We amplified the DNA of the three sections with two genetic markers already validated in previous studies (Pinho et al., 2018). For invertebrates we used a modified version of the IN16STK-1F/IN16STK-1R primers, targeting the mitochondrial 16S rRNA (Kartzinel & Pringle, 2015; Pinho et al., 2018), and for plants, we used the g/h primers targeting the short P6-loop of chloroplast trnL (UAA) (Taberlet et al., 2007). We performed all PCRs, including blanks, as described in Pinho et al. (2018). Then, we prepared libraries following the Illumina MiSeq protocol "16S Metagenomic Sequencing Library Preparation" (https://support.illumina.com). Finally, we sequenced the samples in the MiSeq sequencer (Illumina) using the MiSeq Reagent Kit V2 (Illumina, San Diego, CA, USA) for an expected average of 17,000 paired-end reads per sample.

We processed the obtained DNA sequences at the bioinformatics level using tools incorporated in the software package OBITtools (http://metabarcoding.org/obitools), which include the alignment of the sequences obtained and the filtering of sequencing errors, to obtain the

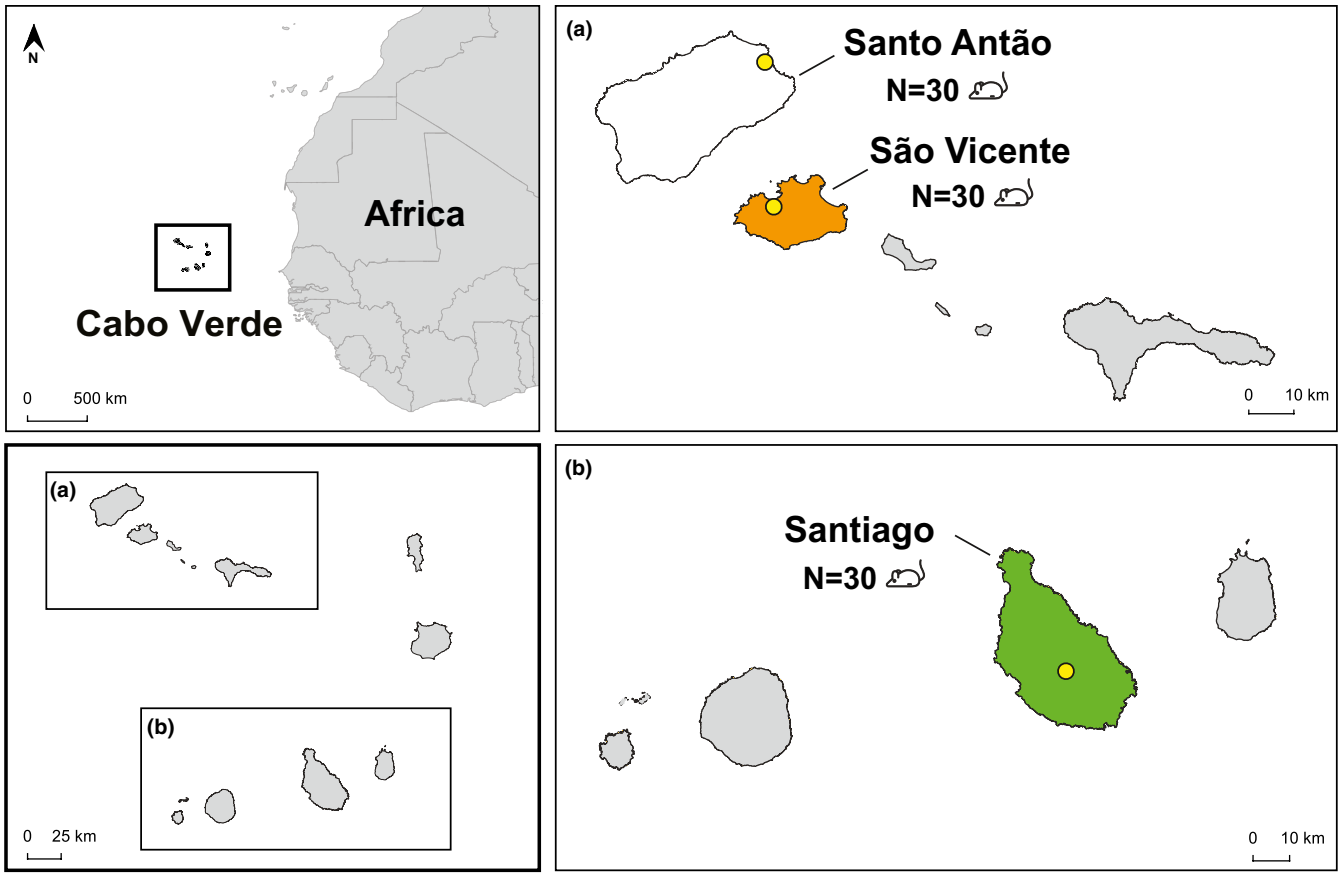

FIGURE 1 Map of Cabo Verde showing the geographic location of the islands and the sampled sites (yellow circles). (a) Two islands were sampled in the Windward group, Santo Antão (Paúl) and São Vicente (Mindelo). (b) One island was sampled in the Leeward group, Santiago (São Lourenço dos Órgãos). N stands for the number of sequenced samples per island

molecular operational taxonomic units, MOTUs (Pinho et al., 2018). In the final dataset, we removed all the samples with less than 500 reads and within kept samples, we also excluded haplotypes representing less than 1% of the total number of reads of that sample (Mata et al., 2016). Finally, to taxonomically identify the MOTUs present in the mice diet, we compared the obtained haplotypes with our reference database and with those available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). We classified sequences that had less than 90% of similarity with known species only to the class level, the ones with values between 90% to 95% to the family level, and the remaining sequences with similarity values above 95% to the genus or species level. We considered only species or genera known to occur on our sampled sites, or the surrounding islands of the archipelago. When a haplotype matched more than one species or genus, then a higher ranking would be attributed (e.g., family). If more than one haplotype corresponds to the same taxon, we attributed a number to each. We removed the haplotypes identified as contaminations (e.g., mice, bait, or human DNA).

2.4 | Data analysis

We estimated the frequencies of occurrence (FO) of plants and invertebrates in samples for the three sections. Using R software version 4.0.2, we performed a permutational multivariate analysis of variance (PERMANOVA) using the vegan package (ADONIS function) to compare the composition of the contents of all sections (https://CRAN.R-project.org/package=vegan). For the same purpose, we performed a pairwise multilevel comparison (parwise.adonis function). We also carried out a homogeneity of dispersion test (PERMDISP) to ensure the importance of the PERMANOVA test, as it presumes an equal dispersion of values among groups. Additionally, we performed a similarity percentage analysis, using the vegan package (simper function), to infer the contribution of each prey to the differentiation among sections.

Lastly, we classified the impact of mice on the taxonomically identified diet items into three categories: negative, positive, or non-identified. We consider it as negative if the items were native or endemic plants/invertebrates, plants of economic importance (e.g., for human or domestic animals' consumption), or invertebrates with essential ecological functions, such as pollinators. We considered it as a putative positive if the items were exotic plants/invertebrates and as non-identified if the items were identified with a low taxonomic resolution or if the impact could not be classified. We compared the FO differences of each category between prey groups (plants vs. invertebrates), gastrointestinal sections (stomach vs. intestines) and islands using chi-square tests.

3 | RESULTS

After data filtering, we obtained 20,000 reads per sample on average. We were able to identify 131 diet items of eight taxonomic classes in the 90 mice from the three islands, 73 of which corresponded to plants and 58 to invertebrates (Table A1). Plants were distributed among three classes, 20 orders, and 30 families, with Poaceae and Fabaceae as most frequent overall. We identified invertebrates from five classes, 15 orders, and 31 families, with Apidae and Aphididae as the most frequent families. Both plant and invertebrate items were present in similar frequencies in the overall diet (63% and 72%, respectively). Several taxa were present exclusively in one section, in total 54% of plants items were only in the intestines (e.g., Arecales and Apiales orders), and 65% of invertebrate items were only in the stomach (e.g., Araneae and Mesostigmata orders). Fifty MOTUs had FOs below 1% and were only present in one sample.

Regarding the gastrointestinal track sections analysed, we obtained significant differences in the diet composition of the stomach in comparison to the two intestinal sections (S vs. SI: $F = 3.46$, $p = .003$; S vs. LI: $F = 7.68$, $p = .003$; SI vs. LI: $F = 1.65$, $p = .120$) and no effect of data dispersion on the results. Frequencies of plants and invertebrates were similar in the small and large intestines (Figure 2), yet both sections' estimates were significantly different from the stomach ones (plants – S vs. SI: $p = .001$; S vs. LI: $p = .001$; SI vs. LI: $p = 1.000$; invertebrates – S vs. SI: $p = .008$; S vs. LI: $p = .001$; SI vs. LI: $p = .062$). These differences were observed at the MOTU, family, and order levels. Additionally, we observed a higher frequency and diversity of invertebrates in the stomach and of plants in the intestines (Figure 2).

The similarity percentage analysis revealed that six MOTUs, five families, and seven orders contributed significantly to differences between sections (Figure 3; Table A1). The MOTUs that contributed significantly to the differences between sections were all invertebrates. In particular, *Apis mellifera* Linnaeus, 1758 and *Aphis gossypii* Glover, 1877 had a high contribution and were also the most frequent preys overall. The bee and aphid species were present in 66% and 26% of the stomachs, respectively, whereas in the intestines the FOs were only 31% and 8%, respectively. Other three introduced plant species, *Mentzelia aspera* L., *Desmanthus virgatus* (L.) Wild., and *Carica papaya* L. had differences in the FO between sections greater than 10%, all presenting higher frequency in the intestines (Figure 3).

Regarding the impact of this rodent, we identified with a high taxonomic resolution 79 items (27 invertebrates and 52 plants), although the reference collection of DNA sequences for Cabo Verde, mostly for invertebrates, is still incomplete. Therefore, we verified that the impact of this invader in the ecosystem is predominantly negative, as at least 49/50% (unbalanced/balanced per FO, respectively) of the ingested items were native, endemic, or economically important taxa, and only 12/19% (unbalanced/balanced per FO, respectively) of the items were exotics (Table A1). The impact pattern was similar across the three sampled islands ($\chi^2 = 1.87$, $p = .761$; Figure A1) and gut sections ($\chi^2 = 0.66$, $p = .719$). Impact patterns were significantly different between invertebrates and plants ($\chi^2 = 8.95$, $p = .011$), with a higher negative impact on plants. The economically important plants included corn *Zea mays* L., banana *Musa* sp., cabbage *Brassica oleracea* L., radish *Raphanus raphanistrum* subsp. *sativus* (L.) Domin., breadfruit *Artocarpus altilis* (Parkinson ex F.A. Zorn) Fosberg, mango *Mangifera indica* L., papaya *Carica papaya* L., and plants of the genus *Solanum* that includes potatoes, tomatoes, and eggplant, among other vegetables (Table A1). Mice also consumed native and endemic invertebrate species including spiders (e.g., *Pardosa aquatilis* Schmidt & Krause 1995), ants (e.g., *Monomorium subopacum* (Smith 1858)) and European honeybees *Apis mellifera*. The data show that they also consumed invasive plants, such as *Lantana camara* L. and Mexican fireplant *Euphorbia heterophylla* L., along with invertebrate pests, such as cockroaches (e.g., *Blattella germanica* (Linnaeus 1767) and *Periplaneta americana* (Linnaeus 1758)) and the cotton aphid *Aphis gossypii* Glover 1877.

4 | DISCUSSION

To our knowledge, this is the first study to explore diet estimate differences along the gastrointestinal tract using DNA-metabarcoding techniques. In this work, we present the first DNA-based data on the diet and impact of *Mus musculus*, using Cabo Verde as a model. Our results show that the house mouse consumed a wide range of prey items in the sampled islands, as the FOs of most items were low. This confirms the generalist diet and opportunistic feeding behavior of this species as observed in other islands (Angel et al., 2009; Le Roux et al., 2002). We were able to observe that the frequency of plants and invertebrates were similar overall; however, plants had a slightly higher incidence. This was expected since we actively sampled individuals in crop areas. Nevertheless, studies that investigated the seasonal variation of the diet of *M. musculus* in other islands showed a similar pattern (Copson, 1986; Le Roux et al., 2002).

The analyses of different sections of the gastrointestinal tract provided different views of the house mouse diet. Although no significant differences were detected between the two sections of the intestines, disparities between the stomach and the intestines were evident. We observed a higher frequency and diversity of invertebrates in the stomach and plants in the intestines. This may be primarily due to differences in the cellular structures between plants and animals, particularly in the cell walls and fibrous tissues. Plant items have very thick cell walls arranged in complex networks predominantly composed of cellulose, hemicellulose, and pectic polysaccharides (Jarvis, 2011; McDougall et al., 1996). This composition makes plant items harder to break down and consequently more resistant to enzymes of the gastrointestinal tract (Holland et al., 2020). This leads to harder digestion of plants, in comparison with animal items, and subsequently slower absorption along the gastrointestinal tract (Tomé, 2013). Since mice consume raw plants, the DNA is protected by the cell wall and remains poorly detected in the stomach. The subsequent gastrointestinal compartments take a much important part

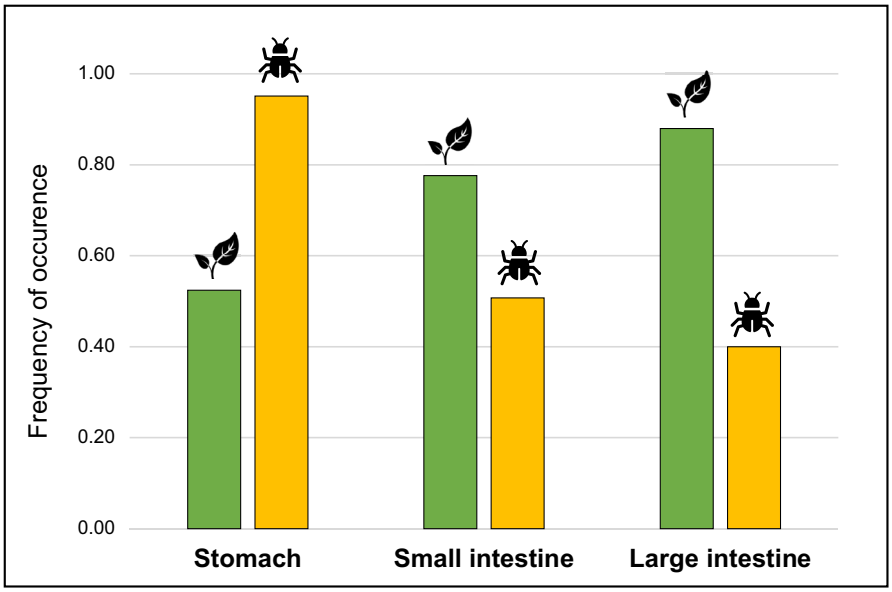

FIGURE 2 Metabarcoding results per gastrointestinal section. Frequencies of occurrence of plants (in green) and invertebrates (in yellow) in the *Mus musculus* stomach and intestines (small and large) samples

in their digestion (Rizzi et al., 2012; Tomé, 2013; Wilcks et al., 2004). Previous studies showed that plant tissues were most effectively digested in the small intestine of rodents, due to the action of pancreatic enzymes (Wilcks et al., 2004). As shown in our results, one is more likely to detect DNA from plants in the intestines rather than the stomach. Moreover, depending on the type, section, and maturation of the plant, the fiber concentration will vary, leading to distinct digestibility rates (Albrecht et al., 1987; Buxton et al., 1995). For instance, grasses usually present more fiber than legumes, especially in the leaves, making them harder to digest and to be absorbed in the gut (Buxton & Redfearn, 1997). In our study, Poaceae was the most frequent plant family and one of the families that contributed to the differences between stomach and intestines. This is probably due to its higher fiber concentration which presumably allowed Poaceae plants to persist longer time in the gastrointestinal tract, therefore having a higher incidence in the intestines. Moreover, when we extracted plant DNA of the intestines, we were also extracting DNA of the faeces within it. Since the DNA detectability half-life is twice as high in faeces compared to stomachs (Egeter et al., 2015), the faeces content in the intestines will also contribute to a higher DNA detectability of plants in that section.

Comparatively, animal DNA will be already further degraded when it reaches the lower sections of the gastrointestinal tract (Tomé, 2013), compromising its detectability. This was probably the reason why we were able to detect higher frequencies of invertebrates in the stomach than in the intestines. Additionally, the most frequent invertebrates consumed by mice, and the ones that contributed the most to differences between sections, seem to be soft-bodied (e.g., *Apis mellifera* and *Aphis gossypii*). These items are probably more easily digested and are already mostly absorbed when they reach the intestines, leading to a lower detection of its DNA in the lower parts of the gastrointestinal tract. Even though metabarcoding has the extraordinary capacity to detect small, soft, and invisible items (Pompanon et al., 2012), based on our results, the detection of these preys will probably be more efficient in stomach contents rather than in the intestines. Therefore, we advise studies targeting the detection of soft-bodied invertebrate preys to use preferentially stomach samples and those targeting plant or harder invertebrate items to use preferentially intestine samples. On the other hand, extraction methods can be adjusted to account for lower digestion of prey. For instance, if only the stomach is available, we advise using specific extraction and amplification methods targeting plants.

In this work, we verified that the general environmental impact of mice in Cabo Verde is predominantly negative similarly to other island ecosystems (Angel et al., 2009). Studies using traditional methods of stomach content analysis on Southern Ocean islands, as the Guillou and Macquarie Islands, similarly showed that mice consume a wide range of native taxa (Copson, 1986; Le Roux et al., 2002). We showed that this invader consumes several plants with human and economic importance. Corn production is one of the most important crops in Cabo Verde, with around 6,000 tons per year (MAA, 2021). We observed that 10% of the individuals ingested corn, which coupled with high population densities could imply significant losses in production, considering that 1 ton of corn is equivalent to 108.100 Cabo Verdean escudos, or 1.190 dollars (I. Gomes pers. comm.). Also summing the losses in other crops, this invader poses a major threat to the national economy of this archipelago. Similarly, in Tanzania and Australia, mice can cause losses of up to 15% of the national agricultural production, equivalent to 40–45 million dollars of losses in the worst seasons (Singleton, 1997). In our results, we were also able to detect that this invader feeds on at least nine native plant species, as *Eleusine indica* (L.) Gaertn and *Sida acuta* Burm. fil. (Table A1). Metabarcoding does not allow us to identify the section of the plant that is consumed; however, this invader can be a great threat for the native flora if it is consuming seeds. In Marion Island, in the sub-Antarctic Indian Ocean, mice almost extirpated the native sedge *Uncinia compacta* R. Br. due to seed predation (Smith & Steenkamp, 1990). The impact on invertebrates is also

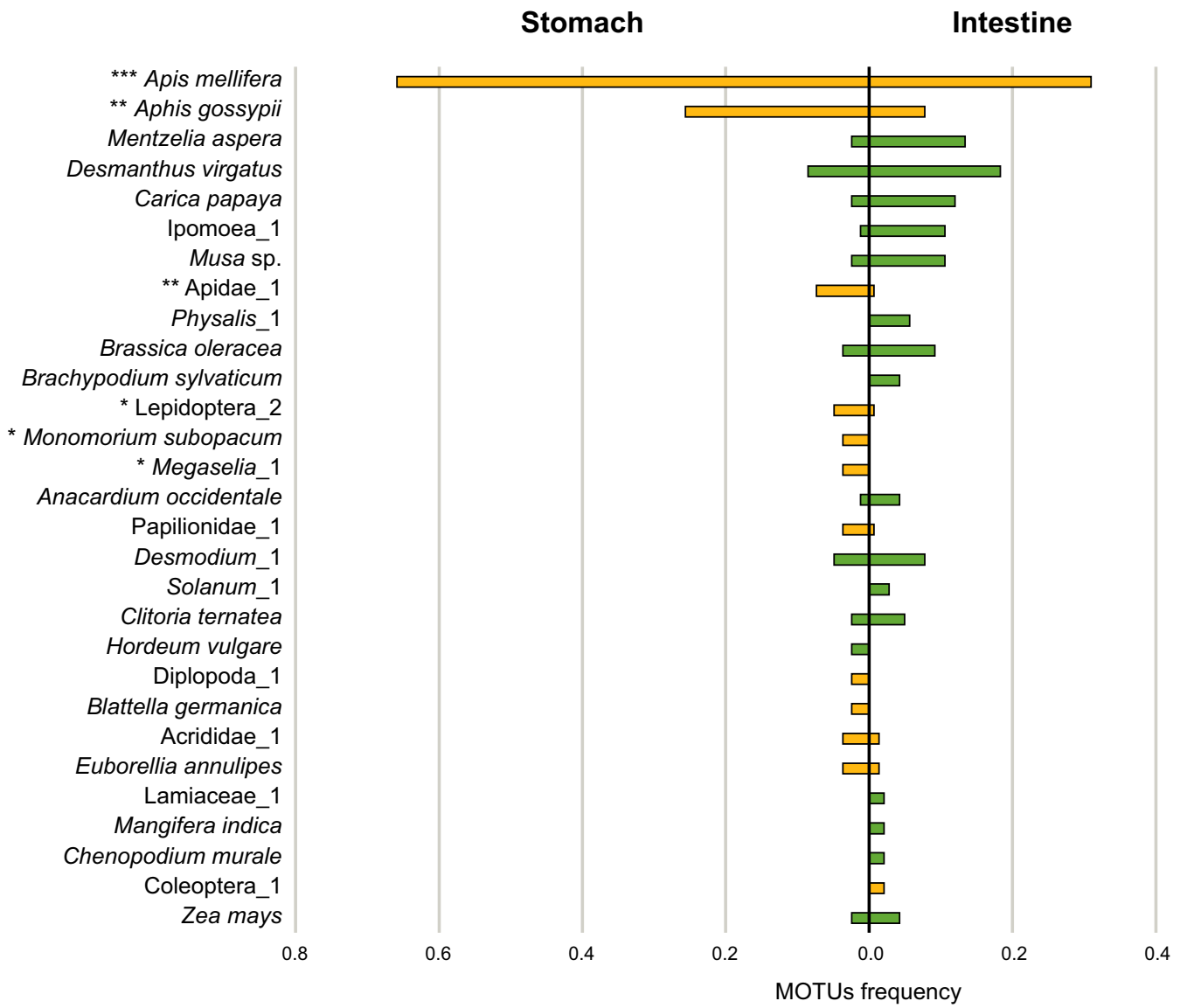

FIGURE 3 Results of the similarity percentage analysis. Frequency of occurrence of 30 Molecular Operational Taxonomic Units (MOTUs) with the highest contribution to differences between the stomach and intestine of *Mus musculus*. Plant MOTUs are represented in green and invertebrates in yellow. Magnitude of significance levels shown with asterisks: ***$p < .001$; **$p < .01$; *$p < .05$

considerable since these rodents consume several native and endemic species, most of them with vital ecological functions. This is the case of the European bee, a species in decline worldwide, that plays an active role in the pollination of several plants, including crops such as cabbage and beans (Themudo et al., 2020). In Antipodes Island, located south of New Zealand, mice were considered accountable for the local extirpation of several invertebrate species (Marris, 2000). Likewise, on Marion Island, they are responsible for the absence of the flightless moth *Pringleophaga kerguelensis* Enderlein 1905 (Vári, 1971).

Mice can also present a putative positive impact by consuming some introduced and invasive herbaceous, as is the case in Cabo Verde, for example, *Lantana camara* and *Euphorbia heterophylla*, two of the main invasive plants in several natural and agricultural tropical regions (Romeiras et al., 2016). The latter is resistant to some herbicides, thus it needs to be manually removed before farming (Wilson, 1981). Mice can also play a role in domestic pest control, such as cockroaches (e.g., *Blattella germanica* and *Periplaneta americana*) and the cotton aphid *Aphis gossypii*, even though some may be resultant of secondary consumption of host plants (Mata et al., 2016). The latter is an important agricultural pest since it can have several hosts and transmit several viruses important to crops (Dedryver et al., 2010). Several crops grown in Cabo Verde can be hosts for this aphid, such as papaya, cashew, breadfruit, and banana, among others (Dedryver et al., 2010).

Optimized measures are needed to control, monitor and eradicate invasive rodents (Stenseth et al., 2003). These measures imply great expenses with equipment and human resources; therefore they must be carried out accurately to avoid resource waste (Mwebaze et al., 2010). The results of Stenseth et al. (2003) suggest that government-level funding for rodent pest control should be devoted to research instead, especially in developing countries. These

authors go even further, stating that not doing so can cost dearly in terms of lost income and food supplies for people. Consequently, additional research is crucial to infer the relative importance of the impacts of this rodent in the Cabo Verdean agriculture and economy, and if eradication is the best solution. If so, there is a need to deliberate on potential surrogates for the positive role of mice. For instance, in this study, we did not consider the impact that this rodent can have on vertebrates. However, we know that in Cabo Verde, mammal invaders already contributed to the extinction of endemic species (Mateo et al., 2005; Medina et al., 2021), therefore, it is important in the future to access the impact that *M. musculus* has on seabirds and terrestrial and marine reptiles. Further studies on other islands, particularly in Seychelles, show that the prevention, eradication, and control of rodents would very positively affect government revenues even after discounting the costs of such actions (Mwebaze et al., 2010). An eradication plan of invasive mammals is already taking place in Cabo Verde on Santa Luzia Island (Alho et al., 2022). This is a particularly promising plan due to the reduced area of the island since in another small island of the Macaronesian region mice were already successfully eradicated (Olivera et al., 2010). On populated islands with a strong agriculture presence, as is the case of this study, the likelihood of eradication failure can be high (Holmes et al., 2015); however, these islands could highly benefit from a rodent control program to reduce the impact of mice on agriculture.

In conclusion, our results showed the need to analyse only two gastrointestinal tract sections (stomach and large intestine) to obtain robust diet representations, increasing the cost-effectiveness of DNA-metabarcoding approaches. In general, the use of this DNA-based approach enabled the identification of a wide variety of mice diet items in a much faster and simplified way compared to other traditional approaches, as shown in previous studies (Gil et al., 2020). Additionally, by uncovering which native prey species are most frequently predated by this invader, this approach allowed to evaluate efficiently which ones are at the highest risk. Thus, our framework can be an asset for studies on the impact of invasive species on other islands or threatened areas.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Catarina J. Pinho: Formal analysis (lead); Investigation (equal); Visualization (lead); Writing – original draft (lead); Writing – review & editing (equal). **Evandro P. Lopes:** Funding acquisition (supporting); Investigation (supporting); Resources (supporting); Writing – review & editing (equal). **Joana Paupério:** Conceptualization (supporting); Formal analysis (supporting); Funding acquisition (supporting); Methodology (equal); Resources (supporting); Validation (equal); Writing – review & editing (equal). **Isildo Gomes:** Funding acquisition (supporting); Investigation (supporting); Resources (supporting); Writing – review & editing (equal). **Maria M. Romeiras:** Data curation (supporting); Funding acquisition (supporting); Methodology (supporting); Resources (supporting); Writing – review & editing (equal). **Raquel Vasconcelos:** Conceptualization (lead); Data curation (lead); Formal analysis (supporting); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Supervision (lead); Writing – original draft (equal); Writing – review & editing (equal).

DATA AVAILABILITY STATEMENT

The datasets supporting this article have been uploaded as part of Appendix A and in the GenBank database (PRJNA801221 accession code; https://www.ncbi.nlm.nih.gov/sra/PRJNA801221).

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APPENDIX A

TABLE A1 Taxonomic identifications, haplotype sequences, and frequency of occurrence (FO) of the MOTUs present in the diet of *Mus musculus*

Phyllum	Class	Order	Family	Final_ID
Tracheophyta	Liliopsida	Arecales	Arecaceae	Arecaceae_1
		Commelinales	Commelinaceae	*Commelina benghalensis*
		Poales	Poaceae	*Aristida adscensionis*
				Brachypodium sylvaticum
				Cenchrus ciliaris
				Chloris_1
				Cynodon dactylon
				Dactylis smithii
				Eleusine indica
				Hordeum vulgare
				Oryza sativa
				Poaceae_3
				Poaceae_6
				Poaceae_7
				Saccharum_1
				Setaria_1
				Urochloa_1
				Zea mays
		Zingiberales	Musaceae	*Musa* sp.
	Magnoliopsida	Apiales	Apiaceae	Apiaceae_1
			Araliaceae	Araliaceae_1
		Asterales	Asteraceae	Asteraceae_1
				Asteraceae_2
				Asteraceae_3
		Boraginales	Heliotropiaceae	*Heliotropium_1*
		Brassicales	Brassicaceae	*Brassica oleracea*
				Raphanus raphanistrum subsp. *sativus*
			Caricaceae	*Carica papaya*
			Cleomaceae	Cleomaceae_1
				Cleome_1
		Caryophyllales	NI	Caryophyllales_1
			Aizoaceae	*Trianthema portulacastrum*
				Zaleya pentandra
			Amaranthaceae	*Amaranthus_1*
				Chenopodium murale
		Cornales	Loasaceae	*Mentzelia aspera*
		Fabales	Fabaceae	*Clitoria ternatea*
				Desmanthus virgatus
				Desmodium_1
				Fabaceae_1
				Lens culinaris
				Leucaena leucocephala
				Lotus_1

DNA sequences	ST	SI	LI	Total	Impact
atctttattttgagaaaacaagggtttataaaactagaataaaaaaag	0.000	0.004	0.004	0.009	–
atccttaagatttttaaaactagaaaaaagg	0.000	0.000	0.004	0.004	+
atctctttttttgaaaaacatgtggttctaaaactagaacccaaaggaaaag	0.000	0.000	0.004	0.004	–
atccgtgttttgagaaaacaaggaggttctcgaactaaaatacaaaggaaaag	0.000	0.000	0.004	0.004	+
atccctttttttgaaaaacaagtggttctaaaactagaacccaaaggaaaag	0.000	0.004	0.000	0.004	–
atccctgttttgaaaaacaagtggttctcaaactagaacccaaaggaaaag	0.000	0.000	0.009	0.009	–
atccctttttttgaaaaacaagtggttctcaaaccagaacccaaaggaaaag	0.004	0.000	0.004	0.009	+
atccgtgttttgagaaaacaaagaaaggggttctcgaactagaatacaaaggaaaag	0.000	0.004	0.004	0.009	–
atccctttttttcattttaaaaacaagtggttctcaaactagaacccaaaggaaaag	0.004	0.000	0.009	0.013	–
atccgtgttttgagagggggattctcgaactagaatacaaaggaaaag	0.009	0.000	0.000	0.009	–
atccatgttttgagaaaacaagcggttctcgaactagaacccaaaggaaaag	0.004	0.000	0.000	0.004	–
atccctttttttaaaaacaagtggttctcaaactggaacccaaaggaaaag	0.000	0.004	0.000	0.004	?
atccgtgttttgagagggggttctcaaactagaatacaaaggaaaag	0.000	0.000	0.004	0.004	?
atccgtgttttgagaaaacaaggggttctcgaactagaatacaaaggaaaag	0.031	0.022	0.000	0.054	?
atccccttttttgaaaaaacaagtggttctcaaactagaacccaaaggaaaag	0.000	0.000	0.009	0.009	–
atccctttttttgaaaaaacaagtggttctcaaactagaacccaaaggaaaag	0.054	0.045	0.045	0.143	?
atccctctttttgaaaaaacaagtggttctcaaactagaacccaaaggaaaag	0.036	0.022	0.045	0.103	–
atccctttttttgaaaaacaagtggttctcaaactagaacccaaaggaaaag	0.009	0.022	0.004	0.036	–
atccttattttgagaaaacaaaggtttataaaactagaatttaaaag	0.009	0.018	0.049	0.076	–
atcctattttccaaaaacaaacaaaggcccagaaggtgaaaaaag	0.000	0.009	0.000	0.009	–
atcctgttttccgaaaacaaacaaaggttcagaaggcgaaaaaagg	0.000	0.000	0.004	0.004	?
atcacgttttccgaaaacaaacaaaggttcagaaagcgaaaataaaaaaag	0.004	0.000	0.009	0.013	–
atcacgttttccgaaaacaaataaaggttcagaaagcgaaaataaaaaag	0.000	0.000	0.013	0.013	?
atcacgttttccgaaaacaaacaacggttcagaaagcgaaaatcaaaaag	0.000	0.004	0.004	0.009	?
atcctgttttccgaaaacaaagaaaggttcagaaagcaaaaaaag	0.000	0.009	0.000	0.009	–
atcctgggttacgcgaacaaaacagagtttagaaagcga	0.004	0.000	0.000	0.004	–
atcctgagttacgcgaacaaaccagagtttagaaagcgg	0.000	0.004	0.000	0.004	–
atcctgttttacgagaacaaacaaaacaagagttcagaaagcgaaaaaagggg	0.000	0.004	0.000	0.004	–
atcctggtttccgcgaacaaacaagagtttagaaagcgagaaaaaagg	0.004	0.000	0.000	0.004	?
atcctggtttacgcgaacaaacaagagtttagaaagcgagaaaaaagg	0.031	0.027	0.031	0.089	+
ctccttttttcaaatcaaaagaaaaaaaaaataaagattcataaagcaagaaaaaag	0.009	0.000	0.004	0.013	?
ctccttttttttttcaaaagcaaaaaacaaaaaataaggattcagaaagcaagaaaaag	0.004	0.004	0.004	0.013	+
ctccttttttttcaaaagcaaaaaaataaggattcagaaagcaagaaaaaag	0.004	0.004	0.004	0.013	–
ctccttttttcaaaagtaaaaaaaaaaaaatacggattcagaaagcaagaataaaaaaaag	0.004	0.000	0.000	0.004	–
ctccttttgtcaaaagcaaaaaatactcaaaagaaaaaaataataaaaaagcaagaaaaaaaaag	0.000	0.004	0.009	0.013	+
atcctgttttccgaaaacaaacaaaggttcagaaagcgaaaataaaaaaag	0.009	0.040	0.045	0.094	+
atcctctttttccaaaattttccaaaaacaaaaaacttaagaaagtgaaaaaagag	0.009	0.022	0.009	0.040	–
atcctgttttccgaaaaccaagaagagttcagaaagggagaatcaaaataaaaaaa	0.000	0.000	0.004	0.004	–
atccttttttccgtaaacgaagaaaagttcagaaaagaaaggtataatcaaaaaaaag	0.000	0.000	0.004	0.004	–
atcctgttttccgaaaacaaagaacaaagaaaaagaagagtttagaaagcgagaataaaaaatcaaag	0.000	0.000	0.004	0.004	?
atccttctttccgaaaacaaatcaaagttcagaaagtgaaaatcaaaaaag	0.004	0.000	0.000	0.004	–
atcctgttttccgaaaagcaagaagagttcagaaagggagaatcaaaataaaaaaag	0.004	0.004	0.009	0.018	–
atcctgctttacgaaaacaagggaaagttcagttaagaaagcgacgagaaaaaagg	0.000	0.000	0.004	0.004	–

(Continues)

TABLE A1 (Continued)

Phyllum	Class	Order	Family	Final_ID
				Prosopis juliflora
				*Vigna*_1
		Gentianales	Apocynaceae	*Catharanthus roseus*
		Lamiales	Acanthaceae	Acanthaceae_1
			Lamiaceae	Lamiaceae_1
				Lamiaceae_2
			Verbenaceae	*Lantana camara*
		Malpighiales	Euphorbiaceae	*Acalypha*_1
				Euphorbia heterophylla
				*Euphorbia*_3
			Passifloraceae	*Passiflora*_1
		Malvales	Malvaceae	*Abutilon*_1
				Grewia villosa
				Malvaceae_1
				Sida acuta
		Myrtales	Myrtaceae	*Psidium guajava*
		Rosales	Moraceae	*Artocarpus altilis*
				*Ficus*_1
				*Morus*_1
			Rosaceae	*Eriobotrya japonica*
		Sapindales	Anacardiaceae	*Anacardium occidentale*
				Mangifera indica
			Rutaceae	*Citrus*_1
		Solanales	Convolvulaceae	*Ipomoea*_1
				*Ipomoea*_2
			Solanaceae	*Physalis*_1
				Sclerophylax spinescens
				*Solanum*_1
	Pinopsida	Pinales	Cupressaceae	*Cupressus sempervirens*
Arthropoda	Arachnida	Araneae*	Araneidae	*Neoscona*_1
			Gnaphosidae	Gnaphosidae_1
			Lycosidae	*Pardosa aquatilis*
			Theridiidae	*Theridion*_1
		Mesostigmata*	Macronyssidae	*Ornithonyssus bacoti*
			Phytoseiidae	*Euseius ovalis*
				Phytoseiidae_1
		Opiliones	NI	Opiliones_1
		Sarcoptiformes	Pyroglyphidae	*Dermatophagoides farinae*

DNA sequences	ST	SI	LI	Total	Impact
atcctgttttcggaaaaccaagaagagttcagaaagggagaataaaaaaag	0.000	0.000	0.004	0.004	?
atcctgttttctgaaaacaaagaaaaattcagaaagttataataaaaaagg	0.000	0.000	0.009	0.009	–
atccagttttccacaaacacaaacaaaggttcagaaaacgaaaaagg	0.004	0.000	0.000	0.004	–
atcctcttttcgcaagacaaaggttcagaaaacgaaaaagg	0.000	0.000	0.009	0.009	?
atcctgttttctcaaaacaaaggttcaaaaaacgaaaaaagg	0.000	0.009	0.004	0.013	?
atcctgttttctcaaaacaaaggtttaaaaaacgaaaaaaaaaag	0.000	0.000	0.004	0.004	?
atcccgttttctcaaaacaaaggttcagaaaacgccaaaggcg	0.000	0.009	0.000	0.009	+
atcctgttttccgaaaacaaaaaaaggttcataaagacagaataaaaaagg	0.000	0.004	0.000	0.004	+
atcctgttttccgaaaacaaaaaaagtttcataaagacagaaaaaaaaaaaaaaaaaagaag	0.013	0.000	0.013	0.027	+
atcctgttttccgaaaacagaaaaagaaaagtttcataaaaacagaaaaaaacaaggaag	0.000	0.004	0.000	0.004	?
atcctgtttttcgaaaacaaaaaaacaacaaaggttcataaagacagaatcagaataaataaag	0.000	0.000	0.004	0.004	?
atcctattattttacgaaaataaacataaacaaaggttcagcaagcgaaaataataaaaaaggaaag	0.000	0.000	0.004	0.004	?
atcctattattttacgaaaataaacataaacaaaggttcagcaagcgagaataaaataaaataataaaaaaag	0.004	0.000	0.000	0.004	–
atcctattattttacgaaaataaacataaacaaaggttcagcaagcgagaataataaaaaaggaaag	0.013	0.022	0.063	0.098	?
atcctattattttacgaaaataaacagaaacaaaggtttagcaagcgagaataataataaaaaaggaaag	0.000	0.000	0.004	0.004	–
atcctgttttacgaaaaccaacaaaacaataagggttcataaagcgagaataaaaaaaggatag	0.000	0.000	0.004	0.004	–
atccggttttctgaacccttgtttgttttcagaaagcgataataaaaaag	0.004	0.004	0.009	0.018	–
atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag	0.000	0.000	0.004	0.004	–
atccggttttctgaaaacaaacaagggttcagaaagcgataatacaaaag	0.000	0.000	0.004	0.004	?
atcctgttttatgaaaataaacaagggtttcataaaccgaaaataaaaaag	0.000	0.000	0.004	0.004	–
atcctattttacgagaacaaaaacaaacaggggtcagaacgggagaaaaaaaag	0.004	0.009	0.018	0.031	–
atcctattttacgagaacaaaaacaaacaaggggtcagaacgggaaaaaaaaag	0.000	0.004	0.000	0.004	–
atcctcttctctttccaagaacaaacaggggttcagaaagcgaaaaagggg	0.000	0.004	0.000	0.004	–
atcctgttttccgaaaacaaacaaaagttcagaaaaaaag	0.004	0.004	0.013	0.022	–
atcctgttttccgaaaacaaacaaaggttcagaaaaaaag	0.000	0.000	0.004	0.004	–
atcctgttttctgaaaacaaacaaaggttcagaaaaaaag	0.000	0.013	0.022	0.036	–
atcctgttttctgaaaacaaacaaaggttcagcaaaaaag	0.004	0.004	0.013	0.022	+
atcctgttttctcaaaacaaacaaaggttcagaaaaaaag	0.000	0.000	0.009	0.009	–
atccgatttctagaaacaataggttcctttccgagaacgg	0.000	0.000	0.004	0.004	?
tgatttaaaggtcgaacagacctacttttaaaattgttgcatctataaagttacattaattcaacatcgag gtcgcaatcatttttttaaataagaactttagaaaaata	0.004	0.000	0.000	0.004	–
atttttataagtcgaacagactttatatattcaatcttgcttgaataggataattaattcaacatcgaggtc gtaaacatttatttttattaggactttaaattaata	0.004	0.000	0.000	0.004	–
ttttttaaaagtcgaacagacttccttttattaactattgcgtaaattaggaaaaattaaatcaacatcgagg tcgcaatctttatttttaaataagatcttataaattata	0.004	0.000	0.000	0.004	–
tatattaaaggtcgaacagacctacttatataactactgcgttattatagaataataattcaacatcgaggt cataaacattatttttaataagagctttaaaataata	0.004	0.000	0.000	0.004	–
tgatttaaaagatgaacaatctttcttatatagtctctccaccatatagacacattaattcaacatcgaggt cgcaaactactatatcaatatgaactctccatagtaa	0.004	0.000	0.000	0.004	?
caatttaaaagatgaacaatctcccttagtaattctctacaacaactaggtgtgtcaaattcaacatcgaa gtcgcaaactactataccaatatgaactctccatagtaa	0.004	0.000	0.000	0.004	?
aaatttaaaagatgaacaatctttctattttaatattcttctattaatagatatcttaattcaacatcgaggt cgtaaactattataccaatatgttctatccataataa	0.004	0.000	0.000	0.004	?
aaaattaatagtcgaacagactaactcatatctatatttctagaaacaatgtttttaattcaacatcgaggt cgcaatcttactcactaataagaactctaaaagtaaa	0.004	0.000	0.000	0.004	?
aaaatcatcagacgaacagtctgactaactctagccccttcgccaagtagtggttttaatccaacatcgag gtcccaaacccccttaaagataagaactcataaagggta	0.004	0.004	0.009	0.018	?

(Continues)

TABLE A1 (Continued)

Phyllum	Class	Order	Family	Final_ID
	Diplopoda	NI	NI	Diplopoda_1
	Entognatha	Collembola	Entomobryidae	Entomobryidae_1
				Entomobryidae_2
				Entomobryidae_3
	Insecta	NI*	NI	Insecta_1
		Blattodea*	Blattidae*	*Blattella germanica*
				Periplaneta americana
				*Symploce*_1
		Coleoptera	NI	Coleoptera_1
			Curculionidae	*Hypothenemus*_1
		Dermaptera	Anisolabididae	*Euborellia annulipes*
		Diptera*	NI	Diptera_1
			Chironomidae	*Cricotopus*_1
			Diopsidae	Diopsidae_1
			Phoridae*	*Megaselia*_1*
			Psychodidae	Psychodidae_1
		Hemiptera*	Aphididae*	Aphididae_1
				*Aphis gossypii**
			Cicadellidae	Cicadellidae_1
				*Nesophrosyne*_1
		Hymenoptera*	NI	Hymenoptera_2
			Aphelinidae	*Eretmocerus mundus*
			Apidae*	Apidae_1*
				*Apis mellifera**
			Braconidae	*Diaeretiella rapae*
			Formicidae*	Formicidae_2

DNA sequences	ST	SI	LI	Total	Impact
gaatttaaaggtcgaacagaccttctttaacaactactgcaccaataagacttcttattccaacatcgaggtcgcaaacattttcgtcaatatgaactctcaaaaaata	0.009	0.000	0.000	0.009	?
aaatttaatagtcgaacagactttcttataaaaatactgctcttttaagaaattttaattcaacatcgaggtcgcaaaaaattttgtcgataagaactctaaaaaatca	0.000	0.000	0.004	0.004	–
aaatttaatggtcgaacagaccatttaatattaactactgcactaaaaaaacttttttaattcaacatcgaggtcgcaaacacatctattaataagaactctcaagatata	0.004	0.000	0.000	0.004	–
atattttatagacgaacagtctagcttttaaaatttctgcactttaaagcgtatttaattcaacatcgaggtcgcaaactaaaatgtcgataagaactctcaattttaa	0.000	0.004	0.000	0.004	–
gaatttaatagtcgaacagactaactcaaaagaaaaactacccccccaaaattatcttaattcaacatcgaggtcgcaaacttaactataaataagaactctccagtaaaa	0.009	0.000	0.000	0.009	?
gattttaaaggtcgaacagacctaacatacttaaacttctacacctaatgtttatcttaatccaacatcgaggtcgcaacccttttttgtcgataagaactcttaaaaaaga	0.009	0.000	0.000	0.009	+
gattttaaaggtcgaacagacctaaacaaattgaaattctacacccaaattaaatcttaatccaacatcgaggtcgcaaccccatttatcaataagaactctcaaaaaaga	0.004	0.000	0.000	0.004	+
gattttaaaggtcgaacagacctaacattattaaacttctacacctaatgtttatcttaatccaacatcgaggtcgcaacccttttttgtcgatattaactctcaaaaagga	0.004	0.000	0.000	0.004	–
aaaattaaaagacgaacagtcctacttctccagccccttcgccaaagagtaattttaatccaacatcgaggtcccaaaccccccctaaagataagaactcacaagaagga	0.000	0.000	0.013	0.013	?
aatttcaaaagtcgaacagactcaatttcccagcttctacaccaagaatttattttaatccaacatcgaggtcgcaatctcttctttcgataagaactctcaagaaaaa	0.000	0.004	0.000	0.004	–
aattttaatagtcgaacagactaaaattataaactcctgcatttatattttattttaatccaacatcgaggtcgcaatctctcttgttaataagttctctctaagagaa	0.013	0.009	0.000	0.022	+
gattttaatagtcgaacagactaaattttcaagcttctgcacctaaaatttatcttaatccaacatcgaggtcgcaatcctctataataataagaactcaaaaaagaga	0.004	0.000	0.000	0.004	?
gaaaatataagtcgaacagactttgaatttaaacttctacacctaaattaattcttagtccaacatcgaggtcgcaatctttttttatcgatttgaactctccaaaaaaa	0.004	0.000	0.000	0.004	?
gatttaaaaagtcgaacagacttaatatttaaaactcttctttttaaatttaaccttaattcaacatcgaggtcataaatatttttttatatacgatctacaaaaaaatt	0.004	0.000	0.000	0.004	?
gaattcaaaggtcgaacagacctaaactttaaacttctacacctaaaaataactcttaatccaacatcgaggtcgcaatctttttttgtcgatatgaactctaaaaaaaaa	0.004	0.000	0.000	0.004	–
gaatttaaaagtcgaacagactttatttttaaactgctgcacctaaaataaatcttaattcaacatcgaggtcgcaatcatttttatcgatatgaactctctaaaaaga	0.000	0.004	0.000	0.004	–
gaatttaaaggtcgaacagacctaacacttaaaattttgcacctaagattaatcttaattcaacatcgaggtcgcaaactaatttttaaatttgaacttaaaaaattaa	0.004	0.000	0.000	0.004	?
gaatttaaaggccgaacagacctaatacttaaaattttgcacctaagattaatcttaattcaacatcgaggtcgcaaactaatttttaaatttgaacttaaaaaatgaa	0.004	0.000	0.000	0.004	+
gaaattaaaagtcgaacagacttaaattataaagaatctacccccctaattttatcttaattcaacatcgaggtcgcaaactttaatatagatatgaactctccattaaaa	0.004	0.000	0.000	0.004	?
gattttaaaagtcgaacagacttaatataaagaattctgcttctaatataaatcttaattcaacatcgaggtcgtaaactttaatatagatatgaactccccattaaaa	0.004	0.000	0.000	0.004	–
gattttaatagtcgaacagactaaataaattattatctccaataattattaatcttaattcaacatcgaggtcgcaaacttaaatatcaatatgatctctccatttaaa	0.004	0.000	0.000	0.004	?
aattttaatagtcgaacagactaaaatttttaatatcttcattaaaattaaatttttaattcaacatcgaggtcgcaaacttttttatcaatatgaactatttaaaaaaa	0.004	0.000	0.000	0.004	?
gattttaaaagtcgaacagacttaaatattaaactccttcatttaatttttatcttaattcaacatcgaggtcgcaaccaccttttattaataggatctctctaaagata	0.022	0.000	0.000	0.022	?
gactttaaaagtcgaacagacttaaatattaaactccttcatttaatttttatcttaattcaacatcgaggtcgcaatcaccttttattaataagatctctctaaagata	0.009	0.000	0.000	0.009	–
gattttaaaagtcgaacagacttaaaatataaaaatttttcattttaattttatcttaattcaacatcgaggtcataaaatttattttaaatttgatcttagaaataaaa	0.009	0.009	0.000	0.018	+
gattttaatagtcgaacagactaaacttttaaacttcttcatttaaattaaatcttaattcaacatcgaggtcgcaaacatttttattaataagatctctccaaaaata	0.004	0.000	0.000	0.004	?

(Continues)

TABLE A1 (Continued)

Phyllum	Class	Order	Family	Final_ID
				Formicidae_3
				*Camponotus*_1
				Cardiocondyla emeryi
				*Monomorium subopacum**
			Thynninae	*Neozeleboria*_1
		Lepidoptera*	NI	Lepidoptera_1
				Lepidoptera_2*
				Lepidoptera_3
				Lepidoptera_6
				Lepidoptera_8
			Bombycidae	*Bombyx*_1
			Noctuidae	Noctuidae_1
			Papilionidae	Papilionidae_1
				Papilionidae_2
			Pieridae	*Colotis*_1
			Pterophoridae	Pterophoridae_1
		Orthoptera	Acrididae	Acrididae_1
				Acrididae_2
			Gryllidae	*Gryllus*_1
			Pyrgomorphidae	*Pyrgomorpha conica*
		Thysanoptera	Phlaeothripidae	Phlaeothripidae_1
	Malacostraca	Decapoda	NI	Decapoda_1
				Decapoda_2

Note: The final ID of MOTUs corresponds to the highest taxonomical classification possible. Information about the putative impact (positive, +, negative, −, or unknown, ?) of predation on each MOTU is also given. The (*) indicates taxa that contributed significantly to differences between sections.

DNA sequences	ST	SI	LI	Total	Impact
gattttaaaagtcgaacagacttaaatattaaatatctccatttaatttttatcttaattcaacatcgaggtcg caaacatttttatcaatatgatctttctaaaaata	0.004	0.000	0.000	0.004	?
aattttaatagtcgaacagactaaaatattaaactcctacgcctaatttttattttaattcaacatcgaggtcg caatcacttttatcaataagatctttctaaaaaga	0.000	0.000	0.004	0.004	–
gattttaaaagtcgaacagacttaaatattaaatttctccatctaatttttatcttaattcaacatcgaggtcg caaacatttttatcaatatgatctctccaaaaata	0.004	0.000	0.000	0.004	?
gattataaaagtcgaacagacttaaatattaaatatctccatttaatttttatcttaattcaacatcgaggtcg caatcacttttatcaatatgaactctctaaaaata	0.013	0.000	0.000	0.013	–
gattttaaaagtcgaacagacttaaatattaaacttctccatctaatttttatcttaattcaacatcgaggtcg caaccatttttatcaatatgaactctccaaaaata	0.004	0.004	0.000	0.009	?
gattttaatgatcgaacagatcaaaaattttaaacttttgcatttaaattttatcttagtccaacatcgaggtc gcaaacttttttttttatttgaactaaaaaaaaaaa	0.013	0.009	0.000	0.022	?
gattttaatgatcgaacagatcaaaaacttaaacttttgcattataaattttatcttaatccaacatcgaggtc gcaaactctttttcttataagaactaaaaaaaaaa	0.004	0.000	0.000	0.004	?
gattttaatgatcgaacagatcaaaaattttaaacttttgcatttaaattttatcttagtccaacatcgaggtc gcaaacttttttttttatttgaactaaaaaaaaa	0.004	0.004	0.000	0.009	?
gattttaatgatcgaacagatcaaaaattttaaacttttgcatttaaattttatcttagtccaacatcgaggtc gcaaacttttttttttatttgaactaaaaaaaaaga	0.000	0.004	0.000	0.004	?
gattttaatgatcgaacagatcaaaattttaaacttctacatttaaattttatcttaatccaacatcgaggtcg caaacttttcttcttatttgaactaaaaaaaaaa	0.004	0.000	0.000	0.004	?
gattttaatgatcgaacagatcaaaattttaaacttctacatttaaattttatcttaatccaacatcgaggtcg caaacttttcttcttatttgaactaaaaaaaaaaa	0.004	0.000	0.000	0.004	?
gattttaatgatcgaacagatcaaaattttaaacttctgcatttaaattttatcttaatccaacatcgaggtcg caaactctttttttttatttgaactaaaaaaaaaa	0.004	0.000	0.000	0.004	–
gattttaatgatcgaacagatcaaaaattttaaacttttgcatttaaattttatcttagtccaacatcgaggtc gcaaacttttttttttatttgaactaaaaaaaaaaa	0.013	0.004	0.000	0.018	?
gattttaatgatcgaacagatcaaaaattttaaacttttgcatttaaattttatcttagtccaacatcgaggtc gcaaactttttttttatttgaactaaaaaaaaaa	0.009	0.004	0.000	0.013	?
gattttaatgatcgaacagatcaaaattttaaacttttgcatttaaattttatcttaatccaacatcgaggtcg caaacttttattttttatttgaactaaaaaaaaaaa	0.004	0.000	0.000	0.004	–
gattttaatgatcgaacagatcaaaattttaaatttttgcatttaaattttatcttaatccaacatcgaggtcg caaacttttattttttatatgaactaaaaataaaaa	0.004	0.000	0.000	0.004	?
gaatttaaaggtcgaacagacctaatcattgggctactgcacccaaaatttttcttaatccaacatcgaggt cgcaatctgctttgtcgatatgagctctcaaaaacga	0.004	0.000	0.000	0.004	–
gaatttaaaggtcgaacagacctaatcattgggctactgcacccaaaatttttcttaatccaacatcgaggt cgcaatctgctttgtcgatatgagctctcaaaaacaa	0.004	0.000	0.000	0.004	–
gaatttaatggtcgaacagaccaattaattaagcttctgcacctaataattttcttaatccaacatcgaggtc gcaatctttattatcaatatgaactctccaataaca	0.000	0.000	0.004	0.004	–
gattttaaaggtcgaacagacctagtttctaaactgatgcacctagaacttatcttaatccaacatcgaggt cgcaatctaatttgttgatatgaactctcaaaattaa	0.004	0.000	0.000	0.004	–
gattttaaaagtcgaacagacttaatttattaaaatttttcttaaaaaatttatcttaattcaacatcgaggtc ataaaaaatatattaaaataaggacttttatataatt	0.004	0.000	0.000	0.004	–
aaatttaatggttgaacaaaccaacttactaggatcctgctcctaatagtaattttaattcaacatcgaggt cgcaacccactctattgatacggactcttcagagtga	0.004	0.000	0.004	0.009	?
atatttattagtcgaacagactgtcttttataaactgctgcttctaaaagaaaatttaattcaacatcgaggt cgcaaaaactttatcgatttgaactctccaaaagaa	0.004	0.000	0.000	0.004	?

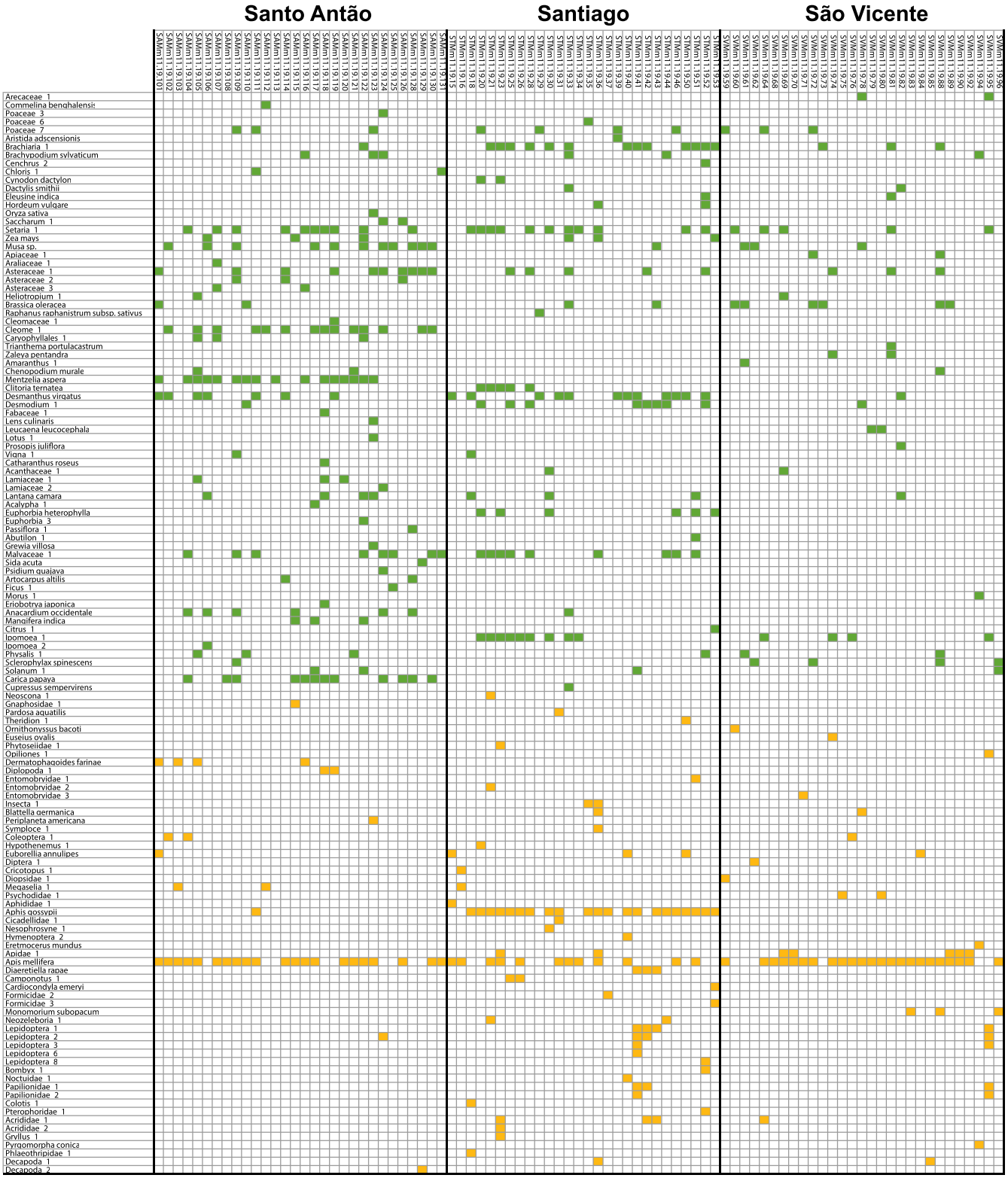

FIGURE A1 Heatmap representing the distribution of the different diet items in the *Mus musculus* samples of the three islands (Santo Antão, Santiago and São Vicente). Occurrences of plant items are represented in green and of invertebrate items in yellow

Article VI

Green matters: Dietary assessment of a reptile community using DNA metabarcoding

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ABSTRACT

DNA metabarcoding is widely used for diet characterization and is becoming increasingly important for biodiversity conservation, allowing the understanding of trophic networks and community assemblies. However, to our knowledge, few studies have used this approach to investigate trophic interactions for whole communities and none for reptiles. In particular, few studies have examined the diet composition of Saudi Arabian reptiles, and all have used classical methods only. Therefore, in this work, a non-invasive approach using DNA metabarcoding of faecal pellets was implemented to investigate the diet composition of the reptile community of Wadi Ashar, in AlUla County, north-western Saudi Arabia Kingdom. In the overall diet composition of the community, arthropods were present in 90% of the samples, and plants were present in 63%, revealing the unforeseen importance of plants to this community as a secondary, but also a primary dietary item. For some species, this is the first time that plants have been reported in their diet. A significant effect of reptile body size on diet composition was also demonstrated, indicating its strong influence on prey selection and resource partitioning in the community. This study highlights the importance of community assessments and the power of combining these with non-invasive DNA metabarcoding to accurately assess biodiversity and feeding habits, revealing unknown ecological interactions of often neglected groups. This revolutionary tool for conservation and management provided rapid and holistic information at relatively low costs, allowing to inform local authorities about which elements are central to the sustainable management of the Wadi Ashar community.

1. Introduction

The traditional methods of morphological characterization of species for biodiversity assessments were revolutionized by DNA-

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based species identification. DNA barcoding is a molecular technique regularized by Hebert et al. (2003) that allows the identification of species using short standardized DNA fragments known as DNA barcodes. Metabarcoding extends DNA-based species identification to complex samples allowing a large-scale taxonomic identification (Pompanon et al., 2012), relying on high-throughput sequencing of DNA using general or group-specific primers. With the power of Next-generation sequencing (NGS) methodologies (Shendure and Ji, 2008), this multispecies identification approach is becoming faster and more cost-effective each day. Metabarcoding has been widely used to characterise species diets by extracting DNA from faeces and gut contents (Pompanon et al., 2012). This technique provides a large amount of data with less effort and time (Taberlet et al., 2012) which is particularly useful for studying the diets of animals with elusive lifestyles. In addition, compared to classical methods, this technique is less reliant on taxonomic expertise (Pompanon et al., 2012), and maximises resolution, detection of rare events, detection of soft, small and difficult-to-detect prey (Symondson, 2002) and ultimately can correct biases in ecological models. It is a particularly useful technique for studying species of conservation concern. The characterization of their dietary compositions allows the understanding of trophic interactions, which are one of the main vectors shaping natural community assemblies and ecosystem processes (Thébault and Loreau, 2005). This data is essential for assessing ecological responses to environmental change, the ecosystems' health, and ultimately the development of sustainable conservation strategies. However, few studies have used metabarcoding of diets to study trophic interactions of whole communities (Ingala et al., 2021; McShea et al., 2019), and to our best knowledge none on reptile communities.

Reptiles are the most diverse group of terrestrial vertebrates in the world, with 11,940 currently described species (Uetz et al., 2023). They are an important component of global biodiversity and are remarkable from an ecological and evolutionary perspective. As ectotherms, reptiles are strongly influenced by the thermal landscapes of their habitat, making them good indicators for studies of climate change (Sinervo et al., 2010). They are also relatively easy to capture and sample for biodiversity and phylogenetic studies, they are widely represented in museum collections worldwide (Shultz et al., 2021), and for many groups, there is abundant and detailed information on their taxonomy, distribution, and ecology (Pincheira-Donoso et al., 2013; Uetz et al., 2023). For these and more reasons, they are excellent models for evolutionary, biogeographic, ecological and conservation studies, and have been used as such for many decades (Camargo et al., 2010; Nordstrom et al., 2022). Since most of the species are adapted to particular environments, they are very good indicators of the ecosystems' health. Therefore, reptiles are a key target group in biodiversity studies, especially in arid areas where they are one of the main components of the diurnal and nocturnal vertebrate fauna, playing a relevant role in the ecosystem (Lopes et al., 2019; Miranda, 2017; Wilms et al., 2010a).

In Arabia, reptiles are the terrestrial vertebrate group that contains the highest number of endemic species and, therefore, are very important from a biodiversity point of view (Cox et al., 2012). The Arabian Peninsula started its formation around 30 million years ago

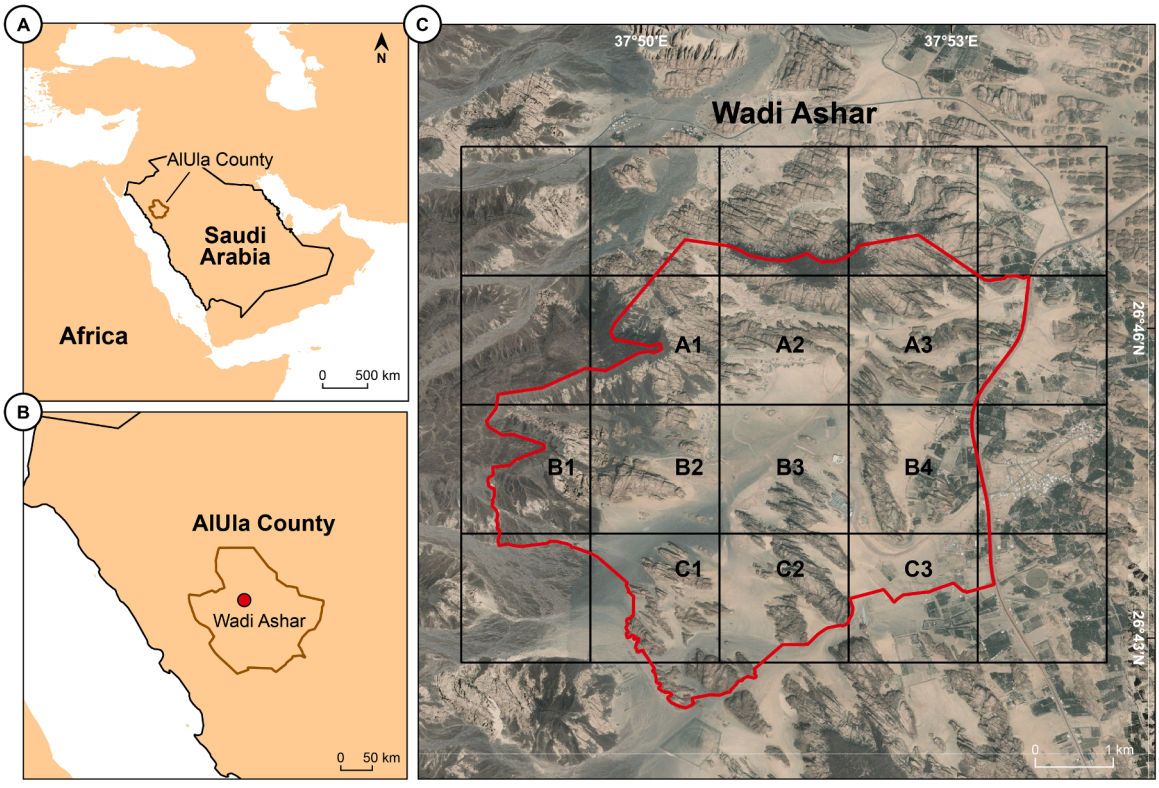

Fig. 1. Map of the study area and study site. **A)** Geographic location of the Kingdom of Saudi Arabia and **B)** the AlUla County, with the location of the study site represented by a red dot. **C)** Wadi Ashar boundary in red, and the 10 sampled squares (Geographic Coordinate System, Datum WGS84).

(Ma), driven by the separation of the Arabian and African tectonic plates (Alsharhan and Nairn, 2003; Bohannon, 1986). The split prompted the formation of the Red Sea about 25 Ma (Bosworth et al., 2005), and played a major role in shaping the geography and history of the region. Accompanying the separation was a shift to an arid climate, which together with the complex and dynamic geologic past, had major biogeographic and evolutionary implications on the unique biodiversity of the region (Kürschner, 1998; Pook et al., 2009; Šmíd et al., 2013; Tejero-Cicuéndez et al., 2021; Zinner et al., 2009). In particular, the Kingdom of Saudi Arabia, covering more than 2.15 million km^2 (Fig. 1A), encompasses a wide range of climates and terrestrial habitats, from mesic, cool, high mountains to arid deserts and hot, semi-arid coastal plains, and thus holds a very rich biodiversity (AbuZinada et al., 2004; Vincent, 2008). The terrestrial reptile fauna of Saudi Arabia includes approximately 118 species (80 lizards, 36 snakes and two freshwater turtles), 8 of which are endemic to the Kingdom (Gasperetti et al., 1993; Šmíd et al., 2017a; Šmíd et al., 2017b; Šmíd et al., 2021; Šmíd et al., 2023; Wilms et al., 2010b). However, to effectively conserve this unique reptile biodiversity, much work remains to be done to fully understand its diversity, distribution, and ecology (Aloufi et al., 2019). Conserving wildlife and maintaining healthy ecosystems in arid habitats is particularly important, as resources are typically limited, and the loss of a single resource can be catastrophic for the stability of the communities (McNeely, 2003). Thus, local authorities are giving increased attention to conservation planning in the region promoting the development of several projects to monitor and protect biodiversity (Alatawi, 2022).

Despite these efforts, few studies have investigated the diet composition of Saudi Arabian reptiles. The few ones focus mostly on the Riyadh region and all rely on the analysis of stomach contents to morphologically identify the different prey items consumed (Al-Sadoon and Al-Otaibi, 2014; Al-Sadoon and Paray, 2016; Al-Sadoon et al., 2016; Al-Sadoon et al., 2020; Ibrahim and Busais, 2016). However, the use of faecal samples is a better approach, mainly when working with threatened species, since they can be obtained with minimum or no impact on individual fitness (Ferreira et al., 2018). Consequently, this work implemented a non-invasive approach, taking advantage of DNA metabarcoding of faecal pellets to investigate the dietary composition and the possibility of resource segregation by the reptile community of Wadi Ashar located in AlUla County, north-western Saudi Arabia. Alongside, a biodiversity survey was carried out to assess the diversity of reptile species in the community. The ultimate goal was to explore ecological patterns through prey items consumed and contribute to a better understanding of the trophic networks involving plants, arthropods, and reptiles at a community level in this restricted area. This data is expected to inform local authorities about which elements are central to the sustainable management of the reptile community.

2. Methods

2.1. Study area

AlUla is a county located 1100 km north-west of Riyadh, in the Medina region of north-western Saudi Arabia (Fig. 1B). It covers a vast area of 22,561 km^2, including desert valleys, sandstone mountains and several ancient heritage sites. Wadi Ashar, located in AlUla, is a valley within the UNESCO Biosphere Reserve of Harrat Uwayrid of about 30 km^2 surrounded by sandstone mountains (Fig. 1C). It is composed of different habitat types such as dunes and sandy areas, gravel plains, rocky slopes, gullies, and agricultural

Table 1
Sampled herpetofauna of Wadi Ashar. List of taxa sampled ordinated by family and their codes, number of sampled individuals (N), of collected pellets (Np), of successfully sequenced pellets (Ns), average snout-vent length (SVL), body size category (class), activity period (Activity) and previous knowledge on diet composition (Diet) based on the literature: [1]Al-Sadoon et al. (2020); [2]Arnold (1980); [3]Arnold (1993); [4]Carranza et al. (2021); [5]Cottone and Bauer (2009); [6]Ibrahim and Busais (2016); [7]Ibrahim and El-Naggar (2013); [8]Kalboussi and Nouira (2004); [9]Ribeiro-Júnior et al. (2022); [10]Roobas and Feulner (2013); [11]Taylor et al. (2012); [12]Tsairi and Bouskila (2004); [13]Yadollahvandmiandoab et al. (2018). *Ptyodactylus* sp1 x sp3 and *Ptyodactylus* sp1 refer to two new species of the *Ptyodactylus hasselquistii* species complex in the process of description.

Family	Taxa	Code	N	Np	Ns	SVL (mm)	Class	Activity	Diet
Agamidae	*Laudakia vulgaris*	LAvu	3	3	3	-	Big	Diurnal	Arthropods, plants[7]
Atractaspididae	*Atractaspis engaddensis*	ATen	1	1	1	-	-	Nocturnal	Vertebrates[1]
Colubridae	*Spalerosophis diadema*	SPdi	1	-	-	-	-	Diurnal/ Nocturnal	Vertebrates[13]
Gekkonidae	*Bunopus tuberculatus*	BUtu	1	1	0	32.96	-	Nocturnal	Arthropods[4]
	Hemidactylus granosus	HMgr	1	-	-	45.08	-	Nocturnal	-
	Stenodactylus doriae	STdo	13	8	4	54.19	Medium	Nocturnal	Arthropods[2,4]
	Tropiocolotes yomtovi	Tryo	10	7	1	24.43	Small	Nocturnal	Arthropods[9]
Lacertidae	*Acanthodactylus boskianus*	ACbo	11	7	5	59.24	Medium	Diurnal	Arthropods[10]
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	9	5	3	55.97	Medium	Nocturnal	Arthropods[6]
	Ptyodactylus sp1	PTsp1	26	12	7	59.51	Medium	Nocturnal	Arthropods[6]
Psammophiidae	*Psammophis schokari*	PSsc	1	-	-	67.40	-	Diurnal	Vertebrates[5]
Scincidae	*Chalcides ocellatus*	CHoc	5	5	4	84.94	Big	Diurnal	Arthropods, plants[8,11]
	Scincus conirostris	SCco	2	2	1	81.87	Big	Diurnal	-
	Trachylepis brevicollis	TCbr	2	1	1	119.68	Big	Diurnal	Arthropods, plants, vertebrates[4]
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	6	3	2	25.48	Small	Diurnal	Arthropods[3]
Viperidae	*Echis coloratus*	ECco	1	1	0	-	-	Nocturnal	Vertebrates[12]

areas. The area is substantially influenced by overgrazing, and it holds several tourist resorts. Recent development has resulted in severe habitat destruction and fragmentation in the area.

2.2. Sampling

Sampling in Wadi Ashar took place between the 15th and 24th of March 2022. The study area was divided into 10 subareas of approximately equal size (Fig. 1C). Each subarea was surveyed both during the day and night to collect representative data on the entire herpetological community by covering the possible activity periods of the different species (Table 1). A total of 23 transects were completed by four observers in teams of two. Each transect was on average 555 m long and sampling took an average of one hour per transect. Active searches during the day included checking under stones and other possible shelters such as bushes, trees, rocks, boulders, caves, and holes. Nocturnal searches consisted mainly of visual searches for active reptiles using flashlights. In addition to the active searches, two fence trap arrays, each with four pitfalls and two funnel traps, were set at appropriate locations in each subarea throughout the study period. Other six pitfalls and three funnel traps were individually placed in appropriate habitats (dunes, shrubs, along long walls). Traps were visited at least two times a day. Individuals were captured by hand, sexed based on the absence or presence of cloacal pouches (Vasconcelos et al., 2012), measured (snout-vent length (SVL) to the nearest mm), geolocated using a GPS device, and photographed. Before releasing each animal, a sample was taken from the tip of the tail for species identification and an abdominal massage was performed to collect fresh pellets for dietary analysis. All samples were preserved in 96% ethanol.

Samples of plant leaves were also collected across the study area, in order to build a DNA reference collection of possible food items. Pictures of each plant were also taken in the field to allow for morphological taxonomic assessment by experts.

2.3. Species identification

For correct taxonomical assignment of the species identified in the field, DNA from the tissue samples was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. DNA samples were PCR-amplified and sequenced for the ribosomal 12 S rRNA (12 S) region, with primers and PCR conditions detailed in Šmíd et al. (2021). The PCR products were sequenced from both directions at Macrogen (Amsterdam, the Netherlands) and raw sequence data were edited and assembled in Geneious v.11 (Kearse et al., 2012). The identification of each specimen was confirmed by employing the BLAST algorithm available at the NCBI website *(https://blast.ncbi.nlm.nih.gov)*.

2.4. Diet analysis

2.4.1. DNA extraction, amplification, and sequencing

For the diet analysis, DNA extractions from the pellets (excluding uric acid) were performed, including one blank per sample batch, using the Stool DNA Isolation Kit (Norgen Biotek Corp., Canada), following the manufacturer's instructions. All DNA samples were amplified with two genetic markers already validated in previous studies (Pinho et al., 2022; Pinho et al., 2018). For invertebrates we used a modified version of the IN16STK-1 F/IN16STK-1R primers, targeting the mitochondrial 16 S rRNA (Kartzinel and Pringle, 2015; Pinho et al., 2018) and for plants, we used the g/h primers targeting the short P6-loop of chloroplast trnL (UAA) (Taberlet et al., 2007). For species that were expected to prey on vertebrates (e.g., *Atractaspis engaddensis* Haas, 1950; Table 1) the primers 12sv5F/12 Ssv5R, targeting the V5-loop fragment of the mitochondrial 12S gene, were also used. All PCRs were performed, including blanks, as described by Pinho et al. (2018). Libraries were prepared following the Illumina MiSeq protocol '16 S Metagenomic Sequencing Library Preparation' (Illumina, 2013). The samples were sequenced in the MiSeq sequencer (Illumina) using the 300-cycle MiSeq Reagent Kit V2 (Illumina, San Diego, CA, USA) for an expected average of 22,000 paired-end reads per sample.

The plant samples for the reference collection were amplified for the chloroplast TrnL (UAA) using two sets of primers. The primer set e/f (Taberlet et al., 1991) was used to further validate the morphological taxonomic assessment, and the primer set c/d (Taberlet et al., 2007) to allow the comparison with diet items. Both markers were sequenced using Sanger sequencing.

2.4.2. Bioinformatics filtering

The obtained DNA sequences for the diet analysis were processed at the bioinformatics level using tools incorporated in the software package OBITtools (http://metabarcoding.org/obitools). This process included the alignment of forward and reverse sequences (command illuminapairedend, –score-min=40), removal of unaligned sequences (command obigrep, mode!="joined"), assignment of reads to samples and removal of primers and adapters (command ngsfilter) and the collapsed of reads into unique sequences (command obiuniq). Unique sequences were discarded if read count was inferior to 10 and if sequence length did not correspond to expected base pair size (16S: ± 110; trnL: 10–143; 12S: 73–110). Finally, potential PCR/ sequencing errors and singletons were removed and the molecular operational taxonomic units (MOTUs) obtained (command obiclean), by removing sequences that differed by 1 bp from the most abundant ones. The MOTU tables were subsequently curated using the R package LULU (Frøslev et al., 2017) in order to remove potential remaining PCR and sequencing errors.

The diet items were taxonomically assigned by comparing the final MOTUs against the online NCBI database using the BLAST algorithm. Sequences that had less than 85% of Percent Identity value with known species were classified only to the class level, the ones with values between 85% and 90% to the order level, between 90% and 95% to the family level, and the remaining sequences with Percent Identity values above 95% to the genus or species level. Only species or genera known to occur in Saudi Arabia or in the broader region of the Middle East were considered (Al-Qthanin et al., 2020; Buttiker, 1979; POWO, 2021; Walker et al., 1987). When a

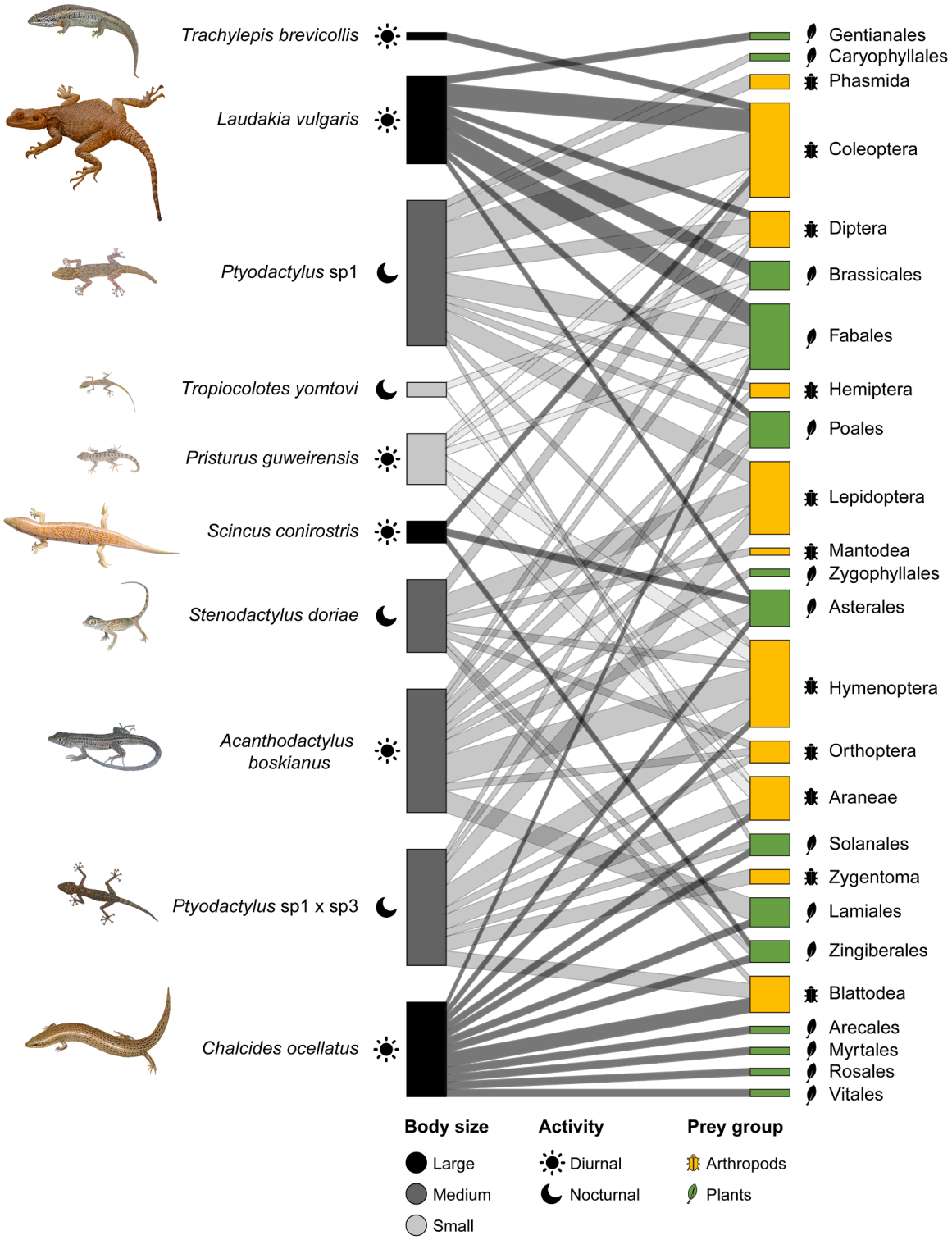

Fig. 2. Bipartite network representing associations between the reptile community of Wadi Ashar on the left and arthropod (yellow) and plant (green) prey orders on the right. The width of the connecting bars represents the frequency of occurrence of each order for each reptile species. Reptile species are represented proportionally according to their sizes, and with darker colour boxes indicating their increasing body size category. Information on reptiles' activity pattern (diurnal versus nocturnal) is also given.

MOTU matched more than one species or genus with the same identity scores or presented low query coverage (<80%), a higher taxonomic ranking would be attributed (e.g., family). If more than one MOTU corresponded to the same taxon at the same taxonomic level, a different number was attributed to each (e.g., Poaceae_1, Poaceae_2), considering genetic differences among MOTUs. The MOTUs detected in extraction blanks were identified as contaminations and removed from the corresponding batch of samples (e.g., human DNA). In the final dataset, based on PCR blanks counts, samples with less than 650 reads were removed and within kept samples, MOTUs representing less than 1% of the total number of reads of that sample were also discarded (Mata et al., 2016).

2.4.3. Data analysis

Frequencies of occurrence (FO) of plants, arthropods and vertebrates were estimated for the reptile community and for each species individually at the order, family, and MOTU level. The sample of *A. engaddensis* (CN20291) was excluded from further statistical analysis, as it was the only exclusively carnivorous species. All analysis were performed using R software version 4.2.2 (R Core Team, 2022). The package bipartite v2.17 (Dormann et al., 2008) was used to visualize the dietary networks using the function 'plotWeb'. Adjacency plots were calculated at the order level using the function 'metaComputeModules' employing the "Beckett" method and visualized using 'plotModuleWeb'. A total of 1000 null model networks were generated using 'nullmodel' function with the "r2dtable" method. To assess the statistical significance of the observed modularity, z-scores were calculated and *p*-values were obtained using the 'pnorm' function. Due to the low resolution at the MOTU level, a binary matrix (of the presence/ absence of prey) was prepared at the order and family level considering individual samples. Reptile species were divided into three body size categories following our SVL average measures per species and the literature: large > 80 mm; medium 50 – 80 mm; and small < 50 mm (Table 1). Generalized linear models (GLM) were carried out to test for diet composition differences among size categories and activity period (diurnal vs nocturnal), accounting for the unbalanced sample sizes. GLMs for multivariate binary data were fitted using the *mvabund* package (Wang et al., 2022), using the *manyglm* function. The significance of the GLMs was tested using the *anova.manyglm* function. The same function was implemented with the argument p.uni= 'adjusted' to perform univariate tests, identifying prey items responsible for differences among size categories. Using the *metaMDS* function from the vegan package (Oksanen et al., 2022), a non-metric multi-dimensional scaling (NMDS) was performed on a Jaccard distance matrix to ordinate samples according to diet dissimilarity. NMDS were calculated at the order-level and visualised in two-dimensional space.

3. Results

A total of 93 individuals of 16 reptile species, including four snakes, were observed (Table 1). From these, a total of 82 individuals were caught and a tissue sample was collected (Table S1). The most common taxon was the candidate species *Ptyodactylus* sp1 (a new species of the *Ptyodactylus hasselquistii* species complex in the process of description), with 24 observations, followed by *Stenodactylus doriae* (Blanford, 1874), *Acanthodactylus boskianus* (Daudin, 1802), and *Tropiocolotes yomtovi*, with less than 15 observations. The least common species, with only one individual recorded during the sampling effort, were *Atractaspis engaddensis*, *Bunopus tuberculatus* Blanford, 1874, *Echis coloratus* Günther, 1878, *Hemidactylus granosus* Heyden, 1827, *Psammophis schokari* (Forskål, 1775), and *Spalerosophis diadema* (Schlegel, 1837).

A total of 2.7 million reads were obtained from the reptile community faecal samples. After bioinformatic filtering, a total of

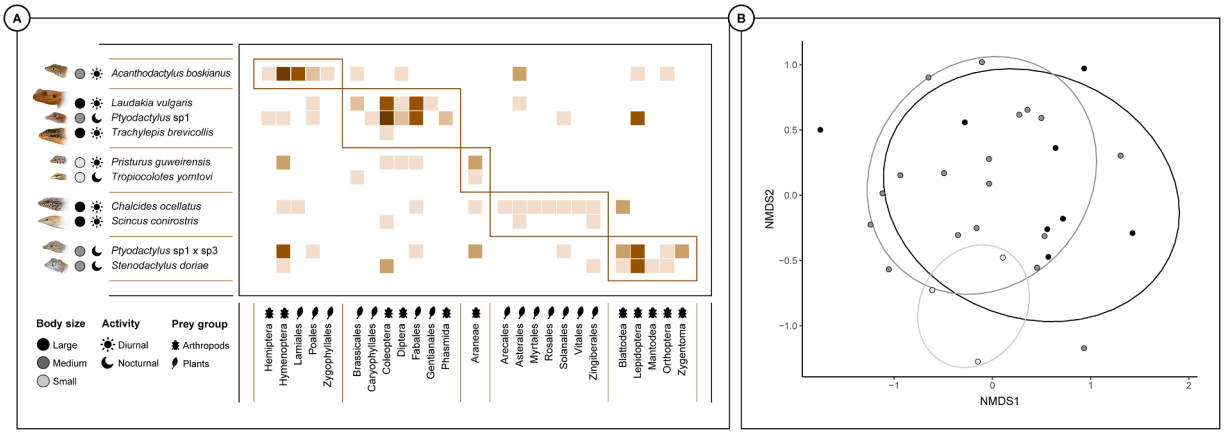

Fig. 3. Dietary composition of the reptile community of Wadi Ashar according to the body size categories. **A)** Adjacency matrix summarizing the reptile community subgroups according to their identified diet item orders. Reptile species are represented proportionally according to their sizes along the vertical axis with darker colour circles indicating their increasing body size category, and plant and arthropod orders are shown along the horizontal axis. Subgroups are delineated by brown boxes. The darker the brown squares the more frequent the association between a diet item and its predator. Information on reptiles' activity pattern (diurnal versus nocturnal) is also given. **B)** Non-metric multidimensional scaling (NMDS) of the prey composition of the Wadi Ashar reptile community by body size categories. Points closer together correspond to individuals with a more similar diet item composition. Ellipses represent the standard error of the dietary composition centroid for each body size category at 90% confidence interval.

467,831 reads were retained, of which 11% were considered non-target (e.g., host DNA, human, bacteria), resulting in a final average of approximately 13,000 reads per sample. Of the 56 faecal samples collected, the final dataset comprised 32 samples from 11 reptile species from the Wadi Ashar community. The remaining 23 samples failed bioinformatic filtering due to low read counts or contamination (Table 1). Within these, a total of 80 MOTUs from five taxonomic classes were identified, with an average of three families and three orders per sample and seven families and six orders per reptile species (Table S2). Arthropods were distributed among two classes, 11 orders, 23 families and 44 MOTUs (Table S2). Plants were distributed among two classes, 14 orders, 18 families and 29 MOTUs (Table S2). The most frequent arthropod orders were Coleoptera (FO= 40.63%) and Hymenoptera (FO= 37.50%), both present in the diet of six reptile species of the community (Fig. 2; Table S3). In particular, *Pimelia arabica* (Klug, 1830) was the most frequent arthropod MOTU (FO= 15.63%), present in the diet of three reptile species, followed by *Monomorium pharaonis* (Linnaeus, 1758) (FO= 12.50%) present in the diet of four reptile species (Table S2). For plants, the most frequent order was Fabales (FO= 28.13%), present in the diet of five reptile species (Fig. 2; Table S3). At the MOTU level, *Vachellia_1* was the most frequent (FO= 25.00%), consumed by four reptile species (Table S2). The only vertebrate detected was *Scincus conirostris* (Blanford, 1881) in the pellet of *Atractaspis engaddensis* (Table S2). In the overall diet composition of the community, arthropods were present in 90% of the samples, whereas plants were present in 63% of them. Lepidoptera was the order with the highest diversity with 12 MOTUs, almost exclusively consumed by nocturnal reptile species (Fig. 2, Table S2).

The adjacency matrix defined a total of five modules in the community (Fig. 3A). Reptiles seem to be grouped by body size and consumption of arthropod orders (e.g., candidate species *Ptyodactylus* sp1 x sp3 – a new species of the *Ptyodactylus hasselquistii* species complex in the process of description – and *S. doriae*) or plant orders (e.g., *Chalcides ocellatus* (Forskål, 1775) and *S. conirostris*). The observed modularity was significantly different from the null model distribution ($P = 0.027$), indicating that the observed pattern deviates from what would be expected by random chance. The multivariate analysis showed a significant effect of body size in the diet composition at the order level (LRT= 70.06, $P = 0.016$), but not at the family level (LRT= 84.78, $P = 0.103$). The orders that contributed significantly for the differences among size categories were Aranae ($P = 0.043$), more frequent in small sized species, and Lepidoptera ($P = 0.035$), only present in medium sized species. The activity period also showed a significant effect at the order (LRT= 45.51, $P = 0.049$) and family level (LRT= 75.69, $P = 0.003$), however, a significant interaction between body size and activity period was also observed for both (order: LRT= 16.35, $P = 0.007$; family: LRT= 9.32, $P = 0.031$). This indicates a relationship between body size and activity period, possibly explained by big sized species being also diurnal. The NMDS plot showed three groups corresponding to the body size categories in the community at the order level ($k = 2$; stress= 0.138; Fig. 3B). Species from the three body size categories exhibit distinct dietary compositions with some overlap between medium and large species.

In terms of the reference collection, 46 samples from Wadi Ashar plants were successfully taxonomically identified and sequenced (Table S4). These samples were compared with the dietary items improving taxonomic resolution.

4. Discussion

To effectively monitor and conserve communities, the first step is to know their composition (Groves et al., 2002). Hence, this study provides, for the first time, a characterisation of the herpetofauna inhabiting Wadi Ashar. In this small valley, we found 17 reptile species of 11 different families, some with the first distribution records for the area (*Hemidactylus granosus*, *Pristurus guweirensis* Haas, 1943, *Trachylepis brevicollis*; (Aloufi et al., 2021; Badiane et al., 2014; Burriel-Carranza et al., 2023; Šmíd et al., 2023). This is one of the few studies to investigate the diet composition of Saudi Arabian reptiles and, to our best knowledge, the first to use non-invasive DNA metabarcoding techniques for this purpose in the region. The same applies to community-level studies in general, a few similar studies are using faecal metabarcoding to access the diet composition of mammal communities (Gordon et al., 2019; Ingala et al., 2021; McShea et al., 2019), but none on reptile communities. In fact, reptiles are still widely underrepresented in molecular diet studies compared to other groups (Ando et al., 2020), emphasizing the importance of not overlooking some less popular vertebrate groups.

Despite what was previously described for some species, plants show an important presence in the diets of the community. *Acanthodactylus boskianus*, *Stenodactylus doriae*, and species from the genus *Ptyodactylus*, *Tropiocolotes* and *Pristurus* were previously reported to prey exclusively on insects and other arthropods (Arnold, 1980, 1984, 1993, 2009; Bar and Haimovitch, 2011; Carranza et al., 2021; Disi et al., 2001; Ibrahim and Busais, 2016; Ribeiro-Júnior et al., 2022; Roobas and Feulner, 2013). However, this study confirms the presence of plant items in their diets with a significant frequency, occurring in half or more of the samples, except for *Stenodactylus doriae* where only one of the four samples presented plant items (Fig. 2, Table S2). Alternatively, this could be explained by secondary predation or inadvertent consumption of plants when preying on arthropods (Tercel et al., 2021), and if this is the case, the consumption of plant items may be overestimated. Nevertheless, there were samples containing only plant DNA for all the species mentioned, except for *Tropiocolotes yomtovi*, where only one sample was successfully amplified, which suggests active consumption of plant items for the remaining species. Then again, since plant DNA persists longer in the gastrointestinal tract than animal DNA (Holland et al., 2020; Pinho et al., 2022) the trophic role of plants in the diet of these reptiles should be further investigated. For *Chalcides ocellatus* and *Laudakia vulgaris* (Sonnini & Latreille, 1801) previous studies reported the presence of plant matter in their diet in other regions (Ibrahim and El-Naggar, 2013; Kalboussi and Nouira, 2004; Taylor et al., 2012). These results could support the findings for *Chalcides ocellatus* and *Laudakia vulgaris*, and substantially improve the taxonomic resolution of the plants consumed, which was not possible in previous studies, as they were based on classical morphological identification methods (Ibrahim and El-Naggar, 2013; Kalboussi and Nouira, 2004; Taylor et al., 2012). In particular, *Chalcides ocellatus* was the species that consumed a higher diversity of plant orders in the community, which can be related to all individuals being found near agricultural areas (mainly palm tree plantations), hence the presence of Arecaceae in its diet. For *Scincus conirostris* no information was available on their diet, thus the present results demonstrate for the first time its consumption of both plants and arthropods (Fig. 2, Table S2). Therefore, this

study reveals the variety of plants consumed and highlights their importance for this community as a secondary and possibly also a primary diet item. It was also possible to improve the taxonomic resolution of the plant items consumed, which was leveraged by the DNA sequences obtained from the collected Wadi Ashar plants, emphasizing the importance of building a complete reference database of the potential diet items available (Pompanon et al., 2012). Even though plant material is harder to digest (Holland et al., 2020), it can be easier to access in arid ecosystems like this, where the availability of arthropods can be very low during some periods. Furthermore, there is a possibility that these reptiles can have important ecological roles as seed dispersers, which is of great importance for the structure of the plant communities in arid human-altered habitats such as this one. This pattern was already observed for other desert environments, with the Mesopotamian spiny-tailed lizard *Saara loricata* (Blanford, 1874), in human-disturbed areas of Iran (Qashqaei et al., 2023).

Through the visualisation of the adjacency matrix, it is possible to observe that the reptiles of Wadi Ashar can be broadly divided into five subgroups, associating body size categories, and in some cases activity periods, with the primary consumption of plants or arthropods (Fig. 3A). This body size association is further confirmed by the ordination of NMDS plots where points closer together correspond to individuals with more similar prey composition (Fig. 3B). This is expected since the body size and head size and shape of reptiles are strongly correlated with their bite force and therefore the hardness and size of prey items consumed (Herrel et al., 2001; Verwaijen et al., 2002). Consequently, the differences in bite force can have a strong influence on prey selection and resource partitioning in the Wadi Ashar reptile community. Likewise, activity period can be correlated with body size as larger reptiles often present higher thermoregulation capacity compared to smaller reptiles (Angilletta et al., 2002; Bogert, 1949; O'Connor et al., 2000) and can benefit from diurnal habits. The first subgroup is only composed of *Acanthodactylus boskianus* with a high frequency of Hymenoptera in the diet, in particular Formicidae, which was a strong preference previously reported for the species (Roobas and Feulner, 2013). The second subgroup, constituted by *Laudakia vulgaris*, *Ptyodactylus* sp1 and *Trachylepis brevicollis*, seems to be grouped mainly by the presence of Coleoptera. Both *Laudakia vulgaris* and *Trachylepis brevicollis* are large reptiles that may have the strength to bite harder and consume hard prey, and the first was previously reported to strongly prey on Coleoptera (Ibrahim and El-Naggar, 2013). Interestingly, although *Ptyodactylus* sp1 is a medium-sized species it does not group with the other medium-sized reptiles, in particular with *Ptyodactylus* sp1 x sp3 from the same genus, which is grouped with *Stenodactylus doriae* seemingly by the consumption of smaller and softer arthropods. This may give us some important lines of evidence, regarding their trophic or behavioural ecology, for the pending description of the unrecognized diversity within this genus. It would be important to investigate the differences in habitat use, body size, and head size and shape between the two *Ptyodactylus* that may influence these differences in resource use. The two small species, *Pristurus guweirensis* and *Tropiocolotes yomtovi*, also form a subgroup associated with the consumption of Araneae. Even though they have different activity periods (*Pristurus guweirensis* being diurnal while *Tropiocolotes yomtovi* is nocturnal), they have similar sizes and select similar refuges, as small rocks, which may lead to a similar resource selection. The large-sized and diurnal *Chalcides ocellatus* and *Scincus conirostris* are grouped by the main consumption of plants, which is expected since larger reptiles usually possess metabolic adaptations that facilitate the digestion of plant items (Dearing, 1993), facilitating their higher intake (but see Cooper and Vitt, 2002 on other factors affecting body size). This emerging pattern is consistent with the expected ecological roles and phylogenetic grouping of most species, however this hypothesis needs to be further explored at lower taxonomic levels to identify detailed patterns.

Although we only had one sample of the snake species *Atractaspis engaddensis*, this study was able to show its predation on Scincidae, similar to what was observed for its congeneric species in Nigeria, based on classical stomach content analysis (Akani et al., 2001), and previous reports on the species (Kochva, 2002). Conversely, a recent study that analysed the seasonal diet of *Atractaspis engaddensis* in the region, by examining more than 50 stomach contents, described small rodents, geckos, worm lizards, and snakes as the main prey items (Al-Sadoon et al., 2020), was not able to detect skinks. This highlights the usefulness of rapid metabarcoding sampling of inconspicuous species using non-invasive methods even with only punctual surveys, or small sample sizes (Gil et al., 2020; Neves et al., 2022). It also points to the fact that the species feeds opportunistically on locally and seasonally available prey items and does not specialize in specific groups.

This study demonstrates the potential of metabarcoding approaches associated with non-invasive sampling for conservation management. Through this method, it was possible to obtain an accurate representation of the diet of the Wadi Ashar reptile community by detecting a wider range of taxa than previous classic approaches that are unable to detect soft, small prey items (Taberlet et al., 2012). Additionally, this technique delivered rapid and holistic results on species composition, diversity, and ecological networks, among others, at relatively low costs (Taylor and Gemmell, 2016). Moreover, metabarcoding can provide a large amount of data in a short period, which is of great help to increase the success of biodiversity conservation actions by responsible institutions (Ji et al., 2013), especially in arid areas that are difficult to access and require urgent action to guide sustainable management on predicted anthropogenically highly-impacted areas, like the study area. Furthermore, accessing diet composition through faecal DNA is particularly advantageous as samples can be collected with minimal impact on individuals (De Barba et al., 2014; Pompanon et al., 2012) what is of particular importance for the Wadi Ashar community, which has already been impacted by anthropogenic activities. However, some constraints may have limited our results and need to be considered in future diet studies in arid ecosystems. Despite the potential of metabarcoding, some methodological limitations need to be taken into account. For instance, when faecal samples are used, the extracted DNA is often degraded which affects amplification and sequencing efficiency (Pompanon et al., 2012). Furthermore, variation in prey digestibility rates and primer bias can lead to over or underrepresentation of prey species (Deagle et al., 2014; Pinho et al., 2022; Pompanon et al., 2012). The lack of comprehensive and up-to-date reference databases of potential prey DNA can difficult species assignments and lead to incorrect or low taxonomic identification (Cuff et al., 2022; Nielsen et al., 2018). An attempt was made to address this shortfall by building a reference database for Wadi Ashar plants, resulting in a notable enhancement of taxonomic resolution. However, for arthropods this was not possible and needs to be considered in the future as well as a multiple

primer approach to increase detection power within this group (Corse et al., 2010). Besides, adequate sample sizes are important to obtain representative diet data. From the 83 individuals that were caught by hand, we were only able to collect faecal samples from 56. This may be related to the low availability and abundance of prey items which can lead to less feeding frequency and, consequently, to low faeces production. Moreover, small reptiles, particularly *Tropiocolotes yomtovi*, are extremally difficult to sample due to their small dimensions and minute faecal pellets, which led to the successful amplification of only one sample for *Tropiocolotes yomtovi* from the seven collected. Yet, with only one sample it was possible to disclose the plant component in the diet of this species, highlighting the power of metabarcoding approaches for diet characterization. In addition, this study was carried out in only one season, so it was not possible to test for seasonal variations in the diet of the community. Besides the presented biases this study provides a basis for long-term monitoring projects of these species that should be implemented in the future.

This study shows that the reptile community at Wadi Ashar is diverse and of great ecological value but may be negatively affected by habitat degradation due to recent construction work in the area. Several plants directly related to anthropogenic activities were found in the diet of the community, such as plants from the families Arecaceae, Musaceae, Oleaceae and Vitaceae and *Festuca* sp. which is often used as a supplementary feed for camels. This emphasizes the potential impact of human disturbance on the natural diet of reptiles. It is demonstrated that anthropogenic-induced deteriorated areas can have an immense impact on the body condition of reptiles (Amo et al., 2007). Consequently, the development of sustainable conservation measures to mitigate the recent human impact in the area is crucial. This study also demonstrates the importance of plant items in the diet of the reptile community, thus, it would be also recommended to fence off some areas with different soil types, protecting vegetation from overgrazing and increasing its diversity, density, and cover (Ayyad and El-Kadi, 1982; Shaltout et al., 2021). This will create different refuges for some reptile species that depend on plants and can also benefit other vertebrate and arthropod species, as well as plant species that are mostly affected by grazers. Moreover, during fieldwork it was observed that due to the recent construction work there is a lot of rubble scattered along the area which is used as sub-optimal refuge sites by several reptiles, including venomous snakes, affecting their fitness, and increasing the risks of accidents with humans. The presence of optimal refuges, such as trees, bushes, rocks, and burrows is essential for efficient thermoregulation, protection against predators (Bauwens et al., 1996; Pereira et al., 2019) and to limit access to human settlements when searching for cover to avoid high temperatures. For that reason, we alert local authorities for the critical necessity to remove debris and place several large rocks and/or piles of smaller rocks, in areas away from tourist accommodation, to mitigate the impact on fitness and promote an increase in the abundance and diversity of reptiles. Finally, it would be beneficial for local authorities to take advantage of tourist visitors to develop educational activities to raise awareness of the links between the reptile community and other elements of fauna and flora. Thus, demonstrating their importance to the ecosystem through the control of micromammal and arthropod populations, as predators and as pollinators and seed dispersers (Miranda, 2017). These public engagement actions have been proven to increase positive attitudes towards reptiles and their conservation (Fonseca et al., 2021). Some of the Wadi Ashar resorts are very close to natural areas with cliffs and dunes and it would be easy to spot some geckos with headlights and provide visitors with unique guided walks to show Wadi Ashar's natural heritage.

In conclusion, our study highlights the importance of community assessments and the power of combining these with non-invasive DNA metabarcoding to accurately assess biodiversity and its feeding habits, revealing unknown ecological interactions of often neglected groups, such as reptiles. This information can be important for understanding the ecology of some of the more inconspicuous and understudied species, and for learning about the taxonomic groups that are most important to the diet of this community. Uncovering the trophic networks in a community can help to shift the perspective of conservation efforts from focusing solely on specific species to a more holistic approach (García and Vasconcelos, 2017), in this study highlighting that vegetation restoration could benefit not only reptiles but the entire ecosystem.

Declaration of Competing Interest

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Data availability

Datasets supporting this research can be accessed through NCBI BioProject PRJNA1018879 and GenBank accession codes OR573700–OR573791 and OR616286–OR616362.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2023.e02667.

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Supplementary Material Article VI

Table S1. List of herpetofauna samples used in this study. The taxa and tissue sample codes, sampled square (check Fig. 1) and GPS coordinates of origin (latitude, Lat, and longitude, Lon), snout-vent length (SVL) in mm, indication of collection of tissue and faecal samples and GenBank accession numbers are given.

Family	Taxa	Code	Sample code	Square	Lat	Lon	SVL (mm)	Tissue	GenBank	Pellet
Agamidae	*Laudakia vulgaris*	LAvu	CN20279	C1	26.730	37.840	-	No	-	Yes
Agamidae	*Laudakia vulgaris*	LAvu	CN20280	C1	26.732	37.842	-	No	-	Yes
Agamidae	*Laudakia vulgaris*	Lavu	CN20282	A1	26.754	37.850	-	No	-	Yes
Atractaspididae	*Atractaspis engaddensis*	ATen	CN20291	A1	26.751	37.849	-	Yes	-	Yes
Colubridae	*Spalerosophis diadema*	SPdi	CN20261	B4	26.749	37.879	-	No	-	No
Gekkonidae	*Bunopus tuberculatus*	BUtu	CN20268	B2	26.738	37.844	32.96	Yes	OR616293	Yes
Gekkonidae	*Hemidactylus granosus*	HMgr	CN20213	C4	26.733	37.884	45.08	Yes	OR616300	No
Gekkonidae	*Stenodactylus doriae*	STdo	CN20220	C3	26.728	37.875	57.95	Yes	OR616341	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20230	C3	26.727	37.874	68.98	Yes	OR616342	No
Gekkonidae	*Stenodactylus doriae*	STdo	CN20242	A3	26.757	37.872	27.76	Yes	-	No
Gekkonidae	*Stenodactylus doriae*	STdo	CN20244	C1	26.725	37.849	45.68	Yes	OR616343	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20246	C1	26.726	37.848	57.85	Yes	OR616344	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20284	A1	26.751	37.850	34.12	Yes	OR616345	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20290	A1	26.754	37.849	65.19	Yes	OR616346	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20306	B4	26.745	37.876	52.72	Yes	OR616347	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20307	B4	26.745	37.876	68.64	Yes	OR616348	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20308	B4	26.746	37.876	60.91	Yes	OR616349	No
Gekkonidae	*Stenodactylus doriae*	STdo	CN20309	B4	26.743	37.874	59.47	Yes	OR616350	Yes
Gekkonidae	*Stenodactylus doriae*	STdo	CN20312	B4	26.748	37.877	38.49	Yes	OR616351	No
Gekkonidae	*Stenodactylus doriae*	STdo	CN20313	B4	26.748	37.877	66.70	Yes	OR616352	No
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20210	A4	26.764	37.890	22.64	Yes	OR616355	No
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20222	A2	26.757	37.858	24.58	Yes	OR616356	Yes
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20225	A2	26.757	37.861	21.9	Yes	OR616357	Yes
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20226	A2	26.757	37.861	19.12	Yes	OR616358	Yes
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20233	C2	26.729	37.856	27.13	Yes	OR616359	Yes
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20241	A3	26.758	37.872	31.61	Yes	-	No
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20251	C1	26.727	37.847	-	No	-	No
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20270	B2	26.738	37.844	-	Yes	OR616360	Yes
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20275	A2	26.757	37.852	24.97	Yes	OR616361	Yes
Gekkonidae	*Tropiocolotes yomtovi*	TRyo	CN20276	A2	26.757	37.852	23.50	Yes	OR616362	Yes
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20217	C3	26.733	37.880	61.81	Yes	OR616286	Yes
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20219	C3	26.730	37.879	-	No	-	No
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20229	C3	26.731	37.878	56.80	Yes	OR616287	Yes
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20232	B4	26.749	37.881	62.32	Yes	OR616288	Yes

Family	Taxa	Code	Sample code	Square	Lat	Lon	SVL (mm)	Tissue	GenBank	Pellet
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20235	C2	26.730	37.854	-	No	-	No
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20256	B4	26.746	37.878	-	No	-	No
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20257	B4	26.746	37.878	71.56	Yes	OR616289	Yes
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20259	B4	26.749	37.879	44.32	Yes	OR616290	Yes
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20262	B3	26.740	37.860	71.12	Yes	OR616291	Yes
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20264	C4	26.730	37.860	-	No	-	No
Lacertidae	*Acanthodactylus boskianus*	ACbo	CN20315	B4	26.749	37.882	46.78	Yes	OR616292	Yes
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20205	B3	26.742	37.859	36.60	Yes	OR616307	No
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20207	A4	26.764	37.890	68.50	Yes	OR616308	Yes
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20208	A4	26.764	37.890	73.40	Yes	OR616309	No
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20209	A4	26.764	37.890	53.54	Yes	OR616310	Yes
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20248	C1	26.726	37.847	40.81	Yes	OR616311	No
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20258	B4	26.744	37.880	-	Yes	OR616312	No
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20266	B2	26.745	37.844	46.24	Yes	OR616313	Yes
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20310	B4	26.746	37.872	71.95	Yes	OR616314	Yes
Phyllodactylidae	*Ptyodactylus* sp1 x sp3	PTsp1x3	CN20311	B4	26.746	37.872	56.75	Yes	OR616315	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20231	C3	26.728	37.867	59.19	Yes	OR616316	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20239	A3	26.758	37.873	81.19	Yes	OR616317	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20243	A3	26.756	37.871	26.62	Yes	-	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20245	C1	26.725	37.850	38.68	Yes	OR616318	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20247	C1	26.727	37.847	38.88	Yes	OR616319	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20252	C2	26.733	37.857	48.54	Yes	OR616320	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20253	C2	26.729	37.855	62.87	Yes	OR616321	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20254	C2	26.732	37.856	-	No	-	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20255	B4	26.748	37.878	62.32	Yes	OR616322	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20267	B2	26.739	37.842	-	Yes	OR616323	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20269	B2	26.739	37.841	56.91	Yes	OR616324	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20271	B2	26.738	37.844	49.37	No	-	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20272	B2	26.738	37.844	50.50	Yes	OR616325	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20273	A2	26.756	37.853	60.48	Yes	OR616326	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20274	A2	26.755	37.852	80.78	Yes	OR616327	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20285	A1	26.754	37.849	41.90	Yes	OR616328	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20286	A1	26.752	37.848	70.88	Yes	OR616329	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20287	A1	26.752	37.848	60.25	Yes	OR616330	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20288	A1	26.752	37.848	58.57	Yes	OR616331	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20289	A1	26.754	37.848	-	Yes	OR616332	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20292	B1	26.738	37.833	-	Yes	OR616333	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20293	B1	26.738	37.836	76.13	Yes	OR616334	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20294	B1	26.738	37.836	68.03	Yes	OR616335	No
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20295	B1	26.738	37.833	52.37	Yes	OR616336	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20296	B1	26.738	37.833	79.26	Yes	OR616337	Yes
Phyllodactylidae	*Ptyodactylus* sp1	PTsp1	CN20314	B4	26.742	37.873	75.37	Yes	OR616338	Yes

Family	Taxa	Code	Sample code	Square	Lat	Lon	SVL (mm)	Tissue	GenBank	Pellet
Psammophiidae	*Psammophis schokari*	PSsc	CN20260	B4	26.749	37.879	67.40	Yes	-	No
Scincidae	*Chalcides ocellatus*	CHoc	CN20212	C4	26.733	37.884	85.47	Yes	OR616294	Yes
Scincidae	*Chalcides ocellatus*	CHoc	CN20214	C4	26.733	37.884	80.05	Yes	OR616295	Yes
Scincidae	*Chalcides ocellatus*	CHoc	CN20215	C4	26.733	37.884	85.00	Yes	OR616296	Yes
Scincidae	*Chalcides ocellatus*	CHoc	CN20216	C4	26.733	37.884	89.22	Yes	OR616297	Yes
Scincidae	*Chalcides ocellatus*	CHoc	CN20265	C4	26.730	37.884	-	Yes	OR616298	Yes
Scincidae	*Scincus conirostris*	SCco	CN20332	B4	26.748	37.877	84.46	Yes	OR616339	Yes
Scincidae	*Scincus conirostris*	SCco	CN20334	B4	26.748	37.877	79.27	Yes	OR616340	Yes
Scincidae	*Trachylepis brevicollis*	TCbr	CN20316	B4	26.749	37.881	122.22	Yes	OR616353	Yes
Scincidae	*Trachylepis brevicollis*	TCbr	CN20317	B4	26.749	37.881	117.14	Yes	OR616354	No
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	CN20221	A2	26.758	37.857	23.69	Yes	OR616301	Yes
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	CN20227	A2	26.758	37.861	24.66	Yes	OR616302	Yes
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	CN20238	A3	26.758	37.871	25.50	Yes	OR616303	No
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	CN20240	A3	26.758	37.872	23.76	Yes	OR616304	No
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	CN20277	B1	26.737	37.831	28.52	Yes	OR616305	No
Sphaerodactylidae	*Pristurus guweirensis*	PRgu	CN20278	B1	26.735	37.831	26.77	Yes	OR616306	Yes
Viperidae	*Echis coloratus*	ECco	CN20206	C1	26.727	37.844	-	Yes	OR616299	Yes

Table S2. List of the molecular operational taxonomic units (MOTUs) identified in the diet of reptiles to the maximum taxonomic resolution. MOTU's frequency of occurrence (FO) in the overall diet of the reptile community of Wadi Ashar is given. NI stands for non-identified. Please check Table 1 for correspondence of taxa codes.

Phyllum	Class	Order	Family	Final MOTUs ID	FO	Taxa code
Tracheophyta	Liliopsida	Arecales	Arecaceae	Arecaceae_1	0.03	CHoc
		Poales	Poaceae	Poaceae_1	0.03	PTsp1x3
				Poaceae_2	0.03	LAvu
				Centropodia_1	0.03	ACbo
				Festuca_1	0.03	PTsp1
				Schismus barbatus	0.03	ACbo
		Zingiberales	Musaceae	*Musa* sp	0.09	CHoc; SCco; STdo
	Magnoliopsida	Asterales	Asteraceae	Asteraceae_1	0.13	ACbo; CHoc; LAvu; SCco
				Asteraceae_2	0.03	ACbo
				Asteraceae_3	0.03	ACbo
				Asteraceae_4	0.03	LAvu
		Brassicales	Brassicaceae	Brassicaceae_1	0.03	ACbo
				Brassicaceae_2	0.03	ACbo
				Nasturtium_1	0.03	ACbo
			Resedaceae	*Ochradenus baccatus*	0.09	LAvu; TRsp
		Caryophyllales	Caryophyllaceae	*Gymnocarpos decandrus*	0.03	PTsp1
		Fabales	Fabaceae	*Astragalus spinosus*	0.03	LAvu
				Medicago_1	0.03	PTsp1x3
				Vachellia_1	0.25	CHoc; LAvu; PRsp1; PTsp1
		Gentianales	Apocynaceae	*Pergularia tomentosa*	0.03	LAvu
		Lamiales	Oleaceae	Oleaceae_1	0.03	CHoc
			Orobanchaceae	*Cistanche phelypaea*	0.03	ACbo
			Plantaginaceae	Plantaginaceae_1	0.06	ACbo
		Myrtales	Lythraceae	Lythraceae_1	0.03	CHoc
		Rosales	Rhamnaceae	Rhamnaceae_1	0.03	CHoc
		Solanales	Convolvulaceae	*Convolvulus bushchiricus*	0.06	CHoc; PTsp1x3
			Solanaceae	*Lycium shawii*	0.03	PTsp1
		Vitales	Vitaceae	Vitis_1	0.03	CHoc
		Zygophyllales	Zygophyllaceae	Fagonia_1	0.03	ACbo
Arthropoda	Arachnida	Araneae	NI	Araneae_1	0.03	CHoc
			Araneidae	Araneidae_1	0.03	TRsp
			Salticidae	Salticidae_1	0.03	PRsp1
				Salticidae_2	0.06	PTsp1x3

Phyllum	Class	Order	Family	Final MOTUs ID	FO	Taxa code
				Salticidae_3	0.03	PTsp1x3
				Salticidae_4	0.03	PRsp1
	Insecta	Blattodea	NI	Blattodea_1	0.03	PTsp1x3
				Blattodea_2	0.03	STdo
			Ectobiidae	Blattella_1	0.09	CHoc; PTsp1x3
		Coleoptera	NI	Coleoptera_1	0.16	LAvu; PTsp1; SCco; STdo
			Curculionidae	Curculionidae_1	0.03	STdo
			Laemophloeidae	Laemophloeidae_1	0.03	PTsp1
			Lampyridae	Lampyridae_1	0.03	PRsp1
			Tenebrionidae	Gonocephalum_1	0.03	PTsp1
				Pimelia arabica	0.16	LAvu; PTsp1; TRbr
				Tenebrionidae_1	0.03	PTsp1
				Trachyderma philistinum	0.03	TRbr
		Diptera	NI	Diptera_1	0.06	LAvu; PTsp1
				Diptera_2	0.03	PTsp1
			Asilidae	Asilidae_1	0.03	ACbo
			Tephritidae	Tephritidae_1	0.03	PRsp1
		Hemiptera	NI	Hemiptera_1	0.03	PTsp1
			Delphacidae	Metadelphax_1	0.03	ACbo
		Hymenoptera	NI	Hymenoptera_1	0.03	PTsp1x3
			Formicidae	Formicidae_1	0.09	ACbo; PRsp1
				Formicidae_2	0.03	ACbo
				Camponotus_1	0.09	ACbo; CHoc; PTsp1x3
				Messor_1	0.06	Acbo; PTsp1x3
				Monomorium pharaonis	0.13	ACbo; PRsp1; PTsp1; STdo
		Lepidoptera	NI	Lepidoptera_1	0.03	STdo
				Lepidoptera_2	0.03	PTsp1
			Crambidae	Loxostege_1	0.03	PTsp1x3
			Erebidae	Erebidae_1	0.03	STdo
			Gelechiidae	*Tuta absoluta*	0.03	PTsp1x3
			Hesperiidae	Hesperiidae_1	0.03	PTsp1
			Noctuidae	Noctuidae_1	0.03	STdo
				Noctuidae_2	0.03	STdo
			Pieridae	Pieridae_1	0.03	ACbo
			Pyralidae	Pyralidae_1	0.03	PTsp1x3
				Pyralidae_2	0.03	PTsp1

Phyllum	Class	Order	Family	Final MOTUs ID	FO	Taxa code
			Tortricidae	Tortricidae_1	0.03	PTsp1
		Mantodea	NI	Mantodea_1	0.03	STdo
			Eremiaphilidae	Eremiaphila_1	0.03	STdo
			Rivetinidae	Rivetinidae_1	0.03	STdo
		Orthoptera	Acrididae	Dociostaurus_1	0.03	ACbo
				Ochrilidia_1	0.03	ACbo
			Gryllidae	Gryllus_1	0.06	PTsp1x3; Stdo
		Phasmida	Phasmatidae	Phasmatidae_1	0.06	PTsp1
		Zygentoma	Lepismatidae	Lepismatidae_1	0.03	PTsp1x3
				Thermobia domestica	0.06	PTsp1x3
Chordata	Reptilia	Squamata	Scincidae	*Scincus conirostris*	0.03	Aten

Table S3. List of the orders identified in the diet of each reptile of the Wadi Ashar community. Frequency of occurrence of each order is given for the overall diet of the reptile community (Total) and for each sampled reptile species. Please check Table 1 for correspondence of taxa codes.

			Total	LAvu	ATen	STdo	Tryo	ACbo	PTsp1x3	PTsp1	CHoc	SCco	TCbr	PRgu
Phyllum	**Class**	**Order**												
Tracheophyta	Liliopsida	Arecales	0.03	-	-	-	-	-	-	-	0.25	-	-	-
		Poales	0.16	0.33	-	-	-	0.40	0.33	0.14	-	-	-	-
		Zingiberales	0.09	-	-	0.25	-	-	-	-	0.25	1.00	-	-
	Magnoliopsida	Asterales	0.16	0.33	-	-	-	0.40	-	-	0.25	1.00	-	-
		Brassicales	0.13	0.67	-	-	1.00	0.20	-	-	-	-	-	-
		Caryophyllales	0.03	-	-	-	-	-	-	0.14	-	-	-	-
		Fabales	0.28	1.00	-	-	-	-	0.33	0.43	0.25	-	-	0.50
		Gentianales	0.03	0.33	-	-	-	-	-	-	-	-	-	-
		Lamiales	0.13	-	-	-	-	0.60	-	-	0.25	-	-	-
		Myrtales	0.03	-	-	-	-	-	-	-	0.25	-	-	-
		Rosales	0.03	-	-	-	-	-	-	-	0.25	-	-	-
		Solanales	0.09	-	-	-	-	-	0.33	0.14	0.25	-	-	-
		Vitales	0.03	-	-	-	-	-	-	-	0.25	-	-	-
		Zygophyllales	0.03	-	-	-	-	0.20	-	-	-	-	-	-
Arthropoda	Arachnida	Araneae	0.19	-	-	-	1.00	-	0.67	-	0.25	-	-	1.00
	Insecta	Blattodea	0.16	-	-	0.25	-	-	0.67	-	0.50	-	-	-
		Coleoptera	0.41	1.00	-	0.50	-	-	-	0.71	-	1.00	1.00	0.50
		Diptera	0.16	0.33	-	-	-	0.20	-	0.29	-	-	-	0.50
		Hemiptera	0.06	-	-	-	-	0.20	-	0.14	-	-	-	-
		Hymenoptera	0.38	-	-	0.25	-	0.80	1.00	0.14	0.25	-	-	1.00
		Lepidoptera	0.31	-	-	0.75	-	0.20	1.00	0.43	-	-	-	-
		Mantodea	0.03	-	-	0.25	-	-	-	-	-	-	-	-
		Orthoptera	0.09	-	-	0.25	-	0.20	0.33	-	-	-	-	-
		Phasmida	0.06	-	-	-	-	-	-	0.29	-	-	-	-
		Zygentoma	0.06	-	-	-	-	-	0.67	-	-	-	-	-
Chordata	Reptilia	Squamata	0.03	-	1.00	-	-	-	-	-	-	-	-	-

Table S4. List of plant samples collected for the DNA reference collection of possible food items. Their taxonomic identification, sample code, GPS coordinates of origin (latitude, Lat, and longitude, Lon), and GenBank accession numbers for both markers used are given.

Family	Taxa	Sample code	Lat	Lon	GenBank	
					TrnL e-f	TrnL c-d
Amaranthaceae	*Haloxylon salicornicum*	CN20362	26.727	37.847	OR573746	OR573700
Poaceae	*Stipagrostis ciliata*	CN20363	26.728	37.867	OR573747	OR573701
Caryophyllaceae	*Polycarpaea repens*	CN20364	26.727	37.847	OR573748	OR573702
Zygophyllaceae	*Fagonia* sp.	CN20365	26.743	37.858	OR573749	OR573703
Asteraceae	*Atractylis mernephthae*	CN20366	26.727	37.847	OR573750	OR573704
Fabaceae	*Senna italica*	CN20367	26.748	37.878	OR573751	OR573705
Zygophyllaceae	*Fagonia bruguieri*	CN20372	26.712	26.712	OR573752	OR573706
Ephedraceae	*Ephedra foliata*	CN20373	26.757	37.798	OR573753	OR573707
Lamiacea	*Ballota undulata*	CN20374	26.734	37.867	OR573754	OR573708
Poaceae	*Schismus barbatus*	CN20375	26.727	37.867	OR573755	OR573709
Brassicaceae	*Farsetia aegyptiaca*	CN20376	26.731	37.878	OR573756	OR573710
Brassicaceae	*Farsetia longisiliqua*	CN20377	26.757	37.867	OR573757	OR573711
Poaceae	*Panicum turgidum*	CN20382	26.738	37.836	OR573758	OR573712
Cucurbitaceae	*Citrullus colocynthis*	CN20383	26.748	37.878	OR573759	OR573713
Resedaceae	*Ochradenus baccatus*	CN20385	26.748	37.878	OR573760	OR573714
Asteraceae	*Pulicaria incisa*	CN20386	26.743	37.858	OR573761	OR573715
Acanthaceae	*Blepharis ciliaris*	CN20387	26.712	37.857	OR573762	OR573716
Poaceae	*Stipagrostis plumosa*	CN20392	26.728	37.867	OR573763	OR573717
Apocynaceae	*Pergularia tomentosa*	CN20395	26.744	37.845	OR573764	OR573718
Zygophyllaceae	*Fagonia arabica*	CN20396	26.744	37.845	OR573765	OR573719
Poaceae	*Centropodia forskaolii*	CN20397	26.723	37.831	OR573766	OR573720
Poaceae	*Stipagrostis* sp.	CN20402	26.723	37.831	OR573767	OR573721
Zygophyllaceae	*Seetzenia lanata*	CN20403	26.731	37.878	OR573768	OR573722
Apocynaceae	*Rhazya stricta*	CN20404	26.712	37.857	OR573769	OR573723
Amaranthaceae	*Aerva javanica*	CN20405	26.738	37.836	OR573770	OR573724
Lamiaceae	*Teucrium polium*	CN20406	26.754	37.850	OR573771	OR573725
Lamiaceae	*Lavandula coronopifolia*	CN20407	26.754	37.850	OR573772	OR573726
Cistaceae	*Helianthemum lippii*	CN20412	26.749	37.878	OR573773	OR573727
Asteraceae	*Asteriscus hierochunticus*	CN20413	26.731	37.878	OR573774	OR573728
Solanaceae	*Lycium shawii*	CN20414	26.738	37.836	OR573775	OR573729
Malvaceae	*Hibiscus deflersii*	CN20415	26.744	37.845	OR573776	OR573730
Brassicaceae	*Zilla spinosa*	CN20416	26.738	37.836	OR573777	OR573731
Fabaceae	*Crotalaria aegyptiaca*	CN20417	26.741	37.850	OR573778	OR573732
Geraniaceae	*Monsonia nivea*	CN20422	26.741	37.850	OR573779	OR573733
Asphodelaceae	*Asphodelus tenuifolius*	CN20423	26.738	37.833	OR573780	OR573734
Fabaceae	*Retama raetam*	CN20424	26.738	37.833	OR573781	OR573735
Fabaceae	*Vachellia tortilis* subsp. *raddiana*	CN20425	26.741	37.850	OR573782	OR573736
Boraginaceae	*Heliotropium digynum*	CN20426	26.743	37.840	OR573783	OR573737
Neuradaceae	*Neurada procumbens*	CN20427	26.738	37.862	OR573784	OR573738
Convolvulaceae	*Convolvulus bushiricus*	CN20432	26.749	37.878	OR573785	OR573739
Nyctaginaceae	*Boerhavia sp.*	CN20433	26.732	37.842	OR573786	OR573740
Asteraceae	*Anvillea garcinii*	CN20435	26.732	37.842	OR573787	OR573741
Fabaceae	*Vachellia tortilis*	CN20436	26.738	37.836	OR573788	OR573742
Caryophyllaceae	*Gymnocarpos sclerocephalus*	CN20437	26.749	37.878	OR573789	OR573743
Amaranthaceae	*Anabasis setifera*	CN20438	26.731	37.878	OR573790	OR573744
Amaranthaceae	*Suaeda vermiculata*	CN20439	26.735	37.876	OR573791	OR573745

Chapter 5

General Discussion

5.1. Main results and conclusions

This thesis provides important insights into the mechanisms of species coexistence in arid environments and the dietary and morphological adaptations behind them. It also provides new biodiversity records, a new understanding of the ecology of previously poorly studied and/ or threatened species from remote areas, discusses possible threats that can contribute to increased species extinction risk, and highlights the negative impact of invasive mammalian species within insular ecosystems. Ultimately, this thesis offers relevant information for the development and implementation of adequate management strategies in arid, remote and insular areas.

For most of the species included in this work, this is the first comprehensive study of their dietary composition. For reptiles in particular, most previous studies have been based on anecdotal observations (e.g., Alexander, 1898; Schleich, 1982, 1987) or morphological analysis of faeces and stomach contents (e.g., Al-Sadoon *et al.*, 2020; Ibrahim & El-Naggar, 2013; Mateo *et al.*, 2016). Therefore, this work significantly contributes to filling the existing gap in molecular diet studies of reptiles, which is particularly evident compared to more extensively studied vertebrate groups (Ando *et al.*, 2020). The use of DNA metabarcoding allied with the use of faecal samples not only allowed the identification of a wider range of prey taxa, some new for the study areas (Article III), with finer resolution compared to traditional methods, but also ensured minimal impact on individual fitness (Ferreira *et al.*, 2018). This is particularly important in the context of this thesis, where several of the study species are threatened with no conservation action plans. It also allowed faster results (Gil *et al.*, 2020) that are essential when governmental authorities need information on invasive species with unknown impacts. For example, Tercel *et al.* (2022) showed that the diet of a threatened endemic Telfair's skink from Round Island was dominated by introduced species, suggesting important ecological implications and potential adaptation of skinks to these species as a replacement for lost native prey due to habitat destruction. Similarly, introduced plant species on a Japanese oceanic island have been shown to be very important in the diet of the Critically Endangered red-headed wood pigeon, so hasty eradication of these invasives would have a major impact on this species (Ando *et al.*, 2013). Therefore, understanding the feeding ecology of threatened and invasive species is crucial to designing and implementing accurate conservation strategies (Nordstrom *et al.*, 2022).

Furthermore, the metabarcoding approach revealed unexpected dietary components, such as vertebrates in the diets of *Tarentola raziana* and *Chioninia* species (Articles I and II), and the consumption of plants by species previously characterised as exclusively

insectivorous (Article VI). Regarding vertebrates, the ingestion of seabird remains is a confirmed behaviour for *T. gigas* (Lopes *et al.*, 2019; Pinho *et al.*, 2018) and has been observed in other reptiles from other insular systems such as the Selvagens and the Azores (Alcover & McMinn, 1994; Matias *et al.*, 2009; Neves *et al.*, 2022). Vertebrate remains could be ingested involuntarily when feeding on invertebrates present in bird regurgitations and faeces (Cuff *et al.*, 2022; Tercel *et al.*, 2021). In respect to plants, both could be cases of secondary or inadvertent consumption since plant DNA can be detected through the consumption of phytophagous arthropods. However, an increase in plant consumption by insular reptiles is also a common behaviour (Van Damme, 1999), which can be observed for instance in several *Podarcis* species of the Mediterranean islands (Brock *et al.*, 2014; Pérez-Mellado & Corti, 1993). Hence, as the presence of samples containing only plant or vertebrate DNA was confirmed in the presented studies, a common pattern of peculiar diets in insular systems was highlighting with this work.

The potential of DNA metabarcoding for dietary characterisation was further highlighted when old and degraded digestive contents from museum specimens were used to effectively describe the diet of the emblematic extinct Cabo Verde giant skink and discuss its extinction drivers (Article IV). In addition, this thesis highlights the importance of considering appropriate samples when investigating specific ecological questions, as variations in dietary estimates were observed depending on the gut section analysed, attributed to differences in the digestibility of invertebrate prey and plant items (Article V). Furthermore, a deeper understanding of the trophic networks of the species studied, and consequently of their ecology and their role within the community, has been facilitated.

Some similar ecological and evolutionary patterns were observed across the reptile species and scenarios analysed. This work has shown that larger reptile species tend to include more plants in their diet and that this is often underestimated when using classical dietary characterisation methods. Although plant material is more difficult to digest (Holland *et al.*, 2020), it may be more accessible in arid ecosystems, such as those in Cabo Verde and Saudi Arabia, where arthropod abundance tends to be low. This is expected since larger reptiles usually hold morphological adaptations that facilitate the digestion of plant items (Dearing, 1993; Van Damme, 1999), facilitating their higher intake. These adaptations certainly reflect the evolutionary responses to optimise energy intake in resource-limited environments (Sagonas *et al.*, 2015). This is exemplified by the significant positive relationship between the length of the small intestine and body size in *T. gigas*, which is not observed in the smaller *T. raziana* (Article I), and the dietary partitioning of the Wadi Ashar reptile community into body size

categories, with larger species being associated with a primary consumption of plants (Article VI). Consequently, these reptiles may play an important ecological role as seed dispersers and/ or pollinisers, contributing to the structuring of plant communities in arid, remote and insular habitats as the ones presented (Miranda, 2017; Nogales *et al.*, 2016).

Character displacement has previously been suggested to partially explain these discrepancies for both *Tarentola* and *Chioninia* genera (Carranza *et al.*, 2000, 2001), but this thesis represents the first time that this question has been explored in detail. In the studied cases, the evident differences in body size observed in sympatric species pairs are a clear manifestation of historical competitive interactions (Connell, 1980) that have caused them to diverge morphologically in opposite directions from their common ancestor. While *T. gigas* and *C. vaillantii* developed large body sizes, *T. raziana* and *C. delallandi* body sizes were reduced and maintained, respectively. The observed differences in head morphology of the smaller sympatric species compared to allopatric populations provide support for the character displacement hypothesis. These morphological variations, combined with significant differences in diet composition between sympatric species compared to allopatric populations, suggest that dietary niche segregation is a mechanism that allows stable coexistence between *T. gigas* and *T. raziana*, and between *C. delalandii* and *C. vaillantii* (du Preez *et al.*, 2017; Razgour *et al.*, 2011; Westeen *et al.*, 2023). Similar patterns can be observed for instance on Dominica Island where the native *Anolis oculatus* (Cope, 1879) shows significant morphological and habitat variation when in sympatry with the recently introduced invasive *Anolis cristatellus* Duméril & Bibron, 1837 (Dufour *et al.*, 2017).

Additionally, this work revealed how reduced and isolated areas can drive diet and/ or morphological (Articles I and II). Both sympatric species pairs on the small islets of Raso and Cima presented generalist habits and more diverse diets when compared to the allopatric population on Santa Luzia and Fogo, feeding on plants, invertebrates and even vertebrates. On both islets, *Tarentola* and *Chioninia* species coexist within a context of limited resource availability, where preferred food items are very restricted in abundance and area. Such a situation would select for individuals to expand their diet to include previously overlooked prey items (Bonesi *et al.*, 2004), leading to niche expansion as a strategy to decrease competition. These findings align with the optimal foraging theory, which indicates that a decrease in the availability of preferred resources is associated with an expansion of the dietary niche breadth (Perry & Pianka, 1997). Morphological differentiation was also more pronounced in the sympatric species of *Chioninia* from Cima compared to Fogo (Article II), and also in Raso, where *T. raziana* differed more from *T. gigas* than the Santa Luzia population (Supplementary Article A), suggesting that

scenarios of increased competitive pressure may accentuate morphological divergence. This is supported by the example of *Podarcis erhardii* (Bedriaga, 1882), introduced to small uninhabited islets in Greece, which showed rapid changes in body size and bite force, indicating intense competition in these restricted area environments (Donihue *et al.*, 2023).

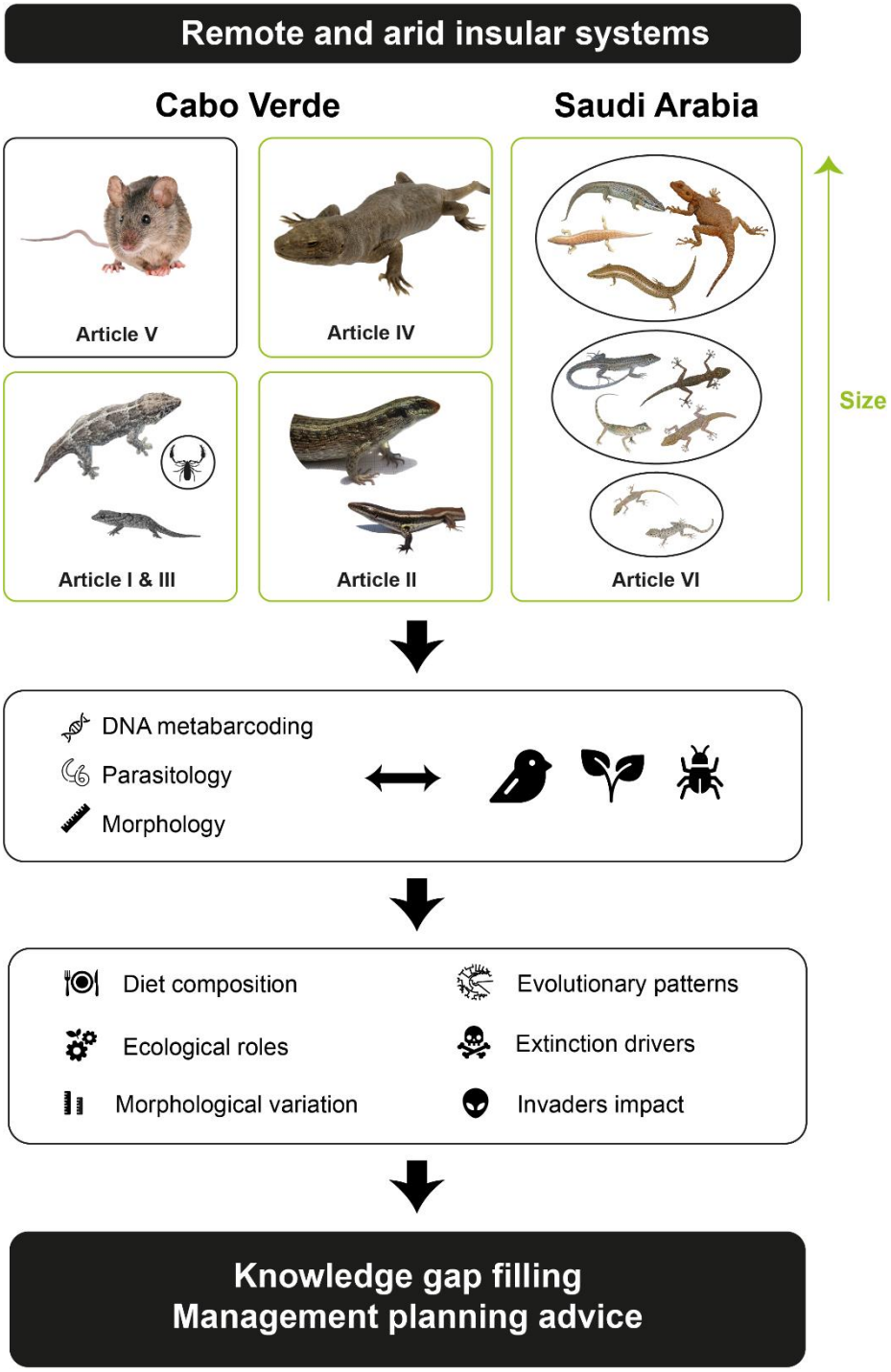

Figure 1. Integrated overview of general discussion of the thesis showing the main results and conclusions.

5.2. Conservation implications

In order to develop effective management strategies, it is essential to collect relevant data on the ecology and evolution of species. The metabarcoding approach used in this work has revealed, at a reasonable taxonomic resolution, a range of prey items that were previously unknown within the diet of the studied reptile species. Such data contributes to a general understanding of the specific roles that these species play within the particular ecosystem they live, and the precise trophic items that are essential for their survival. Or, in the case of invasive species, which are the taxa affected by their introduction into the ecosystem. By highlighting specific trophic relationships, this thesis provides a contribution to the sustainable management of plant, invertebrate and vertebrate communities as a whole. In small and isolated islands, studies such as those executed within this thesis are of particular importance because, due to the limited complexity of trophic networks, the loss of one item can be detrimental to the equilibrium of the whole community.

The data presented in this thesis reveals an urgent need to implement measures to monitor, control and eradicate the house mouse in Cabo Verde. It showed the high negative impact on agriculture and native species of this invasive rodent on populated islands, which could greatly benefit from a rodent control programme (Article V). On the smaller uninhabited islands, the impact on endemic/ native fauna and flora is expected to be more pronounced due to limited resources and extremely high mouse population densities. Although it was not possible to quantify the predation of this invader on the endemic vertebrates, its negative impact on the reptiles is obvious. On Santa Luzia, mice have probably already contributed to the local extirpation of *T. gigas* and the extinction of *C. coctei* (Mateo *et al.*, 2005); nowadays these invaders share activity patterns and refuges with *T. raziana* (Vasconcelos & Pinho, 2021), most likely causing harm and increased competitive pressure to this Near Threatened species. In other island populations in the Caribe, the frequency of tail-break of the West Indian rock iguanas *Cyclura* was significantly higher in the presence of invasive rodents, as a result of failed predation attempts (Hayes *et al.*, 2012). On Cima Islet, mice have most likely contributed to a change to a more aggressive behaviour in the *Chioninia* species (Article II) that may affect the individual fitness (Berger *et al.*, 2007; Calsbeek, 2009; Hayes *et al.*, 2012). For instance, on Dominica Island native *Anolis oculatus* showed an increase in aggressive behaviour when in sympatry with the recently introduced invasive *Anolis cristatellus* (Dufour *et al.*, 2020). However, to our knowledge, this pattern has never been investigated for mice. On populated islands with a strong agricultural presence, such as

Santo Antão, São Vicente and Santiago, the likelihood of eradication failure may be high (Holmes *et al.*, 2015), but the smaller islands of Desertas and Rombo's Islets in Cabo Verde would strongly benefit from these. It has been projected that proper management of invasive species would highly improve the survival prospects of the world's most threatened terrestrial insular vertebrates (Holmes *et al.*, 2019; Spatz *et al.*, 2017). On the Selvagem Grande, house mice and European rabbits have been successfully eradicated and the ecosystem is responding positively with, for instance, an increase in the abundances of the endemic gecko (Olivera *et al.*, 2010).

New knowledge of the ecological needs of the *Tarentola* species (Article I and Supplementary Article A) supports a possible reintroduction of *T. gigas* on Santa Luzia. The limited distribution and ecological needs of *T. raziana* must be taken into account in such reintroduction to avoid wasting resources on unsuccessful outcomes due to competitive exclusion. In addition, the reintroduction of *T. gigas* must be planned in a way that does not interfere with the recent successful reintroduction of the Critically Endangered Raso Lark (Brooke *et al.*, 2020), which is particularly relevant to the diet of this giant gecko on Raso Islet (Lopes *et al.*, 2019; Pinho *et al.*, 2018). Moreover, plans have recently emerged to develop Santa Luzia as a tourist destination, which could have a major impact on its already threatened biodiversity. In this thesis, it is discussed how human activities disturb the reptile community of Wadi Ashar (Saudi Arabia), a tourist destination (Article VI), and how they may have contributed to the extinction of *C. coctei* on Santa Luzia (Article IV), reinforcing how insular delicate ecosystem balance can easily be disrupted (Belsoy *et al.*, 2012). Taking all of this into account, and considering that Santa Luzia Island is a protected Nature Reserve and Wadi Ashar in the buffer zone of an UNESCO Biosphere Reserve, the viability of touristic development plans should be carefully assessed, although those tourism plans provide an economic opportunity for both countries. If not regulated and monitored, tourist activities can lead to habitat destruction with the creation of new infrastructures, the production of waste, and ultimately the alteration of the ecosystems that may lead to the disappearance of unique taxa (Ballantyne & Pickering, 2013; Belsoy *et al.*, 2012). Some characteristics observed in Wadi Ashar for which this thesis recommends the creation of optimal refugia, restoration of vegetation and raising public awareness. This will not only benefit the reptile community, but also the ecosystem as a whole. Furthermore, the increased influx of people also increases the likelihood of the introduction of new exotic species, as has been seen in the islands of the Western Pacific, where tourism has supported populations of introduced rats (Davis *et al.*, 2021).

Finally, insights into the extinction drivers of *C. coctei* (Article IV) are pertinent for formulating conservation strategies for the six extant *Chioninia* species, two of which are classified as Endangered in the IUCN Red List of Threatened Species (Vasconcelos *et al.*, 2013). Given the vulnerability of this taxonomic group, identified as among the most globally threatened taxa in the reptile genera (Mi *et al.*, 2023), understanding extinction drivers is crucial for their conservation in the archipelago.

5.3. Limitations

The global COVID-19 pandemic, which began in the first year of this thesis, represents the single greatest constraint and influence in the development and direction of this thesis, significantly influencing the work plan. As a result of the restrictive measures imposed during the first years of the pandemic, it was simply not possible to undertake the fieldwork within the timeframe, or to the extent, originally planned. Similarly, laboratory processing of pre-existing samples was extremely limited due to access restrictions associated with the pandemic, causing delays in research activities. To meet these challenges, alternative approaches and directions were required, resulting in a different thesis to that initially planned, but within the same overarching framework. Finally, due to COVID restrictions, it proved to not be possible to involve the local Cabo Verdean community, particularly students, in the fieldwork activities as originally planned. This has hampered the possibility of knowledge transfer in both ways by reducing the possibility of getting to know different cultures and share knowledge about these often stigmatised reptile species, which are often the subject of misconceptions.

Regarding fieldwork, both the remoteness and arid nature of the study areas contribute to logistical challenges that manifest themselves in sample size constraints. Small sample sizes can affect the robustness of results by limiting the detection of less common prey species and underestimating the total diversity of prey items (Mata *et al.*, 2019). In addition, dietary studies based on faecal samples provide only a snapshot of the last meal consumed. As diet composition may vary with prey availability, which varies seasonally, it remains to be seen how increased sampling effort over several periods may impact the present understanding of diet of the species. However, these areas are difficult to access and are thus very demanding and costly to sample over several seasons. This could eventually be overcome by involving local students and researchers, but there is sometimes a lack of specialised human resources in these areas and COVID has made this even more difficult. Nevertheless, the use of DNA metabarcoding has

allowed the retrieve of a significant amount of data to be recovered in a relatively short period of time and new taxa to be observed. This is illustrated by the cases of the new records of pseudoscorpions found in the faeces of *T. gigas* (Article III), and the cases of the snake *Atractaspis engaddensis* and the gecko *Tropiocolotes yomtovi*, where only one sample was needed to reveal dietary components previously overlooked by classical methods (Article VI). This further supports the power of this technique even in rapid surveys for understudied taxa in remote areas, which conventional methods cannot accomplish (Gil *et al.*, 2020). Furthermore, the large amount of data provided within a short period can significantly help to increase the success of biodiversity conservation actions (Ji *et al.*, 2013), by meeting the tight time demands of local authorities.

Despite the significant advantages offered by DNA metabarcoding in dietary studies and its broader implications for species conservation, certain challenges persist from the laboratory to bioinformatics. In addition to the challenges mentioned in the general introduction, in the selection of accurate primer sets, quantitative issues and the possible bias caused by errors along the laboratory procedures (e.g., amplification and sequencing errors; Cuff *et al.*, 2022), metabarcoding dietary studies are affected by the lack of reference sequences. The accuracy and taxonomic resolution of taxa identification would substantially improve with comprehensive DNA reference collections. This study initiated the establishment of reference databases of plants and invertebrates for all study areas, which improved the accuracy of dietary characterisation. Nevertheless, more extensive efforts are required to establish reliable and more complete DNA reference collection of the flora and fauna in these understudied regions. Several efforts have been made to describe plant biodiversity in both Cabo Verde (Romeiras *et al.*, 2014, 2015, 2019) and Saudi Arabia (Al-Qthanin *et al.*, 2020), but little genetic information is available for the latter. In addition, little is known about arthropod biodiversity in both areas. Additional surveys are therefore essential to fill these knowledge gaps.

Other constraints that need consideration have emerged as a consequence of this work. When stomach and intestine sections were examined separately, significant differences in composition were found in diet items, with a higher incidence of invertebrates in the stomach and plants in the intestines (Article V). Therefore, dietary studies using faecal samples should take this information into account, as they may overestimate the presence of plants and underestimate the presence of invertebrates, which may be completely absorbed during the digestive process (Tomé, 2013). However, the use of stomach contents or forced regurgitation is not advisable for threatened/ endemic species as it is detrimental to individual fitness (Pérez-Mellado *et al.*, 2011). In addition,

although not mentioned in Article V, an attempt was made to amplify possible vertebrate items from the mouse gut. However, despite using blocking primers specifically designed for the mouse, the sequences obtained were almost exclusively from the host. This was most likely due to the type of samples used and the higher amount of host DNA present in the stomach and intestinal samples when compared to faeces. However, this made it difficult to conclude the impact of this invasive species on the vertebrate fauna of the Cabo Verde Archipelago and should be taken into account in future studies focusing on this issue. The use of blocking primers in metabarcoding studies is very advantageous, as it increases the chance of detecting prey by blocking predator DNA, but it can present some challenges (Vestheim & Jarman, 2008). In addition, the use of these primers inhibits the detection of cases of cannibalism or ingestion of other closely related species, thus preventing the possibility of finding cases of predation in the sympatric species studied (Articles I & II).

To conclude, as technology evolves, ongoing efforts are solving the challenges of DNA metabarcoding. It is expected that this DNA-based tool will become more cost-effective and provide high-quality data to support excellent management plans. This work provides valuable insights into the further development of these approaches to biodiversity conservation.

5.4. Future studies

Much remains to be done in these fascinating ecosystems, which offer unparalleled opportunities to explore ecological and evolutionary dynamics that have broader global relevance. Long-term, comprehensive studies are needed to fill gaps in basic ecological knowledge for the species presented and others that are also at risk of extinction. This is essential to conserve the unique biodiversity of these areas.

It would be important to develop continuity studies over several seasons and years to further support the dietary findings in all studies of this thesis and to better understand inherent variation. In particular, it is essential to improve the sample size for several species found in Wadi Ashar (Article VI), as this was a pilot study. In addition, prey selection studies would be important to understand the feeding strategies of these species (Manicom & Schwarzkopf, 2011; Onkonburi & Formanowicz Jr, 1997). To complement the basic knowledge of the ecology of all reptile species, studies on thermal biology, reproductive strategies, and population dynamics are needed. In addition, plants appear to play an important role in the diet of some species, but there is a need to

investigate what role these reptiles play in plant communities, either as pollinators, seed dispersers or both, and to what extent. Likewise, birds appear to be relevant were important in the diet of both *Tarentola* and *Chioninia* species. It would therefore be of great interest to understand how this species interacts with bird colonies, and whether it is sporadically feeding on bird remains, or actually relying on these nutrients. In addition, it would be very interesting to explore whether these reptiles provide a phytosanitary service by feeding on parasites and carcasses, thereby preventing diseases in bird populations. Additionally, it is crucial to study the impact of the house mouse on all the islands of Cabo Verde, as it has been shown to have a high negative impact on both fauna and flora. This is particularly important for the islets, where the impact is expected to be more pronounced and faster.

It would also be interesting to further investigate the patterns of sympatry and allopatry discussed in Chapter 2 and Supplementary Article A. For example, the study of the *Tarentola* species could be extended to the islet of Branco, where *T. gigas* and *T. raziana* also occur in sympatry. Yet, this is very difficult to accomplish due to the inherent danger involved in reaching this islet. The study of *Chioninia* species could also be extended to the islands of Santiago and Brava, where they occur in both sympatry and allopatry, to understand if patterns are consistent. It could also be extended to other pairs of small and large sympatric species in the Cabo Verde archipelago, such as the larger *Tarentola rudis* Boulenger, 1906 and the smaller *Tarentola darwini* Joger, 1984 on Santiago, or even using museum specimens to compare the large extinct *C. coctei* with the smaller *Chioninia stangeri* (Gray, 1845), which were once sympatric on the islands of Santa Luzia and São Vicente. This could even be studied in other insular systems with similar patterns.

Finally, other evolutionary questions have arisen in the course of this work and are worth exploring. The larger species of *Tarentola* and *Chioninia* have different subspecies on different islands (*T. gigas gigas* on Raso Islet, *T. gigas brancoensis* on Branco Islet, *C. vaillantii vaillantii* on Santiago and *C. vaillantii xanthotis* on Fogo and Rombo' Islets), all of which coexist with their respective smaller counterparts (Articles I and II). However, no subspecies have been described for the smaller species. This raises the question of whether larger species face different selective pressures, forcing them to adapt more rapidly to the different competitive scenarios. There is also the alternative hypothesis that there may be undiscovered subspecies among the smaller species, which will require more extensive morphological and genetic studies to be unravelled. A difference in morphology may be easier to see in larger species, where there may be differences of centimetres, than in smaller species, where a difference of millimetres may not be

considered (Supplementary article A). In this work, the possibility of a new subspecies of *C. delalandii* on the Rombo's Islets is already discussed. This is supported by the genetic differences described by the identification of haplotypes unique to these islands (Miralles *et al.*, 2011). The genetic differentiation between these haplotypes and those from Fogo populations are greater than the difference between *C. vaillantii vaillantii* and *C. vaillantii xanthotis*, further supporting the need to investigate this issue. Other similar cases may exist on these islands and should be further investigated.

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Appendix A
Supplementary Articles

Article A

Big and small: how do we fit all? Morphological evolution of sympatric and allopatric populations of small and giant island geckos

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Manuscript in preparation for submission

Abstract

Evolution can occur in sympatric and allopatric populations, i.e., populations that share or not the same geographic area, respectively. Arid islands are great models for the study of competition in sympatric populations due to their scarce and limited resources. A possible consequence of sympatry is the enhancement of morphological differences between populations in opposite directions, i.e., character displacement (CD). Cabo Verde is an arid archipelago located in the Atlantic Ocean, and home to 12 *Tarentola* species, most of them threatened but very little studied. These geckos arrived in Cabo Verde 7.7 million years ago from the Canary Islands in a single colonization event. From the same medium-sized ancestor, they developed into larger- (*Tarentola gigas*) and smaller-sized (*Tarentola raziana*) species. These two sister species were sympatric on Santa Luzia before the local extinction of *T. gigas*, and presently only on the islet of Raso. There, they are expected to compete with each other for the same scarce trophic resources. It was suggested the hypothesis of CD for partially explaining their morphological evolution, but it was never explored in detail. In the present work, this hypothesis was tested using linear and geometric morphometrics on body size, head shape and size of live and museum specimens. Despite showing different conclusions, both approaches displayed significant differences among all populations, and between live and museum individuals for the same species. Besides uncovering a possible shrinkage in vouchers, a collector's bias towards larger animals was also proposed for the latter observation. The higher differences in sympatric populations may indicate some level of CD and ecological release, i.e., niche expansion following a reduction in competition between species, in the allopatric population. Several alternatives for explaining these morphological differences are discussed.

Keywords: Cabo Verde, character displacement, competition, geometric morphometrics, morphology, Natural History Museum, speciation, *Tarentola*

Introduction

Speciation on islands can occur due to several factors, ranging from selective pressures to stochastic genetic events. Among these, competition arises as an important selective force driving population divergence and adaptation and often enhancing morphological differences between competing species (Case & Gilpin, 1974). One relevant evolutionary phenomenon closely linked to competition and morphological evolution is character displacement (CD), a term first introduced by Brown & Wilson (1956). It defines the divergence in morphology between two sympatric species through interspecific competition. Thus, the CD hypothesis can be tested when sympatric species compete for scarce resources, and morphological divergence is observed.

Despite some exploration of these questions in reptiles, including geckos (Lisičić *et al.*, 2012), the effects of competitor removal have rarely been addressed (e.g., Žagar *et al*., 2017; Gray *et al*., 2019). In particular, insular reptiles are ideal models for studying morphological evolution due to their limited dispersal abilities, and high adaptability to environmental pressures (Sagonas *et al*., 2014; Stadler *et al*., 2022)

The Cabo Verde Archipelago, located in the North Atlantic Ocean and part of the Macaronesian region, provides a great framework for investigating these questions. It has never been connected to the African mainland, thus harbours a great number of endemic species (Arechavaleta *et al*. 2005) . Reptiles are its most important group of terrestrial vertebrate endemics, with 32 different taxa from three genera, one of them the *Tarentola*, commonly known as wall geckos (Miralles *et al.*, 2011; Vasconcelos *et al.*, 2012, 2020). These geckos can adapt to dry areas, and are mostly active during the twilight and night (Carranza *et al*., 2000; Arnold & Ovenden, 2002). Phylogenetic studies have allowed a clarification on their colonization route, having arrived in Cabo Verde on a single event, approximately 7.7 million years ago from the Western Canary Islands (Carranza *et al*., 2000, 2002, Vasconcelos, Carranza & James Harris, 2010). Presently, there are 12 endemic *Tarentola* species (Vasconcelos *et al.*, 2012), including the smaller Raso wall gecko *Tarentola raziana* Schleich, 1984 found on Santa Luzia, Branco and Raso; and the much larger giant wall gecko *Tarentola gigas* Bocage, 1986, presently occurring only in the latter two, as it got extinct on the first around 500 years ago due to anthropic actions (Siverio, Mateo & López-Jurado, 2007; Vasconcelos *et al*., 2013).

Tarentola raziana is the smallest wall gecko species of Cabo Verde (average snout-to-vent length, SVL= 49.3 mm; maximum SVL=60 mm) and it is listed as Near Threatened in the IUCN Red List (Vasconcelos, 2013b). *Tarentola gigas* is the largest one (average

SVL=103.6 mm; maximum SVL=155 mm; Vasconcelos *et al*., 2012). It uses rocks and burrows from sea birds as diurnal refuges and it is classified as Endangered by the IUCN (Vasconcelos, 2013a). These two species are sympatric on Raso, and many times syntopic, and thus may be competing directly for the same scarce resources (Silva, 2012; Seguro, 2017). Thus, the question of how they have managed to coexist arises. Gigantism is one phenomenon that has been reported before on insular lizard species as a way of taking the most advantage of the resources (Stadler *et al.*, 2022). Conversely, the sympatric *T. raziana* reduced to the smallest size of all sister species, raising the question if this is a case of CD.

This work aims to provide more information on how competition, or the lack of it, influenced the evolution of the morphological characteristics of these two species. Some level of CD is expected to be found in sympatric species competing for the same resources, as well as differences between the sympatric and allopatric populations since the population of *T. raziana* on Santa Luzia no longer competes with *T. gigas*. To explore this hypothesis, morphological divergences between the sympatric *T. gigas* and *T. raziana* in comparison with the allopatric *T. raziana* population were accessed using linear (LM) and geometric morphometrics (GM) approaches. Ultimately, this work can aid conservation plans by exploring if the two *T. raziana* populations correspond to distinct morphological units and better evaluate the impacts of a possible reintroduction of *T. gigas* on Santa Luzia.

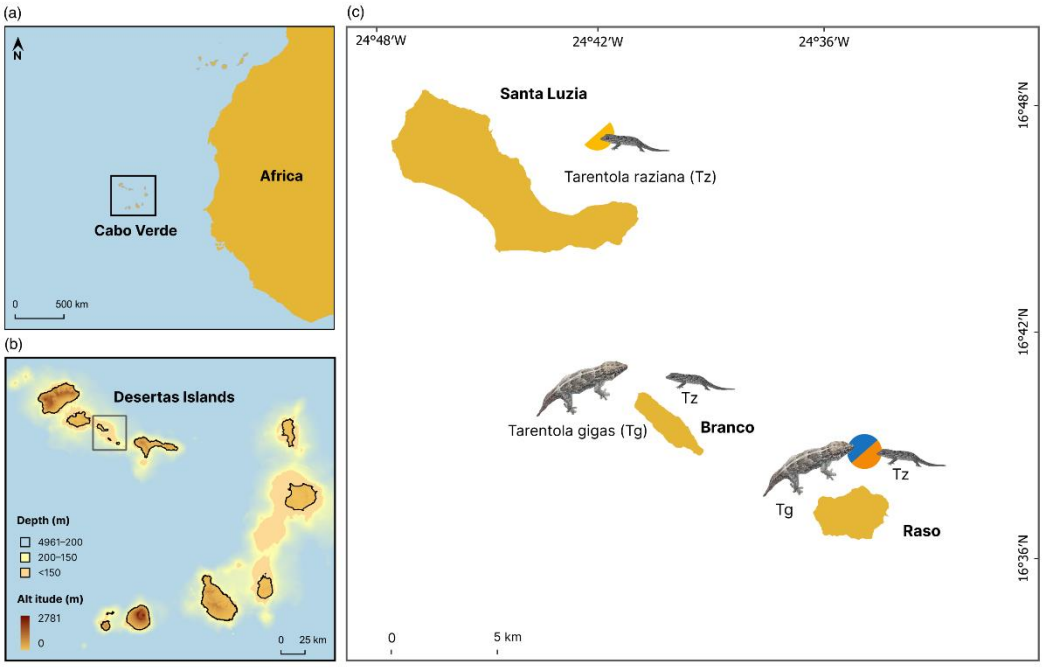

Fig. 1. Study area, sites and species. Map of the Cabo Verde Islands, showing its geographic location (a), of the location studied (b), and the study sites on the Desertas group and, marked with a blue dot, the sampling areas(c), with the current distribution of the studied species depicted.

Methodology

Sampling and Data Preparation

Sampling was performed on the uninhabited Desertas Islands of Santa Luzia and Raso, in the north-western Cabo Verde Islands (Fig. 1). These present a dry tropical Sahelian climate and habitats consisting of flat and arid lowlands, followed by arid medium elevation areas, beaches, dunes and sandy areas, floodplains and dry streams (Diniz & Matos, 1994) . Terrestrial biodiversity is strongly limited by the extremely low precipitation levels, resulting in reduced plant and insect diversity (Vasconcelos, Freitas & Hazevoet, 2015). The study was conducted across the same habitats in both study sites throughout its duration, choosing locations optimal for both species.

Adult (SVL≥ 39 mm for *T. raziana;* SVL≥ 85 mm for *T. gigas*) live specimens of both species, and museum specimens were studied (Table S1). There were no *T. gigas* vouchers from Santa Luzia, and the very few available *T. raziana* specimens were juveniles. Thus, only vouchers from both species from Raso collected in the 1970s, from the National Museum of Natural of History and Science of Lisbon (MUHNAC) were studied. Both LM and GM data were collected for each individual.

For LM, nine linear parameters (Table S2) were measured with a calliper (Mitutoyo 500-753-20 Digital ABS Calliper Coolant Proof IP67 0–200mm 0–8″, accuracy ±0.02 mm, repeatability 0.01mm) following Vasconcelos *et al*. (2012). A total of 83 individuals were used, 30 *T. gigas* vouchers, 19 *T. raziana* vouchers, both from Raso, and 34 live *T. raziana* from Santa Luzia. All geckos were sexed, and during the sampling, there was no preference in capturing males or females.

For GM of the head shapes, individual photos were taken with a digital camera (SONY ILCE-7M2) from a dorsal view of the specimens on top of millimetric paper, parallelly to a flat surface to avoid distortion. Since there are no published GM works on the target species, the choice of landmarks (Table S2; Fig. S1) was based in studies of closely related species, such as *T. mauritanica* (Zuffi *et al.*, 2011; Massetti *et al.*, 2017, 2018). A total of 173 photos, of 99 *T. gigas* individuals, 63 live (TgRaLi herein) and 36 vouchered (TgRaMu herein), and 74 *T. raziana* individuals, 42 from Raso, of which 23 were live (TzRaLi herein) and 19 vouchered (TzRaMu herein), and 32 from Santa Luzia, all live individuals (TzSLLi herein) were used. Photos were loaded onto RStudio v4.3.1 (https://cran.r-project.org) and the R-package *geomorph* v4.0.5 (Baken *et al.*, 2021; Adams *et al.*, 2022) was used for the placement of nine landmarks with the function

digitize2d, and posterior analyses. The scale was provided through the millimetric paper of each photo (10 mm).

Morphometric Analyses

Both scripts for LM and GM analyses of the datasets are available on GitHub (Appendix S.1).

For LM analyses, each of the variables was log-transformed prior to the statistical analyses and three populations were considered: TgRaMu, TzRaMu, and TzSLLi. As there is sexual dimorphism in *T. mauritanica* (Zuffi *et al.*, 2011; Massetti *et al.*, 2017, 2018), females and males in the dataset were firstly analysed separately though a PCA (Principal Component Analysis), checking for this possibility in both *T. gigas* and *T. raziana*. If there were no obvious differences between sexes, all individuals would be used together for subsequent tests. Each of the variables was then tested for normality with Shapiro-Wilk tests for each population, using *dplyr* R-package (Wickham *et al*., 2023), as well as for homogeneity with Levene's tests, using *car* R-package (Fox & Weisberg, 2019). Since *T. gigas* is much larger than *T. raziana*, the effect of size in the data was removed for each variable prior to the significance testing following the procedures from Onn *et al*. (2018) , with the formula: $X_{adj} = X - \beta(SVL - SVL_{mean})$, where X_{adj}= adjusted value; X= measured value; β= unstandardized regression coefficient for each population; SVL= measured SVL; SVL_{mean}= average SVL of all individuals (Lleonart, Salat & Torres, 2000). ANOVAs were performed using each of the adjusted linear parameters as response variables, and the three populations as independent variable, using the *devtools* (Wickham *et al*., 2022) and *factoextra* R-packages (Kassambara & Mundt, 2020). A PCA was performed; subsequent scree plots and quality of representation for the principal components (PC), i.e., PC1 and PC2 were plotted depicting the variables contribution to the variances. The linear PCA was performed using all of the mean values of the SVL-corrected variables. For testing the differences between categories of the predictors and how populations differed from each other, a post-hoc TukeyHSD test was performed on each of the ANOVAs. The ANOVAs and TukeyHSD tests were performed with the base statistics R-package.

For GM analyses, after digitizing all pictures, a Generalized Procrustes Analyses (GPA) aligned and corrected for size all of the specimens (Rohlf & Slice, 1990) in the R-package *geomorph*. From this resulted for each individual a set of 2D shape coordinates, and the centroid size (*Csize*), the square root of the sum of squared distances of each landmark to the centroid of the landmarks, which was log-transformed (*logCsize*) and used as a measure of head size, following Massetti *et al*. (2017). For statistical analyses of the

shapes, ANOVAs were built using residual permutations included in the *procD.lm* function from the *geomorph* package (Collyer & Adams, 2018, 2022). The tests were organized pairwise-like, and firstly, for each of the pairwise sets, a model with *logCsize* as predictor was used, checking for the presence of allometry. In the case of a significant correlation between shape and *logCsize*, the subsequent model was built with *logCsize* as covariate (+*logCsize*), and shape in function of *origin*, when testing vouchers vs live animals, *island* when testing allopatric populations, and *species* when comparing sympatric populations. This model intended in testing if removing the influence from the allometry in the data, shapes among populations would be different. An additional model was tested for each of the previous comparisons, testing for interactions among the predictors *origin*, *island* or *species* and *logCsize* (**logCsize*). The first tests were between live and voucher specimens, TgRaLi vs TgRaMu and TzRaLi vs TzRaMu. If no significant differences were detected on these, correspondent live and vouchered animals of the same species were merged, i.e., TgRaLi and TgRaMu into TgRa, and TzRaLi and TzRaMu into TzRa. The next pairwise tests were performed between allopatric and sympatric populations: TzSLLi vs TzRaLi, TzSLLi vs TgRaLi, TzSLLi vs TzRaMu and TzSLLi vs TgRaMu; TgRaLi vs TzRaLi and TgRaMu vs TzRaMu. This last model was built to test for significant differences among shapes, while taking into account the possibility of different allometric slopes, i.e., if shape varied under different allometric rules.

The GM PCAs were performed on the shape coordinates with the built-in function *gm.prcomp* from the *geomorph* package, for each of the pairwise datasets previously mentioned (data not shown), and one with all of the five groups. For each of the PCAs, PCs 1 and 2 were used to build predictor shapes with the maximum and minimum values of each PC.

Results

Linear Morphometrics

Relating to the adjusted values of the linear measures (Table 1), *T. raziana* showed the highest average values for most variables, while *T. gigas* showed higher standard deviations (SD) and broader ranges. Between TzRaMu and TzSLLi, the first showed highest average values for HW, HH and ED, while the latter showed for HL, EE, ES and TL. TgRaMu had the highest average value for the variable ES2.

Table 1. Average (Avg), standard deviation (SD) and range ([minimum; maximum]) of the linear measurements from the three populations, vouchered T. gigas from Raso, vouchered T. raziana from Raso, and live T. raziana from Santa Luzia. Above are the raw values, and below, marked with an asterisk, are the SVL-adjusted values.

	***T. gigas* from Raso**			***T. raziana* from Raso**			***T. raziana* from Santa Luzia**		
	Avg	**SD**	**Range**	**Avg**	**SD**	**Range**	**Avg**	**SD**	**Range**
HL	32.23	±2.97	[26.34; 39.59]	15.80	±1.45	[13.22; 18.21]	16.99	±0.97	[14.97; 19,17]
HW	22.66	±2.38	[18.96; 27.15]	10.46	±0.97	[9.04; 12.55]	9,79	±0.78	[8.35; 11.60]
HH	15.40	±1.78	[13.25; 19.40]	7.05	±0.66	[5.88; 8.56]	6.35	±0.47	[5.29; 7,59]
EE	11.39	±1.25	[9.67; 14.39]	5.03	±0.41	[4.33; 5.64]	5.07	±0.41	[4.34; 6,11]
ED	6.58	±0.65	[5.52; 7.83]	4.35	±0.43	[3.60; 5.24]	4.35	±0.30	[3.81; 5.00]
ES	13.54	±1.30	[11.63; 16.77]	6.73	±0.44	[5.73; 7.60]	10.64	±0.46	[9.84; 11.79]
ES2	29.16	±2.72	[24.80; 35.44]	13.95	±0.95	[12.53; 15.68]	14.16	±0.70	[12.71; 15.88]
TL	45.91	±4.80	[36.06; 54.09]	17.12	±2.04	[13.53; 21.15]	20.37	±1.91	[16.54; 23.69]
SVL	107.73	±8.91	[91.56; 125.65]	46.63	±4.15	[39.19; 55.11]	48.80	±2.44	[44.00; 53.00]
HL*	3.03	±0.07	[2.89; 3.15]	3.12	±0.03	[3.04; 3.16]	3.14	±0.03	[3.09; 3.20]
HW*	2.65	±0.10	[2.47; 2.91]	2.71	±0.03	[2.66; 2.77]	2.61	±0.05	[2.51; 2.72]
HH*	2.19	±0.11	[1.96; 2.43]	2.33	±0.03	[2.29; 2.38]	2.17	±0.04	[2.08; 2.29]
EE*	1.93	±0.10	[1.70; 2.21]	1.87	±0.05	[1.80; 1.96]	1.98	±0.04	[1.88; 2.07]
ED*	1.49	±0.10	[1.29; 1.63]	1.77	±0.05	[1.68; 1.86]	1.63	±0.06	[1.52; 1.74]
ES*	2.21	±0.09	[2.02; 2.38]	2.16	±0.03	[2.09; 2.21]	2.57	±0.03	[2.53; 2.64]
ES2*	2.93	±0.07	[2.76; 3.10]	2.92	±0.02	[2.88; 2.96]	2.91	±0.03	[2.84; 2.97]
TL*	3.37	±0.10	[3.19; 3.58]	3.29	±0.03	[3.23; 3.35]	3.39	±0.05	[3.32; 3.50]

The PCA for all variables between male and female individuals showed no obvious separation of the data for either species, therefore, for the subsequent analyses, individuals of the same species and island were ensembled. The PCA of the SVL-adjusted variables (Fig. 2(a)) showed a clear separation among the three populations, with a very clear separation between TzRaMu and TzSLLi, more predominantly on PC2. TgRaMu was overall more distributed across the top of PC1. Regarding the PCs contributions, PC1 accounted for 37.18% of the total variance, and PC2 for 27.99%. Analysing the contribution of the variables, EE and ES were the ones with the highest contribution, followed by HL, ED, HH and HW; and ES2 and TL the last two, on PCs 1 to 3 (Fig. 2(b)). The variables HH and HW were the ones with the highest contribution on PC1, 25.70% and 24.40%, respectively, followed by ES2 with 14.29%. On PC2, were ES with 37.42%, and EE with 30.57%.

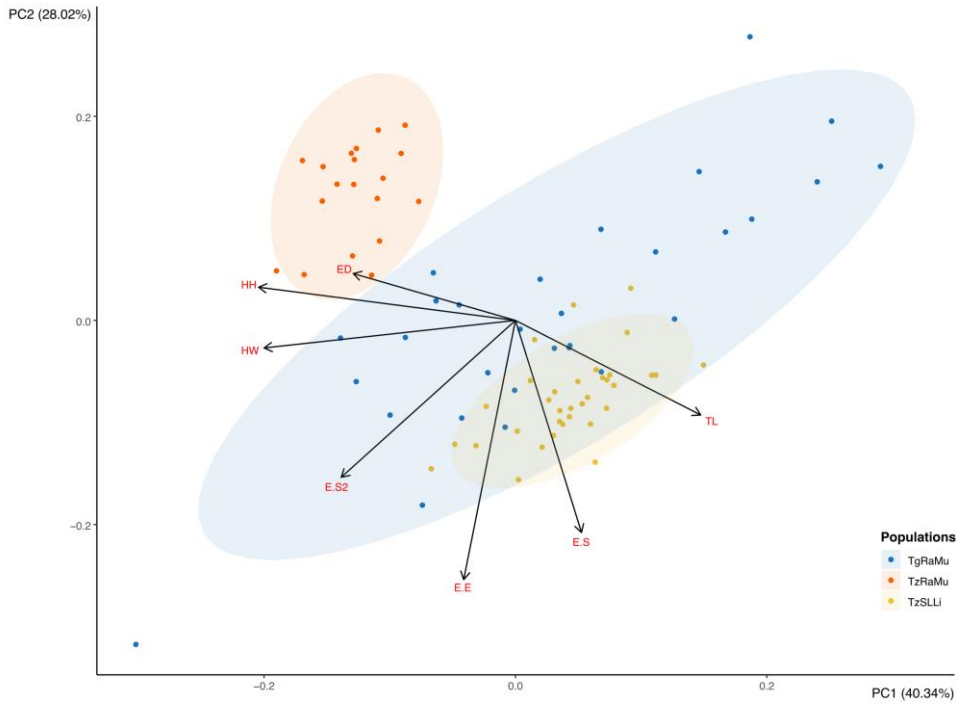

Fig. 2. PCA of the ten linear measurements for the three gecko populations: vouchered T. gigas from Raso, TgRaMu, in blue, vouchered T. raziana from Raso, TzRaMu, in orange, live T. raziana from Santa Luzia, TzSLLi, in yellow. Each individual is represented by a circle in the PCA space, and the 95% confidence interval of each population is circumvented by an ellipse (a). The contribution of the variables for both PCs is also represented (b). See Table S2 for correspondence of the variable codes. The percentage contribution of each PCA component is also given.

Overall, the TukeyHSD tests of the ANOVAs showed several significant differences between the pairs of populations across the linear measures (Fig. 3). Regarding the head variables, HL, showed significant differences in the pairs TgRaMu vs TzRaMu and TzSLLi vs TgRaMu, while both HH and HW showed a significant difference in the pairs TgRaMu vs TzRaMu and TzSLLi vs TzRaMu. Regarding the ocular and ear variables, EE, ED and ES showed significant differences among all of the three pairs of comparisons, while ES2 showed no significant differences among any of the pairs. Lastly, TL showed significant differences between TgRaMu vs TzRaMu and TzSLLi vs TzRaMu. In short, LM results showed significant differences across all variables for TgRaMu vs TzRaMu, except for one, ES2. There were more similarities between TgRaMu and TzSLLi, than between TgRaMu and TzRaMu or TzSLLi and TzRaMu, especially for HW, HH and TL. The sympatric pair TgRaMu vs TzRaMu was the one that had differences across all of the variables, except for ES2. Finally, excluding ES2, TzSLLi and TzRaMu were significantly different in all variables except HL.

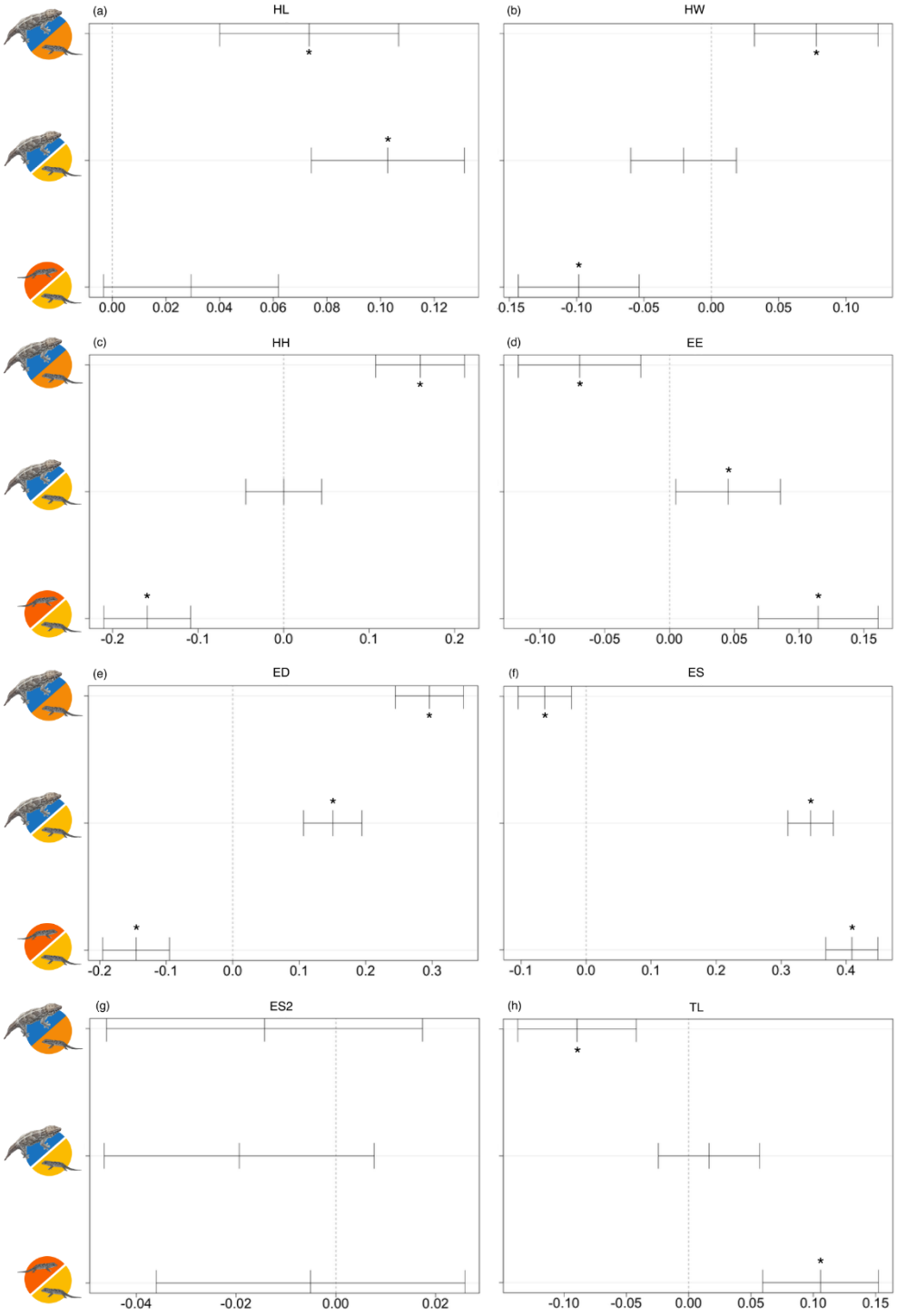

Fig. 3. Visual representation of the results from the TukeyHSD tests of the mean differences in SVL-adjusted values among groups, with 95% confidence interval bars, for the linear variables (check Table S2 for the correspondence for the variable codes), with HL (a), HH (b), HW (c), EE (d), ED (e), ES (f), ES2 (g), TL (h). Marked with an asterisk (*) are the significant differences in mean levels of the SVL-adjusted values among populations (TgRaMu vs TzRaMu; TzSLLi vs TgRaMu; TzSLLi vs TzRaMu; Tg, T. gigas; Tz, T. raziana; Ra, Raso; SL Santa Luzia; Li, live; Mu, museum). If the 95% confidence interval bars cross the vertical dotted line, the comparisons are not significantly different.

Geometric Morphometrics

Pairwise tests showed significant correlations between the shape coordinates and centroid sizes in all groups (Table 2), indicating the presence of allometry between head shapes and their sizes. Therefore, all subsequent models included *logCsize* as a predictor.

Table 2. Results of the ANOVAs performed on the GM data. Each pairwise comparison is identified, and respective SS, F and p values are represented. Marked with an asterisk (*) are the significant p-values. On the first columns are identified the models used in the analyses (check the methods for details), and respective outputs from the procD.lm geomorph function. Different predictors were used according to the pairwise testing, where logCsize is the log-transformed centroid size, og is origin (Li, live or Mu, museum specimens), sp is species (Tg, *T. gigas* or Tz, *T. raziana*), isl is island (Ra, Raso or SL, Santa Luzia).

		TgRaLi vs TgRaMu				TzRaLi vs TzRaMu				TgRaMu vs TzRaMu				TgRaLi vs TzRaLi			
Model	**Output**		**SS**	***F***	***p***	**SS**	***F***	***p***		**SS**	***F***	***p***		**SS**	***F***	***p***
logCsize			0.037	7.350	0.001*	0.013	2.880	0.012*		0.032	8.694	0.001*		0.038	6.605	0.002*
logCsize+og or +sp	*logCsize*		0.029	5.995	0.001*	0.013	3.007	0.011*		0.011	3.096	0.018*		0.028	5.211	0.001*
	+og or +sp	*og*	0.033	6.847	0.001*	0.017	4.001	0.003*	*sp*	0.013	3.757	0.008*		0.040	7.407	0.001*
*logCsize*og or *sp*	*logCsize*		0.019	3.903	0.003*	0.021	5.199	0.002*		0.013	3.892	0.007*		0.019	3.542	0.007*
	og or sp	*og*	0.003	0.676	0.664	0.010	2.419	0.039*	*sp*	0.005	1.522	0.196		0.010	1.959	0.067
	logCsize:og or :sp	*:og*	0.003	0.718	0.630	0.010	2.453	0.037*	*:sp*	0.004	1.212	0.324		0.012	2.215	0.049*

		TzSLLi vs TgRaLi				TzSLLi vs TzRaLi				TzSLLi vs TgRaMu				TzSLLi vs TzRaMu			
Model	**Output**		**SS**	***F***	***p***		**SS**	***F***	***p***		**SS**	***F***	***p***		**SS**	***F***	***p***
logCsize			0.218	38.182	0.001*		0.047	6.397	0.002*		0.142	27.187	0.001*		0.018	3.265	0.017*
logCsize+isl or +sp	*logCsize*		0.039	7.229	0.001*		0.040	6.920	0.001*		0.035	6.985	0.001*		0.018	3.995	0.007*
	isl or sp	*sp*	0.036	6.793	0.001*	*isl*	0.088	15.204	0.001*	*sp*	0.021	4.201	0.003*	*isl*	0.051	11.329	0.001*
*logCsize*isl or sp*	*logCsize*		0.019	3.490	0.008*		0.021	3.604	0.014*		0.026	5.233	0.001*		0.026	5.887	0.993
	isl or sp	*sp*	0.005	0.935	0.428	*isl*	0.007	1.124	0.332	*sp*	0.004	0.705	0.623	*isl*	0.011	2.435	0.038*
	logCsize:isl or :sp	*:sp*	0.006	0.008	0.315	*:isl*	0.007	1.148	0.315	*:sp*	0.005	0.947	0.436	*:isl*	0.009	2.137	0.060

Tests showed significant differences between the shapes of live and voucher individuals of the same species. Henceforth, subsequent tests maintained the five datasets apart (TgRaMu, TgRaLi, TzRaMu, TzRaLi, and TzSLLi). Both comparisons between sympatric populations (TgRaMu vs TzRaMu; TgRaLi vs TzRaLi), showed significant differences between their shapes using *logCsize* as covariate. Pairwise tests with TzSLLi using *logCsize* as cofactor showed significant values between species (TzSLLi vs TgRaLi; TzSLLi vs TgRaMu) and islands (TzSLLi vs TzRaLi; TzSLLi vs TzRaMu).

Tests showed no significant interaction between *logCsize* and *origin* in *T. gigas*, where head shape changes under the same allometric rules both for live and museum individuals, while the contrary happened for *T. raziana*. This means that not only shape is significantly different between live and vouchered specimens in *T. raziana*, but also head size is an important factor on the shape differences between them.

The TgRaLi vs TzRaLi interaction model between *logCsize*, *origin* or *species*, is nearly significant (p= 0.049). In this model, *species* is not significant, thus, allometries of these

populations are in fact significantly different, however, it is not enough to account for differences in head shapes. On the opposite, vouchered *T. gigas* and *T. raziana* showed similar allometries (Table 2).

Pairwise tests for TzSLLi vs TgRaLi and TzSLLi vs TzRaLi showed significant differences between shapes when using *logCsize* as cofactor (Table 2). Both showed no interaction of *logCsize* with *species* or *island*. The same for TzSLLi vs TgRaMu and TzSLLi vs TzRaMu, so in all compared groups their shapes follow the same allometric rules of variation. Conversely, the latter showed a significant value for the *island* (Table 2).

The PCA on shape coordinates of the five groups (Fig. 4) indicated a clear differentiation of TzSLLi from the remaining four Raso groups, mostly across PC1. PC2, while not as clearly, separated *T. gigas* groups from *T. raziana* ones. Regarding the minimum and maximum PC shapes, TzSLLi moved towards a shorter snout (PC1 maximum), while the other four groups moved towards a longer snout (PC1 minimum); *T. gigas* groups moved towards a rounder snout (PC1 maximum), while *T. raziana* towards a pointier one (PC2 minimum).

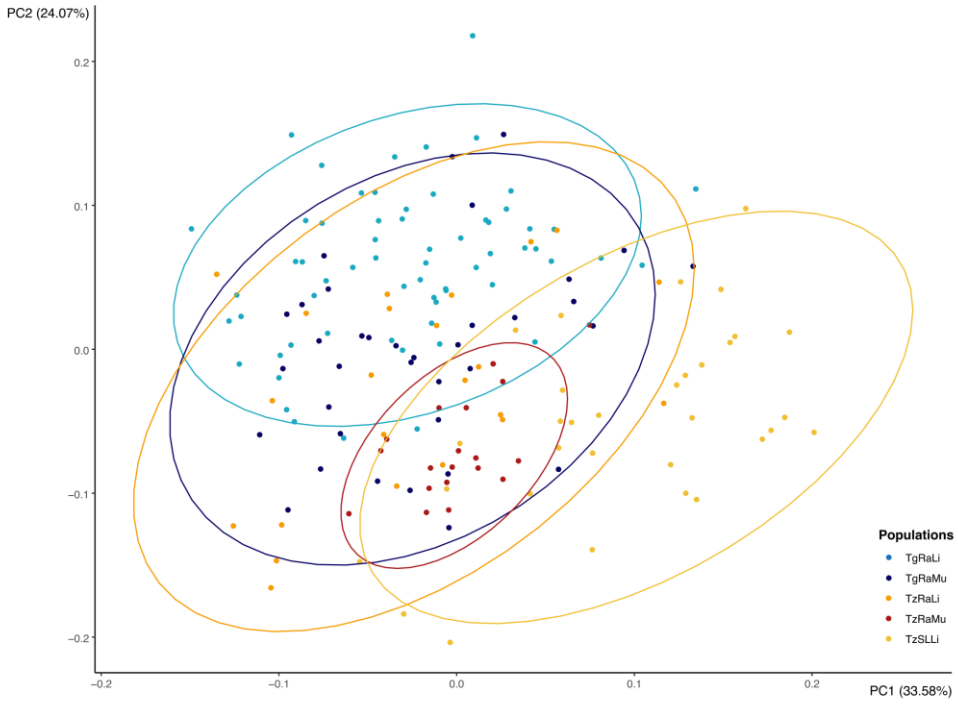

Fig. 4. PCA of the five datasets and GM shape coordinates obtained from the GPA. Legend on the top left corner of the graph identifies the groups and their colours (Tg, *T. gigas*; Tz, *T. raziana*; Ra, Raso; SL, Santa Luzia; Li, live; Mu, museum). On the bottom and top corners of the PCA are represented the shapes correspondent to the minimums and maximums PC values of each axis, respectively.

GM results showed significant differences between voucher and live individuals of the same species and island (Fig. 5(a) and 5(b)). In both species, museum individual shapes appeared to shrink in the ocular landmarks when compared to live individuals. Ear landmarks appeared to move inwards in live specimens. On museum specimens (Fig. 5(a) for TgRaMu, 5(b) for TzRaMu, and 5(c) for both), the head was wider on the neck, with larger and more inwards ocular orbits. Between the anterior edge of the ocular orbits to the nostrils, live individuals presented more volume. The tip of the snout landmarks appeared slightly pointier in *T. raziana* groups, while on *T. gigas* appeared to move towards a more robust and rounder form. Also, shapes of *T. gigas* presented overall smaller eyes, with longer distances between the eyes and the ears. Between both *T. raziana*, Raso geckos showed longer snouts than Santa Luzia ones.

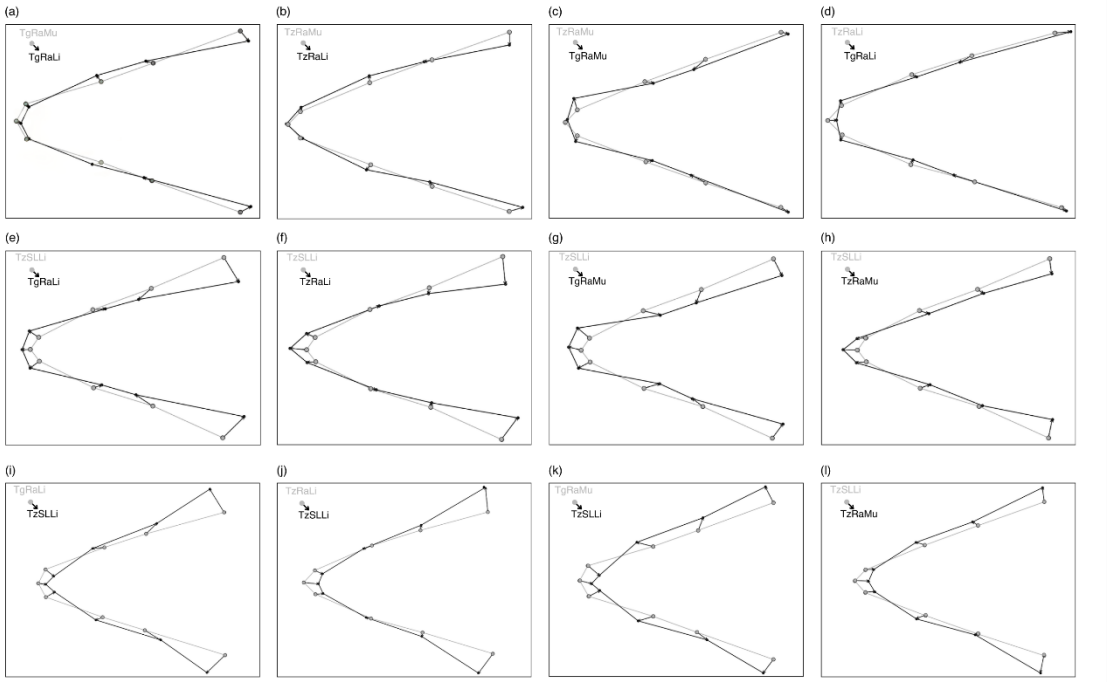

Fig. 5. Mean shape comparison visual representation, using the built-in function *ploRefToTarget* from the geomorph R package, where Tg, *T. gigas*, Tz, *T. raziana*; Ra, Raso, SL, Santa Luzia; Li, live individuals, Mu, museum individuals. Comparisons between TgRaMu vs TgRaLi (A); TzRaMu vs TzRaLi; (B) TzRaMu vs TgRaMu (C); TzRaLi vs TgRaLi (D); TzSLLi vs TgRaLi (E and I); TzSLLi vs TzRaLi (F and J); TzSLLi vs TgRaMu (G and K); and TzSLLi vs TzRaMu (H and L) are depicted. For each of the comparisons, two methods of representation were merged: points (which represents solely the landmarks connected by lines a posteriori) and TPS (representing vectors with the direction of change between populations signalled in the bottom corners), provided by the built-in function of the geomorph R package, both with a magnification of 2. The compared populations are identified on each of the projections, as well as the which one sits at the base of the arrows (grey spots) and which ones are and the end of the arrows (black spots).

Discussion

This study provided insight into the evolution of endemic geckos, by comparing sympatric and allopatric populations. Both linear and geometric tests showed significant differences in head morphology among all populations: between live and museum individuals of *T. raziana* and *T. gigas*; and between populations of different islands, i.e., between Santa Luzia and Raso, for both species. From these findings, a possibility of adaptation through character displacement from the populations can be hypothesized, driven by competition and/ or ecological release. Two different scenarios can be described: one on Raso, where *T. raziana* and *T. gigas*, as natural competitors, have adapted to the feeding habits of each other and diverged morphologically in opposite directions from their common ancestor; and another on Santa Luzia, where *T. raziana* has no direct competition and has changed its diet, and consequently, its head morphology diverged from its sister population on Raso. Alternatively, the populations evolved randomly by drift, fixating certain morphological characters. In this case, it would be expected the two *T. raziana* population to be consistently more similar in those characters than any *T. raziana* and *T. gigas* populations.

Results suggest that the smaller sympatric gecko has developed *in situ* some head and size morphological characteristics, in order to survive with its competitor. Firstly, the fact that these species are syntopic (Seguro, 2017; Pinho *et al.*, 2018) and show huge body size differences may be one evidence accounting of character displacement, *T. gigas* having developed gigantism, and the other reducing to smaller dimensions comparing to the medium-sized ancestral of both for resource partitioning (Žagar *et al.*, 2017; Gray *et al.*, 2019). Secondly, their different sizes resonate in different head morphologies of sympatric species when body size is removed, comparing to the allopatric population (LM showed *T. gigas* head closer to Santa Luzia population than both *T. raziana* populations). This was somewhat unexpected since both *T. raziana* populations are not different subspecies, as supported by phylogenetics (Vasconcelos *et al.*, 2012), thus were expected to be more similar if only drift was acting. These differences could mean a more environmental component and be merely functionally phenotypical, most likely related to their diet.

Head dimensions of lizards are related to bite force, with taller and wider heads relative to body size representing stronger bites (Stayton, 2005, 2006; Daza *et al.*, 2009; Sagonas *et al.*, 2014). Other linear variables, such as ear-eye and eye-snout distances also relate to bite force, as they correlate to the adductor muscles of the jaw, with smaller adductor muscles usually associated with more powerful bites (Stayton, 2005; Daza *et*

al., 2009; Sagonas *et al.*, 2014). A combination of short snouts with wider skulls usually associate to more plant-eating or a more diverse diet in lizards, providing a slow and powerful grip to bite off large pieces of non-moving items, such as plants (Stayton, 2005; Daza *et al.*, 2009; Sagonas *et al.*, 2014). Longer heads allow for a higher volume of jaw adductors, features of faster yet relatively weaker bites, typical of active predators for capturing highly mobile prey (Herrel *et al.*, 1999; Stayton, 2005; Herrel & Holanova, 2008). Both sympatric species have a relatively longer snout shape, which can mean both are active predators. The fact that head height and width from *T. raziana* from Santa Luzia, and *T. gigas* are not different from each other, could mean a similar bite force relative to body size when not competing. Size-adjusted means showed the highest and significantly different values for *T. raziana* from Raso compared to the other two populations in both variables, which could mean the strongest bite and that species differed more in sympatry than in allopatry. Therefore, these measures may be more informative when comparing bite performance, and consequently, dietary characteristics of all populations. However, ear-eye and eye-snout distances were significantly different among the three populations.

Previous results on the diets of the Raso *Tarentola* have shown *T. gigas* as a generalist, feeding on plants, invertebrates and, due to its real larger size, large highly mobile vertebrates as well, such as terrestrial and seabird chicks and eggs (Donald *et al*., 2003; Mateo *et al*., 2016; Pinho *et al*., 2018; Delgado, Lopes & Vasconcelos, 2021; Lopes *et al*., 2021). The feeding habits from *T. raziana* are less known; however, it appears that it is more generalist (Silva, 2012; Mateo *et al.*, 2016). These findings and ongoing work with metabarcoding support this, with both Raso *Tarentola* having head morphological characteristics of a generalist feeder, and the Santa Luzia one preying more on plants, alike *T. gigas*. Furthermore, Raso wall gecko preys more on invertebrates with an overall larger and taller head, pointier snout, shorter trunk, than *T. gigas* and its allopatric population, and also larger eyes than *T. gigas*. It seems that both Raso *Tarentola* have adapted their bite to a wider range of food, due to competition for scarce resources. *Tarentola gigas* showed a head morphology balancing between a longer snout, typical of predation on active prey, but all while not losing bite power for feeding on plants. Conversely *T. raziana* from Raso could need a seemingly stronger bite due to the more chitinous and thus harder constitution of the arthropods it feeds mostly on (Mateo *et al*., 2016; Seguro, 2017).

However, GM results showed the allopatric *T. raziana* to have the widest head at the temporal region and the shortest snout, reinforcing a probable diet mostly comprised of plants. This population has the resources more fully available than on Raso, and

arthropods show an overall low availability in the Desertas (Pinho *et al.*, 2018), and are harder to get. Herbivory in lizards is highly associated to a lack of competitors and predators, as well as arid and hot ecosystems (Szarski, 1962), supporting the possibility of this population shifting towards plant-eating. This, in the end, deviates the Santa Luzia smaller gecko diet towards one more similar to the one of *T. gigas*, hence their similarities in morphology. This could justify the reintroduction of *T. gigas* on Santa Luzia on different areas of *T. raziana* occurrence to prelude mutual exclusion.

Since trunk length differed more if species were in sympatry than allopatry, it may indicate that there may have been a pressure for *T. raziana* to evolve in a different direction of *T. gigas*, with sympatric *T. raziana* presenting a shorter trunk relatively to its size than in the allopatric populations. Žagar *et al*. (2017) reported shorter trunks being associated with higher climbing speeds on lacertids. On Raso, *T. raziana* shorter trunks may be owing to its higher predation on invertebrates, especially on small fast-moving insects, in comparison to Santa Luzia population and *T. gigas* (unpub. data; Mateo *et al*., 2016; Seguro, 2017). However, morphological studies regarding limb length and locomotor performance among the three populations are lacking to test this.

Between both *T. raziana*, it becomes difficult to affirm which would have superior bite force or speed as linear results may point towards *T. raziana* from Raso because of higher adjusted values of head height and width. On the other hand, shape projections seem to point towards allopatric *T. raziana*. These differences may come from the nature of each methodology, where linear variables are analysed independently, but landmarks depend on the overall shape of the head. In LM, measurements were corrected for body size (SVL), while in GM were corrected only for head size (Csize). Shape projections of full body could be a future approach to consider, or taking into account the SVL in the GM models. Sampling more live specimens of all populations, and digitizing more landmarks could also clarify the incongruences between LM and GM results.

Another character differing between species is eye size, highly related to the active time period, where larger eyes are typical of nocturnal activity (Pianka, 1973; Herrel *et al*., 2007; Daza *et al*., 2009), and smaller eyes take a toll on the night vision capacity, y*et al*low for stronger bites (Herrel *et al.*, 2007). While both species are more active during the night (Silva, 2012; Mateo *et al.*, 2016; Pinho *et al.*, 2018), results demonstrate *T. raziana* to have the largest eyes, possibly meaning more active preying during the night than *T. gigas,* as observed by Silva *et al.* (2012). Since *T. gigas* preys more on terrestrial diurnal birds, such as Raso larks and Iago sparrows (Donald *et al*., 2003; Pinho *et al*., 2018), it may have partially adapted to their twilight activities. Conversely, the sympatric

smaller gecko may have adapted to feed mostly at late night on nocturnal invertebrates. Temporal niche displacement was described in predatory lizard species under competition (Pianka, 1973; Pianka & Pianka, 1976). Yet, ecological and behavioural studies from these and other Cabo Verde species are needed to better understand the trophic relationships on the Desertas Islands.

Initially, the main objective in using individuals from museum collections was to gather a larger sample size. Results from LM showed significant differences across the populations, and GM tests proved the vouchers to have different head shapes from their live correspondents. On one hand, the corrected means of the linear measures showed a tendency to be higher in *T. raziana*. Despite having the lowest corrected means in many traits, *T. gigas* vouchers presented generally higher standard deviations and broader ranges. This raises the possibility of a collector's bias towards catching the largest specimens, highly influencing the representation of the average size of the species. Since *T. gigas* is a larger lizard, curators could be less selective in collecting individuals comparing to *T. raziana*, leading to a stricter selection of the latter specimens. This selection of the largest individuals could also justify the difference in sample sizes of the species in the collections. Vouchers from Santa Luzia could help test this possibility, however, despite their limited existence not allowing for further analyses, it also supports the hypothesis of a lack of interest on smaller species. This bias hypothesis may also explain the differences between LM and GM analyses. LM from Raso populations were only obtained in vouchered *T. raziana*, which has already been proposed to present a level of bias for larger animals, thus resulting in higher relative linear values of head height and width. To verify this, measurements of live individuals from all populations are required for future research.

Another possible explanation for a difference between museum vouchers collected in 1970 and live specimens may be the shrinkage and changes in the physical structure of the first. Degradation and shrinkage of preserved animals in fluids were reported in lizards (Maayan *et al.*, 2022), snakes (Reed, 2001), and anurans (Lee, 1982; Deichmann, Boundy & Williamson, 2009). Significant long and short-term effects from ethanol preservation on muscle architecture were also reported (Leonard *et al.*, 2021a, 2021b), and a level of changes in size and shape needs to be considered (Cato *et al*., 2001; Maayan *et al*., 2022). Additionally, live and vouchered *T. raziana* from Raso, were not expected to present significantly different allometries. There can be a higher level of error in *T. raziana* values, both on LM and GM, due to shrinkage and a smaller sample size, due to its smaller size. This is an important question to address in future research, seen how and if there was a change in the allometry of the same species, there may be

strong external pressure that led to this change in allometry (Di Martino & Liow, 2022). Nonetheless, some level of caution is needed when using vouchers.

Investigating other species and pairs of *Tarentola* from Cabo Verde could be pertinent to discern between the existence of a common evolutionary CD pattern, or an alternative stochastic hypothesis. For instance, on Branco, where the subspecies *Tarentola gigas brancoensis* is sympatric with *T. raziana*, the dietary and morphological differences between these species is unknown. With this, extensive morphological studies such the ones performed here could allow for a reconstruction of the ancestral and consequent divergence of the heads of the Cabo Verdean *Tarentola*.

Conclusions

These results can be largely associated with trophic adaptation both in sympatric and allopatric populations, and some level of character displacement. This ultimately showed how the extinction of a competing species 500 years ago may alter the behaviours of other species, to the extent of demonstrating divergence between sister populations. Specific information in the head morphology of *T. gigas* and *T. raziana* was missing, and despite providing more insight in here, some new questions were raised. Further studies will be useful to clarify them, such as biting or locomotor performance, and more detailed information on the geckos' diets. These ecological insights are important to define courses of actions for the conservation of these endemic species, and a possible reintroduction of *T. gigas* on Santa Luzia.

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Supplementary material Article A

Appendix S1. The R script for the linear analyses, landmark digitizing and all analyses is available online on the GitHub platform, on the repository*:* https://github.com/sousasofia/caboverdean.tarentola

Appendix S2. Portuguese translation of the abstract.

A evolução pode ocorrer em populações simpátricas ou alopátricas, ou seja, populações que partilham ou não a mesma área geográfica, respectivamente. As ilhas áridas são frequentemente óptimos modelos para o estudo da competição em populações simpátricas devido aos recursos escassos e limitados destas. Uma possível consequência da simpatria é o aumento das diferenças morfológicas em direcções opostas entre populações, ou seja, o deslocamento de caracteres (DC). Cabo Verde é um arquipélago árido localizado no Oceano Atlântico que alberga 12 espécies de *Tarentola*, a maioria ameaçadas, mas muito pouco estudadas. Estas osgas chegaram a Cabo Verde há 7,7 milhões de anos atrás das ilhas Canárias através de um único evento de colonização. Do mesmo ancestral de tamanho médio, estas desenvolveram-se em espécies de tamanho maior (*Tarentola gigas*) e menor (*Tarentola raziana*). Estas duas espécies irmãs eram simpátricas em Santa Luzia, antes da extinção local da *T. gigas* e presentemente apenas no ilhéu Raso. Aí, é expectável competirem uma com a outra pelos mesmos escassos recursos tróficos. Foi sugerida a hipótese de DC para explicar parcialmente a evolução morfológica das mesmas, mas nunca foi explorada em detalhe. No presente trabalho, esta hipótese foi testada usando morfometria linear e geométrica no tamanho do corpo, forma e tamanho da cabeça de espécimes vivos e de museu. Apesar de mostrarem conclusões diferentes, ambas as abordagens exibiram diferenças significativas entre todas as populações, e entre indivíduos vivos e de museu para a mesma espécie. Para além de revelar um possível encolhimento nos exemplares de museu, um viés de coleccionador em favor de animais maiores foi também proposto para explicar esta última observação. As maiores diferenças nas populações simpátricas podem indicar algum nível de DC e relaxamento ecológico, ou seja, expansão de nicho após uma redução da competição entre espécies, na população alopátrica. Várias alternativas para explicar estas diferenças morfológicas são discutidas

Table S1. Specimens used for the landmark digitizing and analyses. Each individual is identified with their code and respective species (Tg, T. gigas; Tz, T. raziana), island (Ra, Raso; SL, Santa Luzia), origin (Li, live; Mu, museum) and group for the morphological analyses (concatenation of previous codes), and which analyses it was used for (LM for linear morphometrics, and GM for geometric morphometrics).

Specimen code	Species	Island	Origin	Group code	Analyses
Ens_Tg_Ra_06_24	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_06_25	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_06_35	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_06_36	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_06_44	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_08_98	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E030007F1	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000847	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E030008D4	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000903	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000997	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E030009A8	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000A62	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000A88	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000AF1	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000AFC	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000B5A	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000B63	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000BE2	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000CD8	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000D58	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000DA8	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000E4A	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000E51	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000F34	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03000FC6	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E030010BE	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03001102	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03001120	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03001321	*T. gigas*	Raso	Live	TgRaLi	GM
Ens_Tg_Ra_0E03001A2B	*T. gigas*	Raso	Live	TgRaLi	GM
Kat_Tg_Ra_18_01	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_10	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_11	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_12	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_13	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_14	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_20B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_21B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_28B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_29	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_34B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_35B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_36B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_43B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_44B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_45B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_49B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_53B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_58B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_62B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_63B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_64B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_65B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_66B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_67B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_68B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_69B	*T. gigas*	Raso	Live	TgRaLi	GM

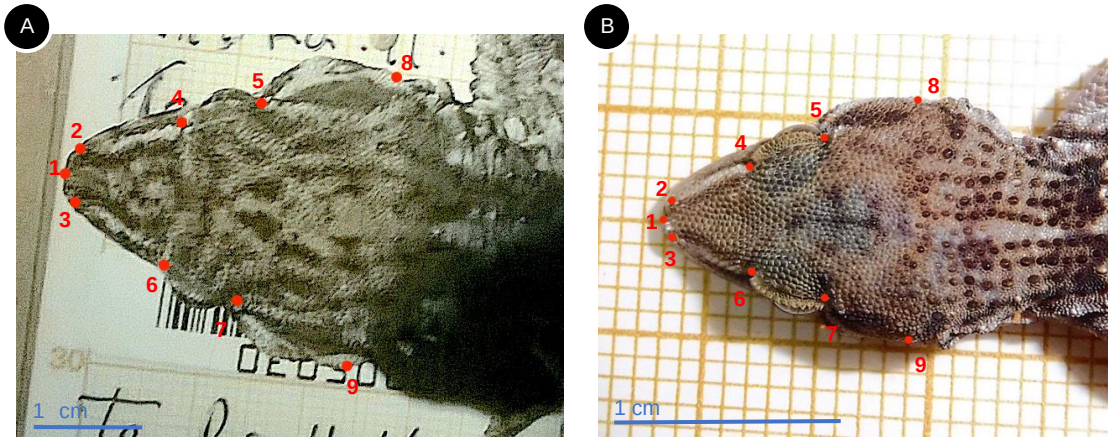
Specimen code	Species	Island	Origin	Group code	Analyses
Nd_Tg_Ra_71B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_8	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_80B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_81B	*T. gigas*	Raso	Live	TgRaLi	GM
Nd_Tg_Ra_9	*T. gigas*	Raso	Live	TgRaLi	GM
MUNHAC-372-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-373-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-374-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-375-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-376-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-377-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-387-1970	*T. gigas*	Raso	Museum	TgRaMu	LM
MUNHAC-388-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-389-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-390-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-403-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-404-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-405-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-406-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-407-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-408-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-409-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-410-1970	*T. gigas*	Raso	Museum	TgRaMu	LM
MUNHAC-411-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-412-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-413-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-414-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-437-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-438-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-439-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-440-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-441-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-442-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-443-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-444-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-445-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-446-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-447-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-448-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-449-1970	*T. gigas*	Raso	Museum	TgRaMu	LM + GM
MUNHAC-450-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-451-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
MUNHAC-452-1970	*T. gigas*	Raso	Museum	TgRaMu	GM
Ens_Tz_Ra_02_159	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_02_172	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_02_174	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_08	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_09	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_10	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_15	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_17	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_19	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_20	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_27	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_28	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_06_32	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_07_73	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_07_75	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_07_76	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_08_88	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_08_91	*T. raziana*	Raso	Live	TzRaLi	GM
Ens_Tz_Ra_08_92	*T. raziana*	Raso	Live	TzRaLi	GM
Nd_Tz_Ra_14B	*T. raziana*	Raso	Live	TzRaLi	GM

Specimen code	Species	Island	Origin	Group code	Analyses
Nd_Tz_Ra_16B	*T. raziana*	Raso	Live	TzRaLi	GM
Nd_Tz_Ra_18B	*T. raziana*	Raso	Live	TzRaLi	GM
Nd_Tz_Ra_21	*T. raziana*	Raso	Live	TzRaLi	GM
MUNHAC-232-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-233-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-234-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-235-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-237-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-238-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-254-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-255-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-256-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-257-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-258-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-364-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-365-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-366-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-470-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-471-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-472-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-473-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
MUNHAC-474-1970	*T. raziana*	Raso	Museum	TzRaMu	LM + GM
Cata_Tz_SL_05_21_10	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_11	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_12	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_15	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_16	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_17	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_18	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_19	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_2	*T. raziana*	Santa Luzia	Live	TzSLLi	GM
Cata_Tz_SL_05_21_20	*T. raziana*	Santa Luzia	Live	TzSLLi	LM
Cata_Tz_SL_05_21_21	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_23	*T. raziana*	Santa Luzia	Live	TzSLLi	GM
Cata_Tz_SL_05_21_24	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_25	*T. raziana*	Santa Luzia	Live	TzSLLi	LM
Cata_Tz_SL_05_21_26	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_27	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_28	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_29	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_3	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_31	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_32	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_33	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_34	*T. raziana*	Santa Luzia	Live	TzSLLi	LM
Cata_Tz_SL_05_21_35	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_36	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_38	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_39	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_4	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_41	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_42	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_5	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_6	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_7	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_8	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM
Cata_Tz_SL_05_21_9	*T. raziana*	Santa Luzia	Live	TzSLLi	LM + GM

Table S2. Variables used in the analysis of the shape and size of the head in both *Tarentola* species. Their type (linear or geometric), code, name and description are given.

Type	Code	Name	Description
Linear	SVL	Snout-to-vent length	from the tip of the snout to the cloaca
	HL	Head length	from the tip of the snout to the beginning of the neck
	HW	Head width	at the widest part of the head, at the temporal region
	HH	Head height	at the widest part of the head, from the jaws to the occiput
	EE	Ear-eye distance	distance between anterior ear and posterior eye
	ED	Eye diameter	at the widest of the ocular orbit
	ES	Eye-snout distance	distance between anterior eye and snout tip
	ES2	Ear-snout distance	distance between anterior ear end and snout tip
	TL	Trunk length	from posterior edge of forelimb to anterior edge of hindlimb
Geometric	1		Tip of snout
	2		Right side nostril
	3		Left side nostril
	4		Frontal edge of the right ocular orbit
	5		Rear edge of the right ocular orbit
	6		Frontal edge of the left ocular orbit
	7		Rear edge of the left ocular orbit
	8		Right side tympanum
	9		Left side tympanum

Fig. S1. Example of the nine landmarks positioning. Landmarks are marked and numbered in red, and the scale in blue, both for a *T. gigas* (A), and *T. raziana* (B) individuals



Article B

Diet of the exotic Madeiran wall lizard: first insights into trophic interactions in an Atlantic seabird sanctuary

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Abstract

The Madeiran wall lizard *Teira dugesii* is a relatively new species to the Azores Archipelago, where it was accidentally introduced about 150 to 200 years ago. This lacertid quickly became naturalised and now occurs in all the nine main islands of the Azores. At Praia Islet, off Graciosa Island, the Madeiran wall lizard was recently observed preying on chicks of the threatened Monteiro's storm-petrel *Hydrobates monteiroi*. To characterise the lizards' trophic niche, we conducted a study of the diet of the Madeiran wall lizard at Praia using stable isotope analysis and next-generation sequencing. Our results indicate that the Madeiran wall lizard has a varied diet, consisting of at least 23 invertebrate taxa, 17 plant species, and occasionally, birds (two species detected, including storm-petrels). Marine derived food items were present in few samples, and it is vital to monitor the trophic interactions at Praia Islet to ensure the conservation of this threatened storm-petrel.

Key Words

Azores, conservation, Macaronesia, next-generation sequencing, Praia Islet, stable isotopes

Introduction

Invasive species can pose a risk to local wildlife as predators (Mack et al. 2000) and disrupt ecosystem dynamics, especially on islands, where ecosystems are more vulnerable to introductions (Butterfield et al. 1997). The Madeiran wall lizard *Teira dugesii* is the only land reptile occurring in the Azores Archipelago (Malkmus 1995; Borges et al. 2022), where it was introduced around 150–200 years ago (Godman 1870) by the shipping trade with the Madeira Islands.

Even though lizards and other reptiles are frequently the only terrestrial vertebrates on oceanic islands, there are few studies documenting interactions between reptiles and seabirds. The interactions between the endemic tuatara *Sphenodon punctatus* and the fairy prion *Pachyptila turtur* in Stephens Island, New Zealand, are perhaps the most studied case (e.g., Wall 1978; Cree et al. 1999). The study of Matias et al. (2009) at Selvagens Islands (Madeira, Portugal) is one of the first documented evidence of a lacertid lizard, the Madeiran wall lizard (in this

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case endemic) preying on seabirds, including storm-petrels, in Macaronesia. Recently, Pinho et al. (2018) found evidence that a common prey of an endemic and Endangered lizard, the giant wall gecko *Tarentola gigas*, is also an endemic and Near Threatened seabird, the Cabo Verde shearwater *Calonectris edwardsii*, showing how intricate these trophic links can be. Also in Macaronesia, Gil et al. (2020) found evidence of a trophic link between the Selvagens gecko *Tarentola* (*boettgeri*) *bischoffi* and Cory's shearwater *Calonectris borealis*.

Ground-breeding seabirds often nest exclusively on small islands, which are probably selected because of a lack of predators (Schreiber and Burger 2001); that is the case for the small Hydrobatidae species breeding in the Azores (Monteiro et al. 1999), such as the endemic Monteiro's storm-petrel, only known to breed in the islets off Graciosa Island (Bolton et al. 2008). Bried and Neves (2014) reported Madeiran wall lizard scavenging dead Monteiro's storm-petrel *Hydrobates monteiroi* chicks on Praia Islet from Graciosa in the Azores. More recently, the killing of Monteiro's storm-petrel chicks by those lizards was confirmed (Neves et al. 2017). This small seabird is classified as globally Vulnerable to extinction on the IUCN Red List (BirdLife International 2018). On those grounds, the lizard predation events detected are a cause of concern and, to our knowledge, are the first documented evidence of an introduced lacertid affecting seabirds.

This study aims to investigate the diet of Madeiran wall lizards at Praia Islet as a first step towards effectively protecting the endemic Monteiro's storm-petrel. To assess the lizards' trophic niche, we studied their diet using Stable Isotopes (SI) in blood and next-generation sequencing in faeces. Stable isotopes analyses provide information on the trophic position and diet (Kartizinel and Pringle 2015) and next-generation sequencing identifies the prey items (Pompanon et al. 2012). Stable isotopes can also provide a time-integrated depiction of consumer diet and trophic relationships (Phillips and Eldridge 2006).

Methods

Praia Islet is located about 1.2 km off Graciosa Island, one of the nine main islands of the Azores Archipelago (Fig. 1) and is a breeding site for several seabird species, including three Charadriiforms and five Procellariiforms – two species are winter breeders (Barolo Shearwater, *Puffinus lherminieri* and Band-rumped storm-petrel, *Hydrobates castro*) and all the others breed during the summer (Monteiro et al. 1999; Bolton et al. 2008). The islet is a natural reserve and Graciosa is classified by UNESCO as a Biosphere Reserve (UNESCO 2007). Fieldwork took place from May to July 2016, coinciding with the breeding season of most seabirds that breed in the islet (Cory´s Shearwater, *Calonectris borealis*; Monteiro's storm-petrel, *H. monteiroi*, Bulwer´s petrel, *Bulweria bulwerii*; Common tern, *Sterna hirundo*; Roseate tern, *S. dougallii* and Sooty tern, *Onichoprion fuscatus*). Lizards were caught by hand or in traps made from 5-litre plastic containers baited with pieces of fresh fruit. Samples for SI analysis were collected in two different areas, approximately 200 m apart to try to minimise the resampling of individuals across sampling areas. One area was around the support house on the west side of the islet (Fig. 1), which was defined as the area with a low density of nesting birds. The other area was on the east side of the islet around an area with 58 artificial nests of Monteiro's storm-petrel (Fig. 1). Sample analysis was divided into two periods: 31 May–8 June, corresponding to the incubation stage of Monteiro's storm-petrels and 3–16 July, corresponding to the chick-rearing stage (Bolton et al. 2008).

Caught lizards were brought to the support house, measured (snout–vent length, SVL) and weighed. Faecal samples were collected by gentle palpation of the abdomen and stored in 70% ethanol. A blood sample (5–10 µL) was drawn with a sterile insulin syringe (0.33 × 12.7 mm, 29G) from the caudal vein (see Divers and Mader 2005) and stored in 70% ethanol. The base of the tail was disinfected using cotton dampened in 90% ethanol. After the procedure, which took approximately 10 minutes, the lizards were released where they were captured.

To provide an isotopic baseline of the food resources, we also sampled 18 food items for SI analysis: six arthropods and two plant species as terrestrial food, as well as blood from 10 Monteiro's storm-petrel chicks as marine-derived food. Blood samples were briefly centrifuged, supernatant (i.e., plasma) was discarded, and the Eppendorf tubes were put into a drying chamber to evaporate the water content of the blood cells. The dried samples were then pulverized using a mortar and pestle to obtain a fine powder, and a subsample of 0.35–0.45 mg was weighed into tin capsules. Some samples were slightly lighter if there was not sufficient blood, but no less than 0.12 mg. Stable isotope ratio for carbon and nitrogen was determined with an Isotope Ratio Mass Spectrometer at the University of La Rochelle, LIENSs SI Facility. Carbon and nitrogen ratios were determined with a continuous-flow mass spectrometer (Delta V Advantage or Delta V Plus with a Conflo IV interface, Thermo Scientific, Bremen, Germany) coupled with an elemental analyser (Flash EA 1112 or Flash 2000, Thermo Scientific, Milan, Italy). Measurements of internal laboratory standards were conducted using acetanilide (Thermo Scientific) and peptone (Sigma-Aldrich) and indicated an experimental precision of ± 0.15‰ for both elements. Results are expressed in parts per thousand (‰), in the usual δ notation relative to Vienna Pee-Dee Belemnite for $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$, following the formula: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. Differences in the mean $\delta^{13}C$ in the different habitats before and after the hatching of the chicks were analysed using Wilcoxon rank sum test with continuity correction because the data were not normally distributed (R v3.3.2, R Core Team 2016). Significance was assumed at $P < 0.05$.

Faecal samples were dried in an incubator at 50 °C to remove the ethanol. The DNA was extracted using the

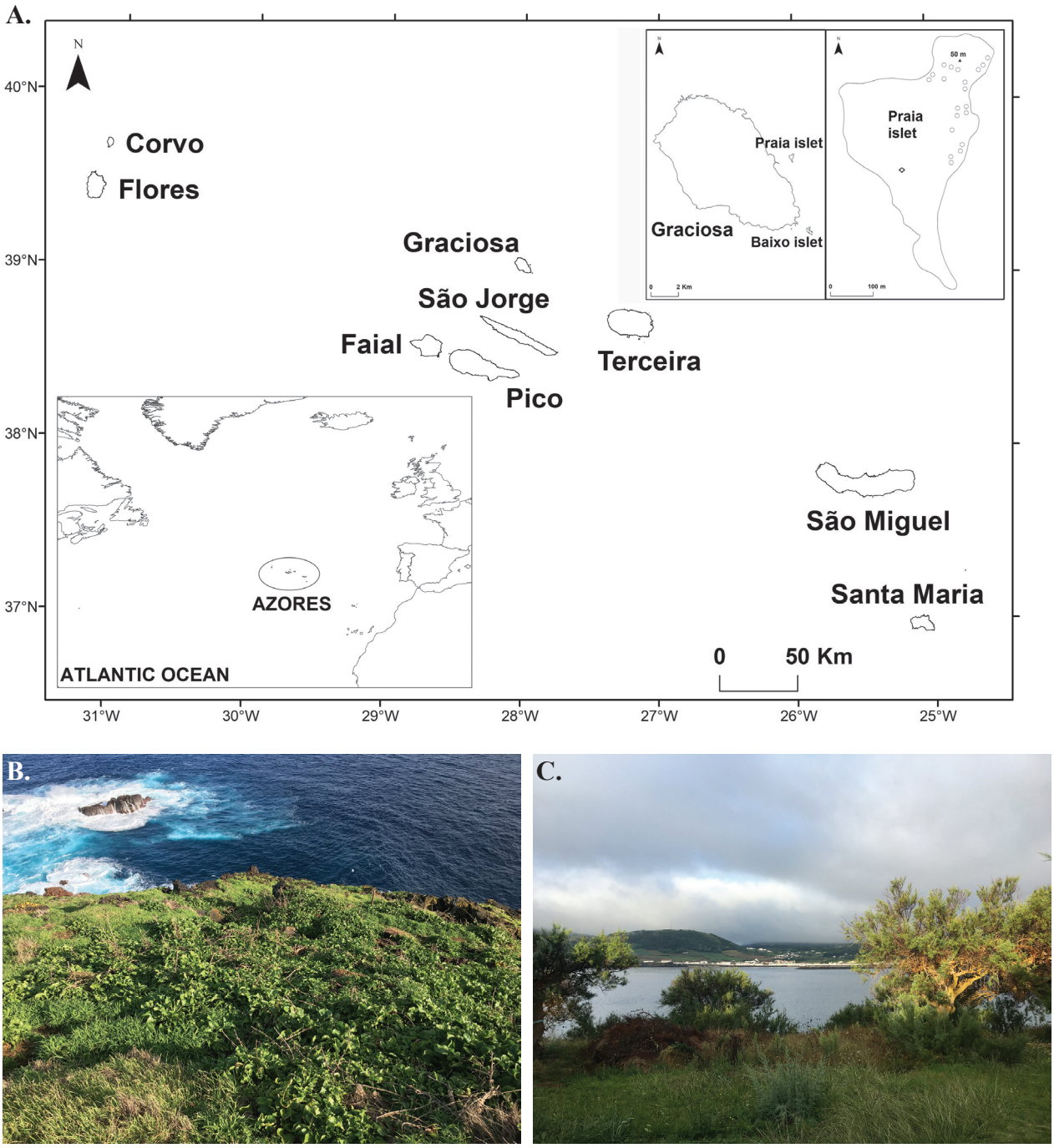

Figure 1. A. Location of Praia Islet in the Azores Archipelago. Squares marked in the east and north-east of the islet indicate the location of artificial nests of Monteiro's storm-petrel, *Hydrobates monteiroi*; the diamond symbol in the west of the islet indicates the location of the house. Adapted from Bried and Neves (2014); **B.** Study area - location 'Nests'; **C.** Study area - location 'House'.

Stool DNA Isolation Kit (Norgen Biotek Corp., Canada), following the manufacturer's instructions. In the end, two DNA elutions were obtained with a final volume of 50 µL each, but only the first elution was used for the next steps. To correctly identify the different prey types (vertebrates, invertebrates, and plants) three different DNA fragments were selected for amplification. For vertebrates, the V5-loop fragment of the mitochondrial 12S gene (Suppl. material 1: Table S1) was selected. The marker selected for invertebrates was the mitochondrial 16S rRNA (Suppl. material 1: Table S1) and for plants the short P6–loop of chloroplast trnL (UAA) intron (Suppl. material 1: Table S1). To allow the individual identification of each sample, all primers were modified with an individual barcode as detailed in Pinho et al. (2018). Each PCR was performed following the same protocol as detailed in Pinho et al. (2018) and the product was tested on a 2% agarose gel.

Library preparation was carried out following Illumina MiSeq protocol "16S Metagenomic Sequencing Library

Preparation" (Illumina 2013) according to the method described in Pinho et al. (2018). OBITools (https://git.metabarcoding.org/obitools/obitools) was used for general sequence processing according to the methods described in Pinho et al. (2018). Results were compared with Azores databases (Borges et al. 2022) and the Encyclopedia of Life database (http://eol.org). We removed a few haplotypes that were considered lab contamination based on published reference lists of species occurring in Azores. When the same haplotype matched more than one species or genera with similar probabilities, we only considered species or genus known to occur at Graciosa, or in other islands of the Azores.

Results

We collected and analysed for SIs 57 Madeiran wall lizard blood samples; mean values and standard deviation for carbon and nitrogen isotopes for the different sampling locations and times are given in Table 1. The food reference material collected on the islet showed clear differences in carbon isotope ratios between terrestrial and marine items with lower $\delta^{13}C$ values for terrestrial items (Table 2), but an overlap in nitrogen isotope ratios. During the incubation period, values of $\delta^{13}C$ isotopes were slightly higher in the location 'Nests' than in the location 'House' but the difference was not significant (Wilcoxon rank sum test with continuity correction, $W = 120.5$, $P = 0.265$; $n_{house} = 16$, $n_{nests} = 12$). During the chick-rearing period, values of $\delta^{13}C$ isotopes were again higher in the location 'Nests' and there was no significant difference between locations ($W = 135$, $P = 0.181$; $n_{house} = 13$, $n_{nests} = 16$). $\delta^{13}C$ isotope values were higher during chick-rearing for the sampling location 'Nests', but also not significant ($W = 61$, $P = 0.109$; $n_{house} = 12$, $n_{nests} = 16$). Around the 'Nests' site, there was also a much greater variance in the stable carbon isotope values (Table 1, Fig. 2). Some individuals ($n = 5$) from this study area reached as high a carbon value as the Monteiro's storm petrel chicks – stable carbon isotope values between -21.49 and -20.10‰. The arthropod samples collected at the islet show an enrichment in $\delta^{13}C$ values compared to plants (Table 2), but the values were still lower than the ones found for Monteiro's storm-petrel chicks, giving support to the hypothesis that some lizards may be feeding on seabirds.

Out of the nine faecal samples analysed, only seven worked for most markers used and two samples failed to be sequenced. In total, 43 molecular operational taxonomic units (MOTU) were identified: 17 plants, 23 invertebrates

Table 1. Results of the isotopic analyses from lizard blood samples. Sample size (n), stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios (mean ± standard error in permillage, ‰) for the different sampling locations and periods. Check Fig. 1 for sample locations.

Location_period	*n*	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
House_incubation	16	-24.53 ± 0.12	12.63 ± 0.12
House_chick-rearing	13	-24.27 ± 0.07	12.90 ± 0.23
Nests_incubation	12	-24.14 ± 0.27	11.46 ± 0.25
Nests_chick-rearing	16	-23.11 ± 0.41	12.03 ± 0.17

Table 2. Results of the isotopic analyses from reference food items samples. Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios (mean ± standard error in permillage, ‰) for reference food resources. Terrestrial items include two plant and six arthropod species, and the marine items consist of blood samples from Monteiro's storm-petrel chicks.

Food items	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Terrestrial	-24.71 ± 0.63	9.62 ± 1.19
Plants	-26.94 ± 0.42	12.74 ± 2.92
Arthropods	-23.97 ± 0.53	8.58 ± 1.09
Marine	-20.23 ± 0.06	11.08 ± 0.07

Figure 2. A. Monteiro's storm-petrel *Hydrobates monteiroi* in an artificial nest at Praia Islet with a recently hatched chick and remnants of eggshell; **B.** Madeiran wall lizard *Teira dugesii* eating a chick of Monteiro's storm-petrel in an artificial nest at Praia Islet.

and 3 vertebrates (Suppl. material 2: Table S2 and Suppl. material 3: Dataset S1). Most faecal samples included invertebrates and/ or plants (seven and six out of seven, respectively), and some of them were composed of only one of these groups. Three out of the seven samples contained vertebrate remnants. The vertebrates matched two birds that breed at Praia Islet, the rock pigeon *Columba livia* and *Hydrobates castro*, which most likely corresponds to *H. monteiroi* considering the small fragment size, the recent taxonomic split and phylogenetic proximity, and the absence of 12S sequences of the latter in public databases. It also included the Madeiran wall lizard itself, indicating a potential failure of the blocking primer, cannibalism or scavenging.

Discussion

Our study indicates that the values for stable carbon isotopes are higher in lizards caught around nest sites than in those caught around the support house, suggesting a possible higher reliance on marine-derived items in the diet of lizards caught in proximity to breeding seabirds. The marine-derived items on the lizards' diet can include remains of hatched eggs (eggshell or membrane), prey dropped in the colony by terns and/ or seabirds (resulting from either scavenging or predation). There was also a higher marker for marine items after the hatching of the chicks than during incubation around the nests, as indicated by the higher carbon isotope levels. Although not significant, this difference is higher than the difference between the two study areas. However, this analysis does not allow distinguishing between marine items consumed and that could have been seabirds in the form of eggs (either whole or remains of eggshell and membrane after hatching), chicks, dead birds, faeces or dropped fish and regurgitate.

The SI analysis also revealed one individual with a stable carbon isotope ratio almost as high as was found in the chicks a month before the hatching started, an indication that marine food sources, other than Monteiro's storm-petrel chicks, are available. These can probably be attributed to Macaronesian shearwater and band-rumped storm-petrel, species that breed during the winter period. In general, there was a wider variance in the stable carbon ratios for the lizards at the nests which indicates that some individuals specialise more in terrestrial food and some more in marine food. Previous studies on lizards and SIs have shown that soil lithology may affect the values of stable carbon isotopes (Martín et al. 2017), but in our case the study islet is small and there is no variation in soil type.

The faecal sample analysis using metabarcoding indicated that these lizards are highly reliant on invertebrates and plants as was found for this species at Selvagens Islands (Sadek 1981; Gil et al. 2020). In addition, that analysis provided a good complement to the SI results and confirmed the presence of seabirds in the diet of Madeiran wall lizards. During fieldwork, we could confirm the deaths of three Monteiro's storm-petrel chicks in the nesting area. Two chicks showed no injuries and the third one was dragged to the nest's entrance and lizards were eating from it, but it is unclear if the bite marks were the cause of death or if the lizards were only taking advantage of the carcass. The next-generation sequencing also showed the presence of seabirds, but we could not confirm if that resulted from scavenging or predation.

It is important to consider that Praia Islet only holds an estimated 150–183 pairs of Monteiro's storm-petrel (Monteiro et al. 1999; Oliveira et al. 2016). The restricted distribution and small breeding numbers of this endemic species make it much less resilient to any impact factors, such as putative predation by lizards. Recently, Lopes et al. (2019) found evidence that the main prey of an endemic and Endangered lizard, the Cabo Verde giant wall gecko *Tarentola gigas*, is the endemic and Critically Endangered bird Raso lark *Alauda razae*, confirming the importance of monitoring the impact of these trophic interactions, even when considering endemic species, especially during drought years when the population of Raso lark can be severely reduced (Lopes et al. 2019).

This study provides a first step towards understanding the trophic links at one of the main seabird sanctuaries in the Azores. Our results also confirm the potential of next-generation sequencing to further our understanding of the lizard/ storm-petrel trophic interactions. Marine-derived food items are not ubiquitous in a lizard's diet and to assess lizards' impact on the breeding success of Monteiro's storm-petrel it would be necessary to increase the sample size and to sample in different seasons. Further studies should also attempt to use wildlife cameras on the nests to study the interactions between lizards and storm-petrels and clarify if the presence of marine items in the diet of the Madeiran wall lizard result from direct predation of viable eggs and nestlings or from scavenging of chicks that died for other reasons or remains of the membrane from hatched eggs.

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Supplementary material 1

Table S1: Forward and reverse primers used in this study. The amplified region, the fragment length (in base pairs, bp), the sequences, and the reference for each primer set is given

Authors: Verónica Neves, Dorothee Rund, Catarina J. Pinho, Raquel Vasconcelos, Paco Bustamante, Petra Quillfeldt

Data type: Word file

Explanation note: This table is referred to in the Methods section and specifies the DNA markers selected for amplification to identify prey types.

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Link: https://doi.org/10.3897/herpetozoa.35.e82096.suppl1

Supplementary material 2

Table S2: Results of the metabarcoding analyses. List of Operational Taxonomic Units (OTUs) sequenced from the faecal pellets of Madeiran wall lizard for the different taxonomic groups and the incidence (n) of the identified taxa

Authors: Verónica Neves, Dorothee Rund, Catarina J. Pinho, Raquel Vasconcelos, Paco Bustamante, Petra Quillfeldt

Data type: Word file

Explanation note: This table is referred to in the results section and lists all the prey items identified through next-generation sequencing of Madeiran wall lizard faeces.

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Supplementary material 3

Dataset S1: Taxonomic identification of the haplotypes sequenced for the three taxonomic groups (plants, invertebrates and vertebrates). Code, sequence, length, amplification primer, number of reads, incidence, taxonomic identification (final, at species, family, order and class level), and percentage of match in blast is given for each haplotype

Authors: Verónica Neves, Dorothee Rund, Catarina J. Pinho, Raquel Vasconcelos, Paco Bustamante, Petra Quillfeldt

Data type: Excel file

Explanation note: This table is referred to in the Results section and contains the taxonomic identification of the haplotypes sequenced for the three taxonomic groups (plants, invertebrates and vertebrates), namely: ID, code, sequence, sequence length, amplification primer, incidence (number of reads, number of samples), taxonomic identification (final, at species, family, order and class level), and percentage of match in blast is given for each haplotype.

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Link: https://doi.org/10.3897/herpetozoa.35.e82096.suppl3

Article C

Aliens on Boats? The Eastern and Western Expansion of the African House Gecko

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Aliens on Boats? The Eastern and Western Expansion of the African House Gecko

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Abstract: Invasive species disrupt relations between endemics and their ecosystem and are an increasing biodiversity conservation problem. The *Hemidactylus* genus comprises the most successful invasive reptile species, including the worldwide-distributed *Hemidactylus mabouia*. In this study, we used 12S and ND2 sequences to taxonomically identify and tentatively determine the diversity and origin of these invaders in Cabo Verde while also clarifying this for several Western Indian Ocean (WIO) populations. By comparing our sequences to recently published ones, we showed, for the first time, that Cabo Verde individuals belong to the *H. mabouia sensu stricto* lineage and that both of its sublineages (a and b) occur there. Both haplotypes are also in Madeira, which indicates a connection between these archipelagos, possibly related to the past Portuguese trading routes. Across the WIO, results clarified the identity of many island and coastal populations, showing that this likely invasive *H. mabouia* lineage is widespread in the region, including northern Madagascar, with important conservation implications. Colonisation origins were difficult to access due to the wide geographical spread of these haplotypes; thus, several possible scenarios were outlined. The introduction of this species throughout western and eastern Africa may threaten endemic taxa and needs to be closely monitored.

Keywords: invasive species; dispersal; distribution; *Hemidactylus mabouia*; *Hemidactlylus mercatorius*; Cabo Verde; Western Indian Ocean islands; mtDNA

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1. Introduction

Since the industrial revolution, the displacement of native species by invasive species and their impact on the introduced ecosystems has been a problem for biodiversity conservation [1]. Hasty anthropogenic globalisation is leading geographically distant areas to become closer than they have ever been. The establishment of trade routes allowed an easy and faster way for species to relocate and enabled the establishment of populations in ecosystems that were otherwise unreachable [2]. Invasive species have been found to disrupt relations between endemic species and their ecosystem, for instance, by competition for resources, predation, and transmission of diseases [3,4]. These invaders can present several attributes that provide them with an advantage over native taxa, such as the possibility of repeated transport or the absence of natural predators and parasites [5]. Native (often endemic) species are at a disadvantage by not being adapted to compete with the invader. In this regard, reptiles are among the most affected groups since they are widely introduced, both unintentionally and through illegal trade, while there is a lack of studies and models to predict such invasions [6]. This problem increases in importance in insular ecosystems, which are often species-poor and disharmonic (i.e., with a biased taxa

representation), although richer in endemic species than continents [7]. On islands, native species have often evolved and adapted to peculiar lifestyles, for example, to have smaller clutches with larger-sized offspring [7]. Additionally, insular populations tend to lose their escape behaviour due to the rarity or lack of predators and, thus, are more susceptible to introduced species [8]. Their usual small ranges make them particularly sensitive to anthropogenic changes and extinction [9].

The *Hemidactylus* genus comprises some of the most successful invasive reptile species in the world [10]. This fact is likely explained by their adaptations to travel great distances, such as fat reserves and calcareous and adhesive eggshells resistant to seawater [11]. The ability of some *Hemidactylus* species to thrive near humans has led to the displacement of endemic geckos in some biodiversity hotspots [12]. One example is *Hemidactylus mabouia* (Moreau de Jonnès, 1818), which currently has a worldwide distribution [13]. This species can easily establish populations in urban and semi-urban habitats by taking advantage of insect clusters that form around artificial light sources [14], inhibiting native species from the possibility of approaching these high food-density places [15,16]. This, combined with aggressive males that create a competitive environment surrounding these food sources, can lead to the displacement of native populations [17]. The morphology of this species, accompanied by its role in the ecosystem, can also be key factors in explaining its success. Combining ambush and pursuit tactics [18], *H. mabouia* has a limb proportion that may give it an advantage over native species [19].

A recent work suggests that *H. mabouia* originated in the Zambezian biogeographic region (Central–East Africa) and includes as many as 20 putative species-level lineages [10]. Of those, *H. mabouia sensu stricto* (native to the Congo Basin, West Africa; herein, *H. mabouia ss*) is invasive throughout Africa and the Americas [10]. According to that study, which focused on the variation of the latter across Africa and parts of the West Indies, Florida, and South America, two shallow subclades were detected. Both subclades arrived in the New World, presumably with the slave trade and recent global trade, likely through multiple colonisations [10]. Further translocations within the western hemisphere may have contributed to its spread, but additional sampling and higher-resolution molecular data are still needed to unveil this more deeply. However, the clear delimitation between *Hemidactylus mercatorius* Gray, 1882 and *H. mabouia* and the identity and origin of some Western Indian Ocean (WIO) islands and both eastern and western African populations remain particularly confusing, leading authors to refer to an '*H. mercatorius–mabouia* species complex' and to suggest that *H. mercatorius* should be the designation applied to the Gulf of Guinea Islands, Malagasy, Comoran, and Seychelles populations pending further studies [20]. This led to the designation of the Cabo Verde populations also as *H. mercatorius* in some recent studies [21]. For simplicity, here we use the designation *H. mabouia*, currently used by the majority of the researchers to refer to populations outside Madagascar (e.g., [10]).

Due to increased globalisation, the number of translocated species has been rising, leading to more introduction events [22]. In fact, it was just found that Madeira Island also harbours both *H. mabouia ss* subclades' most frequent haplotypes, which could have resulted from multiple introductions as well [23]. However, the number and origin of colonisations that explain that pattern remain uncertain with current data. These two subclades are often found in sympatry both in Africa and in South America; thus, multiple or a single colonisation event from a diverse population are plausible alternatives [23].

The presence of *H. mabouia* in Cabo Verde has been known since 2001 [24]. However, no thorough analysis has been performed so far concerning their genetic variation and putative origins. The Cabo Verde Archipelago is situated about 500 km off the west coast of Africa and comprises ten volcanic islands and several islets. It belongs to the biogeographical region of Macaronesia and the Mediterranean Basin biodiversity hotspot. It holds 32 endemic terrestrial reptile taxa from three genera, including four native species of the genus *Hemidactylus* [21,25], in addition to the introduced *Hemidactylus angulatus* Hallowell, 1854 and *H. mabouia*. The latter was first recorded on the island of São Vicente [24] and then on Santo Antão, Brava, and all nearby ports and main villages [21].

Across several WIO islands and eastern African coastal localities, the systematics and distribution of *Hemidactylus* are still particularly unclear. Based on previous works [20] that used different markers from the currently most comprehensive study [10], the Aldabra Archipelago (at the northwest of Madagascar) harbours a set of endemic haplotypes closely related to the most widespread Malagasy ones, assigned to *H. mercatorius*. However, the Comoros Archipelago (Mayotte), Seychelles (Mahé), Mascarenes (Reunion), several eastern African coastal localities throughout Tanzania (including Zanzibar and Pemba islands), and South Africa harbour haplotypes closely related to the ones assigned to a divergent lineage of *H. mercatorius* from North Madagascar [26,27]. Individuals from Uganda and the West African Gulf of Guinea islands, such as S. Tomé, Príncipe, and Annobón, also belong to this latter clade, which is hypothesised to reflect human-aided dispersals [10,28].

Taking advantage of the most recent data [10], the main goal of this work was to place these islands' populations in this new detailed phylogenetic context for this species-complex, finding their closest phylogenetic affinities, as well as characterise their diversity and identify the possible geographical origin(s) of *H. mabouia* in Cabo Verde using molecular tools. Importantly, we compared them to populations from several West African and WIO locations, intending to definitely clarify their species–identity (*H. mabouia* vs. *H. mercatorius*). By sequencing several new samples from Cabo Verde and re-sequencing several representatives of the main haplogroups previously identified across the WIO and comparing them to the recently available phylogenetic data [10], we aim to answer the following questions. Are the introduced populations of *H. mabouia* in Cabo Verde derived from single or multiple events? What is the genetic variation of the species throughout the archipelago? To which of the 20 species-level lineages previously described do they belong? Where do the previously described *H. mercatorius–mabouia* lineages fall within the current phylogenetic framework for this group? With this work, we have clarified the taxonomic identity and origins of several island populations from both the western and eastern sides of the African continent.

2. Materials and Methods

2.1. Data Collection

For Cabo Verde, we collected a total of 11 individuals in the field morphologically assigned to *H. mabouia* from 2006 to 2012. We collected and preserved their tail tips in 96% ethanol and successfully amplified and sequenced the mtDNA 12S ribosomal RNA marker for ten individuals. We further sequenced the mitochondrial gene ND2 in two of those specimens. To further clarify their taxonomy, we re-sequenced representatives of the main *H. mercatorius–mabouia* complex haplogroups previously identified in the WIO region for the ND2 marker [20] to enable data to be directly compared to other studies [10]. We sequenced five individuals from clade A-I, representing five different 16S haplotypes and localities (H1/MY46; H3/TZ12; H5/SA20; H7/MA5; H8/Z40; check [10]), plus three individuals from clade III (endemic to Aldabra Archipelago; check [10]), also from different islands (see details in Table S1 in Supplementary Material). All samples included in these analyses with available geographical coordinates are shown in Figure 1.

We extracted DNA from tissue samples using a standard high-salt protocol [29]. We amplified the 12S marker following a previous study [30]. We amplified the ND2 fragment using the primers MetF1/L4437 and H5934 [31]. For the PCR, we used an initial activation temperature of 95 °C for 15 min, a denaturation temperature of 95 °C for 30 s, annealing at 60 °C for 35 s, extension at 72 °C for 45 s for 35 cycles, and finally, 10 min at 72 °C.

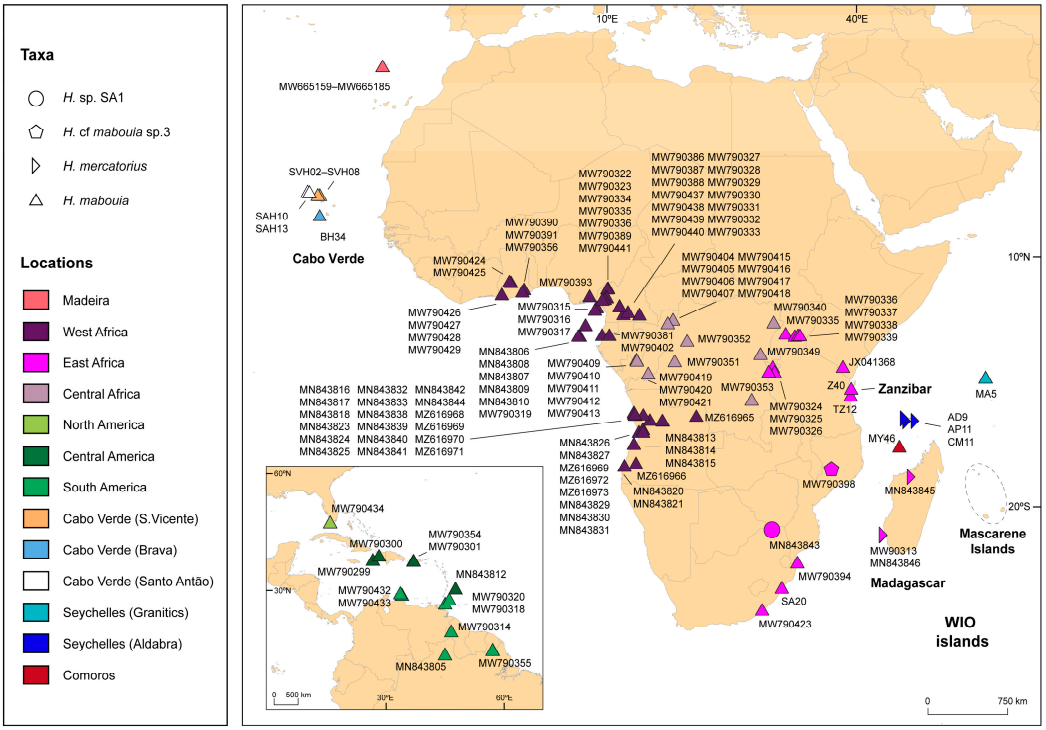

Figure 1. Map representing the geographical location of the samples used in this study for both 12S and ND2 markers. The bottom left corner represents samples from the Americas. Samples obtained from GenBank are represented by accession numbers, and samples obtained in this study are represented by tissue codes. Different symbols represent the different taxa and are coloured according to location. For further details, please check Table S1.

2.2. Alignment and Phylogenetic Inference

For ND2, from a first visual examination of a larger alignment including representatives of the different species-level lineages identified in the previous study [10], it was clear that all our individuals were most similar to those identified as *H. mabouia ss* and *H. mercatorius* (only the ones from Aldabra Archipelago). Thus, for further analyses, we used only the most similar ND2 sequences to ours, 892 base pairs plus a couple of outgroup sequences (see below). For 12S, we collected from GenBank all available *Hemidactylus* 12S sequences that aligned with our fragment of 349 base pairs. In total, we retrieved 63 12S and 133 ND2 sequences and added them to our dataset. Detailed information about the locality and GenBank accession codes is presented in Table S1. We visualised all 12S and ND2 sequences using Geneious [32] and aligned them using MAFFT v7.505 [33] with the -auto option, which automatically selects the appropriate alignment strategy according to the data characteristics.

In detail, first, we built phylogenetic trees for the ND2 dataset with our 10 sequences, plus 127 *H. mabouia* ss sequences, three *H. mercatorius*, and one *Hemidactylus* c.f. *mabouia* sp. 3 plus two *Hemidactylus* sp. SA1 as outgroups. All sequences but ours are from the most recent and complete study on this group [10] (see details in Table S1 in Supplementary Material). These were collapsed into 46 haplotypes using ALTER [34]—only different haplotypes were kept except for the individuals sampled in this study. We used the IQ-TREE 2 [35] to infer a Maximum-Likelihood (ML) tree with 1000 bootstrap replicates. The best model (inferred through ModelFinder [36]) for the ML analyses was the TIM2 + F + G4. Then we used MrBayes 3.2 [37] to construct a phylogenetic tree under Bayesian inference (BI) using the same model. We ran BI analyses twice for 10^7 generations and a sampling frequency of 10^3 to produce 10^4 trees. After examining the loglikelihood curves, we discarded 20% of the initial samples as burn-in. We considered $\geq$70% bootstrap proportion and $\geq$0.95 posterior probability as evidence for highly supported branches. For *H. mabouia ss*, as divergence was

low, we further built a haplotype network with the 134 ND2 sequences to best visualise the relationships between all individuals using TCS [38] with the connection limit set to 40 steps, so that all haplotypes were connected as in the previous reference study [10]. Finally, we also constructed a haplotype network with 72 sequences for the 12S fragment using TCS with default parameters. We used FigTree v1.4.4 (https://github.com/rambaut/figtree, accessed on 12 July 2022) and tcsBU v1.21a [39] to visualise the results.

3. Results

ML and BI phylogenetic trees for the ND2 fragment had largely congruent topologies. The ML tree is shown in Figure 2 with support values from both methods. Both trees with detailed support values can be found in the Supplementary Materials (Figure S1). As in the previous study [10], they showed a clear distinction between the two major clades, *H. mercatorius* and *H. mabouia ss*, which were well-supported clades by both methods. Then two groups were present within *H. mabouia ss*: a highly supported one corresponding to *H. mabouia* ss a [10] and the other corresponding to *H. mabouia ss* b (unsupported; 52% bootstrap at ML). The two individuals from Cabo Verde sequenced for this marker (SVH03 and SVH04) are both well-nested into the *H. mabouia* ss b clade, exhibiting identical haplotypes. The WIO individuals fell in different clades: the ones from Aldabra Archipelago clearly grouped within *H. mercatorius* samples from Madagascar, most closely related to the sample from north-western Madagascar, while all the others (from Seychelles, Comoros, Tanzania, and South Africa) fell both within *H. mabouia* ss a and b.

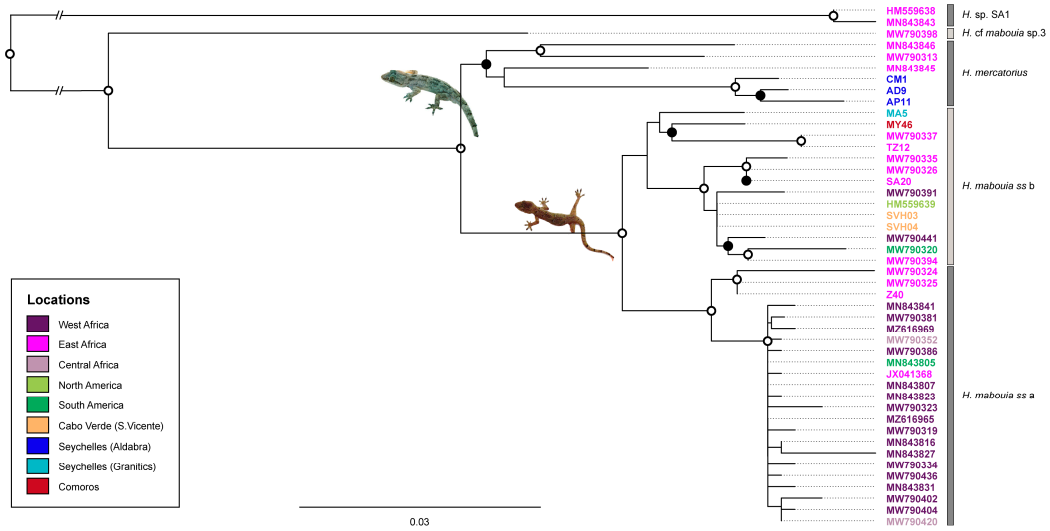

Figure 2. Maximum likelihood (ML) tree for the ND2 fragment showing the relationships between *Hemidactylus* from Cabo Verde and WIO region and their relatives. Accession numbers are colour coded geographically (for details, see Table S1). The designation of *H. mabouia* ss a/b follows a recent study [10]. Bootstrap (≥70%) and posterior probability (BI) support (>95%) are represented at nodes (solid black dots if there was ML support and open white dots if there was both ML and BI support). The two basal branches were shortened for graphical purposes, and *Hemidactylus* sp. SA1 was used as an outgroup [10]. See Figure 1 and Table S1 for detailed locality data and GenBank accession numbers of the new sequences.

The ND2 network (Figure 3) represents the relationships within the two *H. mabouia ss* clades in greater detail. The two major haplotypes widespread across central and western Africa reaching the Americas are clearly identifiable as the most frequent (and roughly central) of each clade. For this marker, only one haplotype was detected in Cabo Verde, precisely the most frequent one of *H. mabouia* ss clade b, in individuals that also shared the same 12S haplotype (Figure 4). For the individuals from the WIO, the situation is slightly different: several haplotypes were detected, belonging to both clades, but none corresponded to the most frequent and widespread of each clade. The sample from

Zanzibar that clustered within *H. mabouia* ss a (Z40) exhibited a haplotype also found in Burundi and the Democratic Republic of Congo (DRC), a few mutation steps from the central one. The remaining samples fell within clade ss b. The South African sample (SA20) shared its haplotype with samples from Burundi, Uganda, and DRC; the Seychelles sample (MA5, from Mahé Island) exhibited a haplotype also found in Uganda, which is also the case of the sample from Tanzania (TZ12). A sample from Comoros Archipelago (MY46, from Mayotte Island) presents a unique haplotype, more or less equally distant from the latter two.

We were able to amplify and sequence a larger number of samples from Cabo Verde for 12S than for ND2, and thus a 12S haplotype network is represented in Figure 4. It further shows the presence also in Cabo Verde of the two major *H. mabouia* ss haplotypes, the same ones spread across the large areas of West Africa and the Americas, as well as in Madeira.

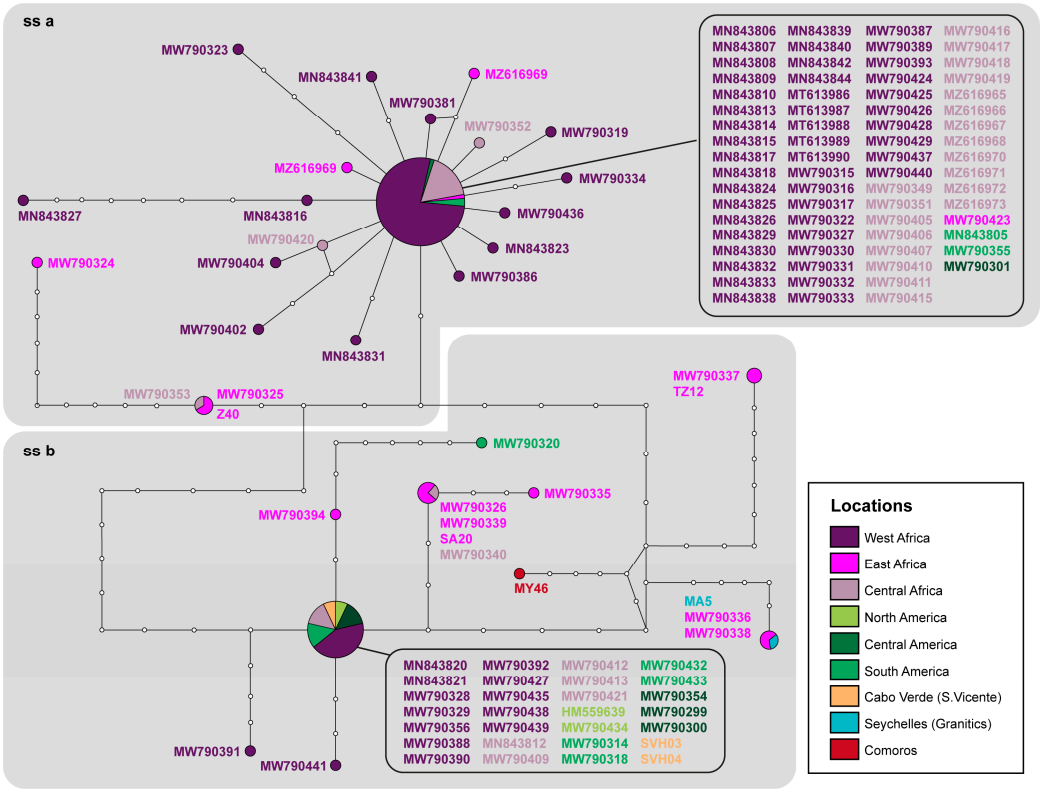

Figure 3. Haplotype network for ND2 mitochondrial marker. White dots along the branches represent missing or unsampled haplotypes. Haplotypes are colour coded geographically (for details, see Table S1) as well as the accession numbers/tissue codes which are given next to the nodes or in boxes. The circle size represents the haplotype frequency. The grey boxes in the network delimit *H. mabouia* ss a (**ss a**) and *H. mabouia* ss b (**ss b**), following the ML tree and a previous study [10].

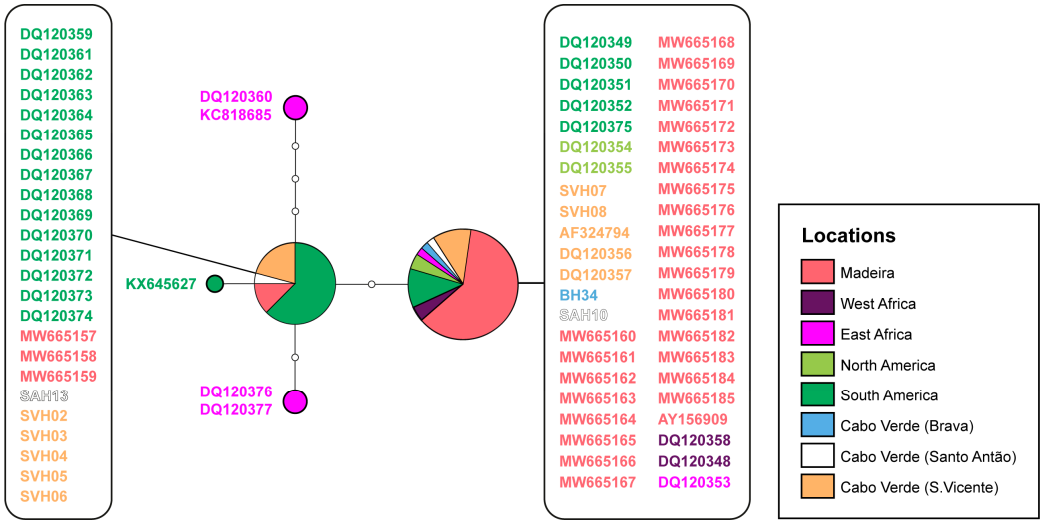

Figure 4. Haplotype network for 12S ribosomal RNA marker. White dots along the branches represent missing or unsampled haplotypes. Haplotypes are colour coded geographically (for details, see Table S1) as well as the accession numbers/tissue codes which are given next to the nodes or in boxes. The circle size represents the haplotype frequency.

4. Discussion

Molecular phylogenetic methods are of great utility to precisely identify cases of cryptic species and lineages and tentatively infer colonisation or invasion routes, as is the case here. The close relationship between *H. mabouia ss* and *H. mercatorius* and the unclear identity of some populations was clearly shown in a previous study [20], to the point that some refer to specimens from Cabo Verde as *H. mercatorius* [21]. While these doubts remained for years due to the use of different gene fragments by different authors—16S rRNA was used [20], leading to incomparable datasets until now—the most recent study showed clearly that *H. mabouia* is a species-complex of circa 20 species-level lineages, as well as that the populations consensually called *H. mercatorius* (from central-south Madagascar) make *H. mabouia* paraphyletic [10]. Importantly, amongst the several *H. mabouia* lineages, they define *H. mabouia ss* as a sister taxon to *H. mercatorius* and a well-supported clade of two sublineages (a and b) widely distributed across sub-Saharan Africa, the West African islands of S. Tomé, Príncipe, and Bioko, and several localities of the Americas. Phylogeographic patterns and biogeographic reconstructions support the origin of *H. mabouia ss* in the East African Valley region, with relatively recent expansions throughout its western range, including to the Gulf of Guinea Islands and South America. At least throughout this western range, this species is most likely invasive, given its clear preference for human-dominated habitats and its proven ability to outcompete and directly prey on both native and other invasive geckos [14,16,17,40].

This new phylogenetic framework provided the ideal opportunity to further clarify the identity of several island and coastal populations on both sides of the African continent, including the ones from Cabo Verde, while at the same time exploring possible colonisation routes and origins (native or introduced) of several island populations, which are of utmost importance for conservation management. As such, the phylogenies now generated by combining representatives of the haplogroups/clades described in previous works [20] with the recently generated ND2 data provided us with important insights: populations from Cabo Verde are clearly assigned to *H. mabouia ss*, as well as all the representatives of the WIO populations, except for the ones of the Aldabra Archipelago, which are undoubtedly a lineage of *H. mercatorius* endemic to that archipelago. This allows us to say that the lineage referred to as *H. mabouia–mercatorius* clade A–I [20] are, in fact, *H. mabouia ss*, implying that these populations found in the islands of Mayotte (Comoros), Mahé (Seychelles), Reunion (Mascarenes) [41], Zanzibar, and Pemba (Tanzania), as well as the ones present in some

(mostly coastal but not only) localities of both Tanzania and South Africa, are highly likely introduced through human-aided transport, which seems to be the predominant pattern of this species. This was, in fact, expected given their apparently restricted distribution and only recent detection across some of these locations (1905 and 2010 for Mahé and Reunion, respectively). With respect to the mainland locations where this species may have spread naturally vs. due to human aid, however, a much more detailed sampling throughout East Africa and higher resolution molecular data will be needed to properly clarify it. Populations from the Gulf of Guinea Island of Annobón (southwest of S. Tomé) [20] also belong to this clade. This is important information in terms of invasive species management, especially for those small islands where these introduced species may have a significant negative impact on native ones. Notably, these new results also allow us to infer that *H. mabouia ss* is present in northern localities of Madagascar (at least in Montagne des Français, Sambava, and Andapa—haplotypes H9, H11 and H37 from clade A–I [20]), corresponding to what has been erroneously interpreted as an *H. mercatorious* divergent lineage from that area [20,26,27]. Again, this adds to the list of invasive reptiles of these biodiversity hotspot islands and certainly has important conservation implications.

Regarding the genetic diversity of the introduced populations across these places, for Cabo Verde, it is now clear, taking together the 12S and ND2 results, that, similarly to what was found for Madeira and South America, both *H. mabouia ss* subclades are present, with individuals from subclade a (the most abundant one also in Madeira) present in S. Vicente, Santo Antão, and Brava and further populations assigned to subclade b in S. Vicente and Santo Antão for now. For ND2, only two individuals from Cabo Verde were successfully amplified (our samples were very old and most of them were too degraded to sequence), these exhibiting the most frequent and widespread haplotype from subclade b. However, for 12S, individuals from different islands were successfully amplified and sequenced, and one haplotype of each clade was found overall, and these were the same ones widespread across Madeira, South America, and also present in S. Tomé, Príncipe, and Bioko islands, clearly pointing to the expected scenario for this species of a relatively recent human-aided invasion of Cabo Verde. The fact that both haplotypes present in Cabo Verde are also present in Madeira indicates a possible connection between these archipelagos [23]. In fact, other studies have shown a possible colonisation route linking Madeira and Cabo Verde mediated by humans in mice *Mus musculus* [42]. This opposes previous studies, which claimed that *H. mabouia* found in Cabo Verde and Madeira all belong to one lineage [43] and that one of the haplotypes found in Madeira was exclusively related to South American individuals [23], and highlights the importance of a comprehensive sampling before any conclusions about dispersal patterns can be made, especially in species with cosmopolitan distributions, such as this one. Colonisation origins of the populations from Cabo Verde are still impossible to resolve due to the wide geographical spread of these haplotypes. Several scenarios are possible according to our results: a single south American origin from a place where both clades exist in sympatry, such as Brazil, as proposed for Madeira [23] or Puerto Rico [10]; a single colonisation from West Africa, again from a place where both clades co-habit; multiple colonisations both from South America or West Africa (or both); or a (single or double) colonisation from Madeira, such as hypothesised for *M. musculus* [42]. Further data with much higher resolution and a much more comprehensive sampling will be needed to distinguish these hypotheses and further clarify the phylogeographic and invasion patterns of this species.

In the WIO islands, the pattern was different. Although both *H. mabouia ss* subclades were again found in the region, none of the haplotypes found was the most frequent (and widespread) of each clade. On the contrary, there, we found low-frequency haplotypes some mutations away from the most frequent one, in most cases shared with East African individuals from Burundi, Cameroon, and Uganda from a previous study [10]. The sequenced representatives of *H. mercatorius–mabouia* clade A–I cover all the variation previously described for 16S within this group [20], which is completely unstructured, with many haplotypes shared between East African, Comoroan, and Seychelles populations.

Only five representatives were sequenced in this work. The 16S data in a previous work [20] provided a more complete picture of the extensive degree of haplotype sharing across these locations. Altogether, this pattern most likely reflects multiple origins along the east part of the East African Rift Valley for these WIO islands populations, from a much more diverse genetic pool that the one on the western African coast. However, precise origins for these introductions of the island populations and clarification of the native vs. introduced origin of continental East African populations must await a much more comprehensive sampling across its East African distribution and, again, higher resolution markers. Given the case that haplotypes are most often identical to other locations, there would be no power in the current data to estimate colonisation ages. For that, a population genetics approach with higher resolution markers should follow.

Importantly, these inferences are so far based on mtDNA only. For a thorough look into dispersal and differentiation patterns and processes and species delimitations across this complex of circa 20 species-level lineages, multiple nuclear markers should be taken into account. These may also be important to understand if any genetic impact was caused in native closely related populations due to hybridisation and/or introgression. However, that was outside the scope of this work.

The introduction of invasive species to both Cabo Verde and the WIO islands can threaten endemic taxa. Since *H. mabouia* is one of the most effective invaders of the *Hemidactylus* genus [10], they should be closely monitored to ensure that they do not outcompete the native species. This is especially important, knowing that the other introduced *Hemidactylus* species in Cabo Verde, *H. angulatus*, has been displacing the native *Hemidactylus boavistensis* (Boulenger 1906) [44]. Additionally, in the WIO Mauritius Island (Mascarenes), food competition was demonstrated between the introduced *Hemidactylus frenatus* and the native diurnal gecko *Phelsuma ornata* [45], as well as its effect in displacing the nocturnal endemic *Nactus* species [12]. Overall, throughout the world, invasive *Hemidactylus* have been constantly associated with the decline in native species, especially other geckos, both through site competition and direct predation (e.g., [46–48]) For this reason, and especially now knowing their specific identity and its usual invasive behaviour, efforts should be made to clearly assess their current distribution and abundance across these different islands, and avoid their spread within and across them. Considering the noticeable fauna of endemic diurnal and nocturnal geckos across all these islands, their impact is expected to be considerable.

5. Conclusions

In summary, we clarify the identity of populations from Cabo Verde as *H. mabouia ss*, describe their current genetic variation across the archipelago, confirm the distinctiveness of the Aldabra Archipelago population as an endemic lineage of *H. mercatorius*, and assign other populations from the WIO islands, including populations from the north of Madagascar also to *H. mabouia ss*. Although precise colonisation sources could not be inferred due to the widespread distribution of identical haplotypes, this preliminary data shows that Cabo Verde is related to West African, Madeira, and South American populations, while WIO island populations have clear affinities with East African populations. Detailed sampling, particularly in East Africa, and higher resolution molecular data are still needed to infer with more detail the biogeographic history and spread of this species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes14020381/s1, Table S1: Details of the *Hemidactylus* samples used in this study. The taxon (Hm SA1, *H.* sp. SA1; Hm sp3, *H.* cf. *mabouia* sp. 3; Hr, *H. mercatorius*; Hm, *H. mabouia sensu stricto*), tissue code, GenBank accession number (GenBank), country of origin (STP, São Tomé and Príncipe; DRC, Democratic Republic of the Congo), locality, geographical coordinates, and colour group of the sequences used for the two markers in this study are given. The * stands for new samples from this study; Figure S1: Phylogenetic trees. (A) Maximum likelihood tree, and (B) Bayesian inference for the ND2 fragment showing the relationships between *Hemidactylus* from Cabo Verde and WIO region and their relatives; File S1: Portuguese version of the abstract.

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Taxon	Voucher code	GeneBank	Marker	Country	Locality	Latitude	Longitude	Group
Hm SA1	CAS 248776	MN843843	ND2	South Africa	Limpopo Prov.	-22.7064	29.8289	East Africa
Hm SA1	AMB 8301	HM559638	ND2	South Africa	Limpopo Prov.	-	-	East Africa
Hm sp3	MVZ 265935	MW790398	ND2	Mozambique	Zambezia	-15.4639	36.9778	East Africa
Hr	AMNH R-16825	MN843845	ND2	Madagascar	Mahajanga	-16.3833	47.0381	East Africa
Hr	AMNH-R-165402	MW790313	ND2	Madagascar	Toliara	-23.3511	43.6870	East Africa
Hr	AMNH R-16826	MN843846	ND2	Madagascar	Toliara	-23.3006	43.6592	East Africa
Hr	AP11*	OQ266924	ND2	Seychelles	Assumption, Aldabra	-9.7342	46.5003	Seychelles (Aldabra)
Hr	AD9*	OQ266923	ND2	Seychelles	Picard, Aldabra	-9.4011	46.2060	Seychelles (Aldabra)
Hr	CM1*	OQ266925	ND2	Seychelles	Menai Island, Aldabra	-9.7100	47.5079	Seychelles (Aldabra)
Hm	YPM 14798	HM559639	ND2	USA	Florida	-	-	North America
Hm	LSUMZ 12403	MN843805	ND2	Brazil	Roraima	1.7958	-61.5281	South America
Hm	MB03-000965	MN843806	ND2	STP	Príncipe	1.6257	7.4138	West Africa
Hm	MB03-000998	MN843807	ND2	STP	São Tomé	0.3382	6.7323	West Africa
Hm	MB03-001025	MN843808	ND2	STP	Príncipe	1.6259	7.4138	West Africa
Hm	MB03-001059	MN843809	ND2	STP	São Tomé	0.3499	6.5411	West Africa
Hm	MB03-001060	MN843810	ND2	STP	São Tomé	0.3499	6.5411	West Africa
Hm	XXXX 267704	MN843812	ND2	Barbados	St. Joseph	13.1988	-59.5403	Central America
Hm	UF 187193	MN843813	ND2	Angola	Benguela	-12.6245	13.2327	West Africa
Hm	CAS 263381	MN843814	ND2	Angola	Benguela	-12.6245	13.2327	West Africa
Hm	CAS 263382	MN843815	ND2	Angola	Benguela	-12.6245	13.2327	West Africa
Hm	CAS 263373	MN843816	ND2	Angola	Luanda	-9.1830	13.3706	West Africa
Hm	CAS 263374	MN843817	ND2	Angola	Luanda	-9.1830	13.3706	West Africa
Hm	CAS 263375	MN843818	ND2	Angola	Luanda	-9.1830	13.3706	West Africa
Hm	CAS 263522	MN843820	ND2	Angola	Namibe	-15.2089	12.1015	West Africa
Hm	CAS 263523	MN843821	ND2	Angola	Namibe	-15.2089	12.1015	West Africa
Hm	CAS 263518	MN843823	ND2	Angola	Luanda	-8.9977	13.2638	West Africa
Hm	INBAC/AMB 1066	MN843824	ND2	Angola	Luanda	-8.8155	13.2359	West Africa
Hm	MHNC-UP/Rep 494	MN843825	ND2	Angola	Malanje	-9.7712	15.1572	West Africa
Hm	CAS 263432	MN843826	ND2	Angola	Kwanza Sul	-11.1981	13.8355	West Africa
Hm	CAS 263433	MN843827	ND2	Angola	Kwanza Sul	-11.1981	13.8355	West Africa
Hm	CAS 263431	MN843829	ND2	Angola	Kwanza Sul	-10.8675	14.3216	West Africa
Hm	CAS 263430	MN843830	ND2	Angola	Kwanza Sul	-10.8672	14.3244	West Africa
Hm	UF 187201	MN843831	ND2	Angola	Kwanza Sul	-10.8675	14.3228	West Africa
Hm	CAS 258427	MN843832	ND2	Angola	Malanje	-9.8727	16.7009	West Africa
Hm	INBAC/JVV 9516	MN843833	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	INBAC/JVV 9524	MN843838	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	INBAC/JVV 9525	MN843839	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	INBAC/JVV 9529	MN843840	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	INBAC/JVV 9531	MN843841	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	INBAC/JVV 9535	MN843842	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	CAS 263443	MN843844	ND2	Angola	Luanda	-9.1839	13.3722	West Africa
Hm	PEM R19461	MZ616965	ND2	Angola	Lunda	-8.4184	20.7419	West Africa
Hm	NB1083	MZ616966	ND2	Angola	Huíla	-14.9189	13.4853	West Africa
Hm	FKH0443	MZ616967	ND2	Angola	Cuanza	-9.1431	14.3672	West Africa
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Hm	FKH0031	MZ616969	ND2	Angola	Cuanza	-11.1972	14.3406	West Africa
Hm	FKH0219	MZ616970	ND2	Angola	Luanda	-9.0606	13.3539	West Africa
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Hm	CAS 207984	MW790317	ND2	Equatorial Guinea	Bioko Norte	3.4613	8.5524	West Africa
Hm	CAS 231714	MW790318	ND2	Trinidad & Tobago	Tunapuna-Piarco	10.5926	-61.3474	South America
Hm	CAS 233427	MW790319	ND2	STP	Príncipe	1.6399	7.4193	West Africa
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Hm	CAS 249873	MW790322	ND2	Cameroon	Northwest	6.0097	10.1295	West Africa
Hm	CAS 249883	MW790323	ND2	Cameroon	Northwest	5.9661	10.0420	West Africa
Hm	CAS 250633	MW790324	ND2	Burundi	Gitega	-3.4271	29.9346	East Africa
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Hm	CRSN84	MW790340	ND2	DRC	Orientale	1.9589	30.0357	Central Africa
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Hm	ELI 2274	MW790352	ND2	DRC	Equateur	-0.2594	19.6358	Central Africa
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Hm	MA5*	OQ266926	ND2	Seychelles	Airport, Mahé	-4.6717	55.5108	Seychelles (Granitics)
Hm	MY46*	OQ266927	ND2	Comoros	Chirongoui, Mayotte	-12.9360	45.1443	Comoros
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Hm	E609.23	DQ120355	12S	USA	Florida	-	-	North America
Hm	Bichoa	AF324794	12S	Cabo Verde	S. Vicente	-	-	Cabo Verde (S. Vicente)
Hm	31HbouSV	DQ120356	12S	Cabo Verde	S. Vicente	-	-	Cabo Verde (S. Vicente)
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		OQ266930	ND2					
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Hm	SVH08*	OQ267606	12S	Cabo Verde	S. Vicente	16.8923	-24.9895	Cabo Verde (S. Vicente)
Hm	BH34*	OQ267597	12S	Cabo Verde	Brava	14.8695	-24.6955	Cabo Verde (Brava)
Hm	SAH10*	OQ267598	12S	Cabo Verde	Santo Antão	17.1819	-25.0648	Cabo Verde (Santo Antão)
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Hm	E609.9	DQ120375	12S	Brazil	Rio Grande do Norte	-	-	South America
Hm	E609.10	DQ120359	12S	Brazil	Fernando de Noronha	-	-	South America

Hm	E1109.8	DQ120361	12S	Trinidad & Tobago	Tobago	-	- South America
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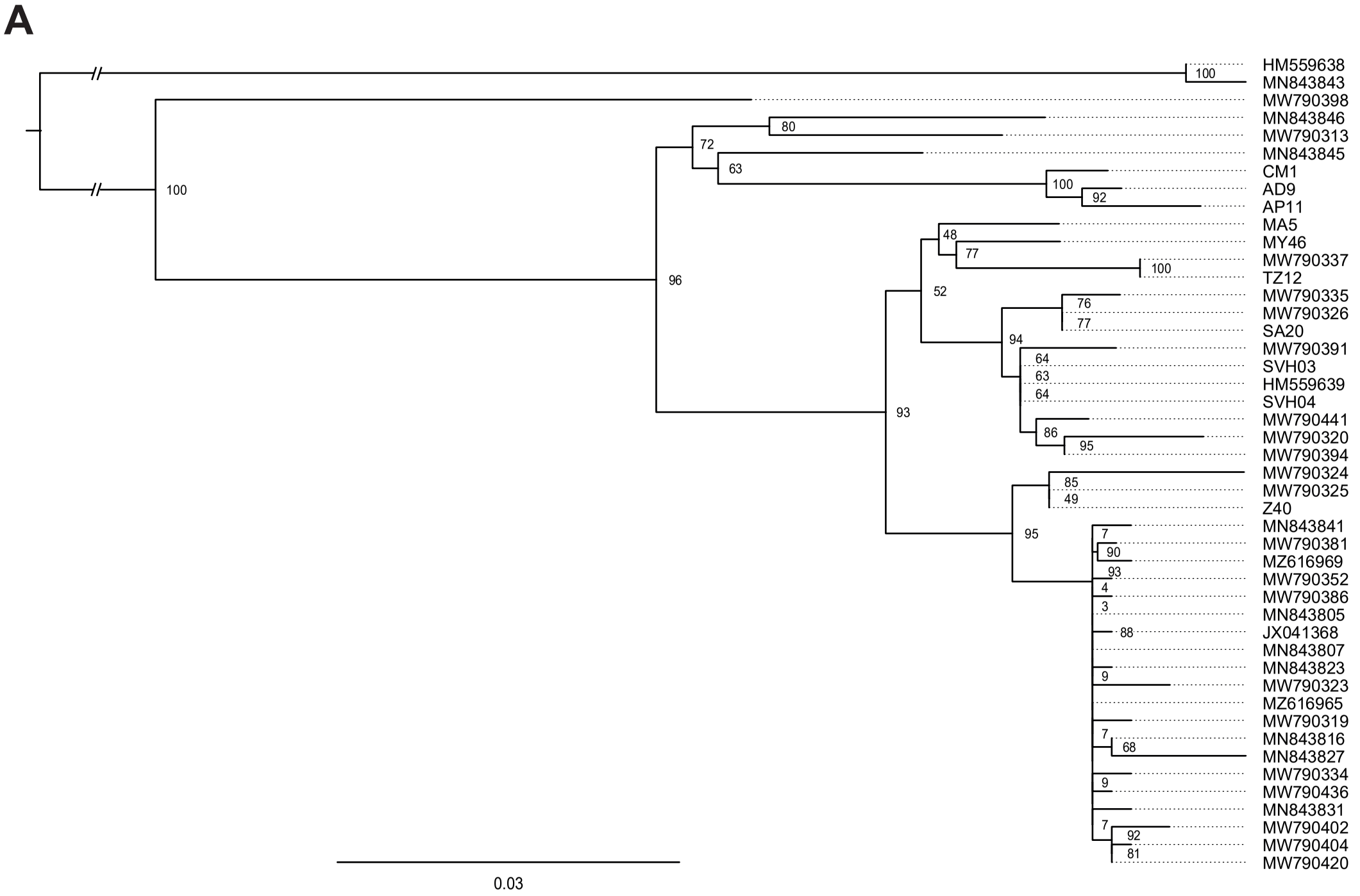
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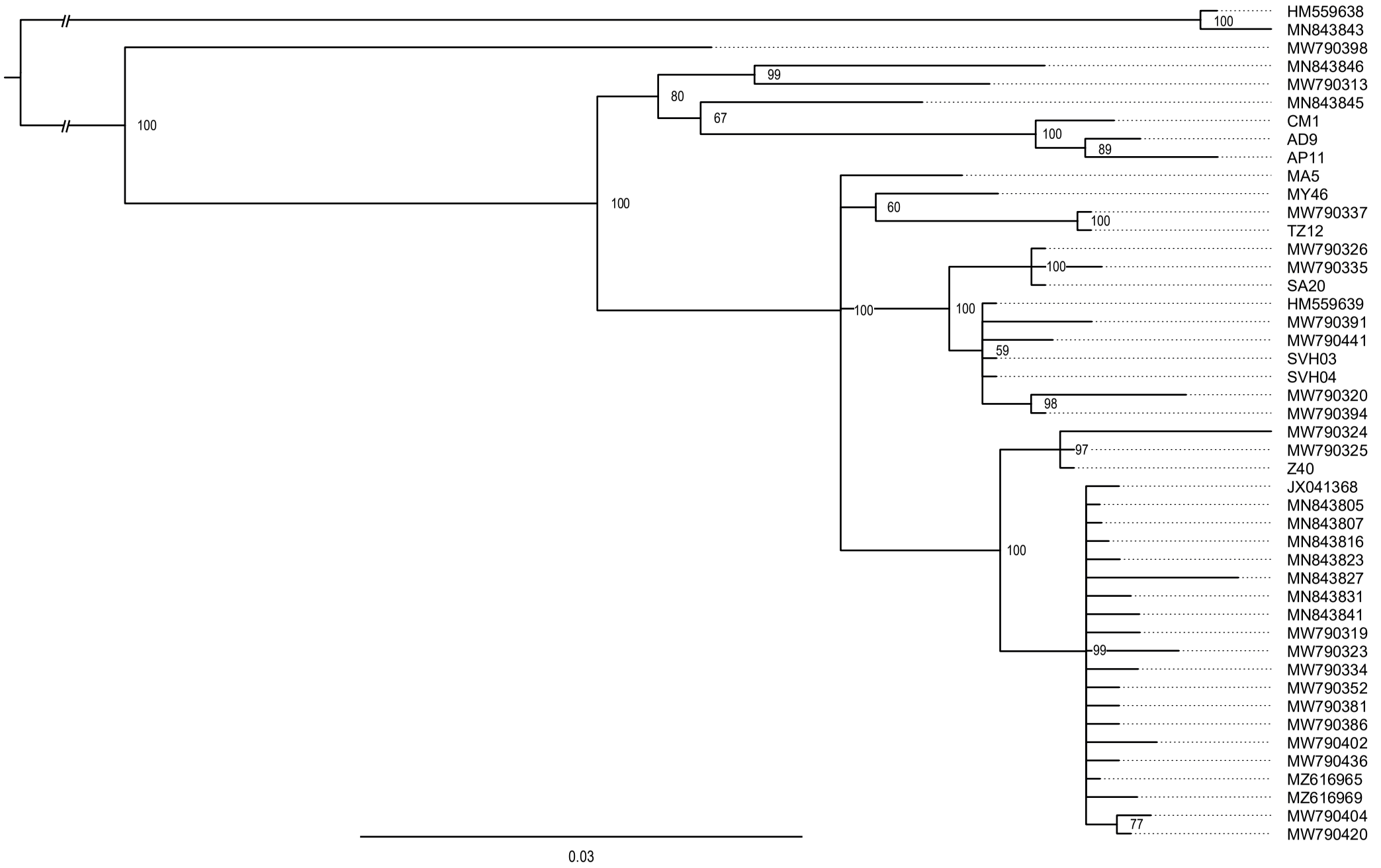
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Abstract in Portuguese

Espécies invasoras perturbam as relações entre espécies endémicas e respectivos ecossistemas e são um problema crescente de conservação da biodiversidade. O género *Hemidactylus* apresenta as espécies de répteis invasores de maior sucesso, como o *H. mabouia* distribuído globalmente. Neste estudo, usámos sequências de 12S e ND2 para identificar taxonomicamente e tentar determinar a diversidade e origem desses invasores em Cabo Verde, enquanto esclarecemos também isso para várias populações do Oceano Índico Ocidental (OIO). Comparando as nossas sequências com as recentemente publicadas, mostrámos, pela primeira vez, que os indivíduos de Cabo Verde pertencem à linhagem *H. mabouia sensu stricto* e que as duas sublinhagens desta (a e b) ocorrem aí. Ambos os haplótipos também se encontram na Madeira, o que indica uma possível ligação entre estes arquipélagos, possivelmente relacionada com as antigas rotas comerciais portuguesas. Em todo o OIO, os resultados esclareceram a identidade de muitas populações insulares e costeiras, mostrando que esta linhagem provavelmente invasora de *H. mabouia* está disseminada na região, incluindo no Norte de Madagáscar, com importantes implicações de conservação. As origens da colonização foram difíceis de definir devido à ampla distribuição geográfica desses haplótipos, assim vários cenários possíveis foram delineados. A introdução desta espécie na África Ocidental e Oriental pode ameaçar taxa endémicos e precisa ser cuidadosamente monitorizada.

Appendix B

Supplementary Reports

Relatório final

Medição do impacto do rato-doméstico nas plantas de Cabo Verde usando sequenciação de nova geração

Catarina J. Pinho [1, 2], Evandro P. Lopes [1, 3], Joana Paupério [1], Lara Almeida [3], Maria Romeiras [4], Isildo Gomes [5], Raquel Vasconcelos [1, 5,*]

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Resumo

As espécies introduzidas são uma das principais ameaças à biodiversidade, principalmente em ilhas. A introdução de ratos nestes sistemas já levou à devastação de alguns habitats e extinção de muitas espécies a nível mundial, causando também grandes perdas a nível económico. No entanto, os impactos destes micromamíferos nas plantas nativas e domésticas raramente são quantificados devido à falta de conhecimento taxonómico e morosidade na obtenção de dados usando métodos tradicionais de identificação morfológica dos itens predados. O objectivo deste projecto foi identificar quais as espécies de plantas nativas silvestres e domésticas que o rato-doméstico *Mus musculus* introduzido nas ilhas de Cabo Verde consome e quantificar a incidência do consumo de plantas relativamente a outros itens de dieta (invertebrados) recorrendo a novos métodos genéticos de sequenciação de ADN em massa, o *metabarcoding*. Tal permitiu obter essas informações de forma rápida e com elevada precisão a nível taxonómico, sem necessidade de envolver muitos especialistas, e avaliar o impacto da predação do *M. musculus.* Observou-se que este invasor consome 131 itens, nomeadamente 73 diferentes taxa de flora e 58 de invertebrados, tendo em geral um impacto negativo no ecossistema. Desta forma recomenda-se que esta espécie seja designada como prioritária para erradicação nos planos de conservação, nomeadamente nas Reservas Integrais dos Rombos e na Reserva Parcial de Santa Luzia, bem como no interior das áreas protegidas do país. O projecto foi ainda bem sucedido na formação de profissionais e estudantes nas metodologias envolvendo a captura de micromamíferos, colheita de amostras genéticas e procedimentos laboratoriais de *metabarcoding*, que capacitou as instituições da UniCV, do INIDA e ONGs locais para a continuação do projecto a nível nacional e a fortaleceu as relações pessoais e institucionais entre todos os membros.

Palavras-chave: ecologia trófica, espécie invasora, ilhas, *metabarcoding* de ADN, *Mus musculus*

Introdução

As espécies exóticas invasoras são uma das causas principais da perda de biodiversidade (Butchart *et al.*, 2010), principalmente em ilhas. Dentro destas, os gatos e os roedores são os que têm causado maiores danos no património natural de ilhas (Doherty *et al.*, 2016; Russell *et al.*, 2017). O rato-doméstico em particular está entre as 15 espécies mais prevalentes em ilhas no mundo (Russell *et al.*, 2017). Estes já contribuíram para a extinção mundial de espécies endémicas em Cabo Verde, como o lagarto gigante de Cocteau *Chioninia coctei* (Mateo et al., 2005) e para a extinção local da osga gigante de Cabo Verde (Siverio *et al.*, 2007).

Estes danos são difíceis de quantificar, pois o valor de uma espécie única em todo o mundo está por definir. Podemos, no entanto, imaginar que um dos escincídeos maiores do mundo, que atraiu a atenção de políticos, como recentemente do príncipe Alberto I do Mónaco, de grandes cientistas, como no século XIX de Barbosa de Bocage, e de exploradores como Leonardo Fea, teria atraído também actualmente a atenção de turistas da natureza. Fenómenos semelhantes foram observados com espécies de aves que só ocorrem noutras ilhas com forte dependência do turismo para o produto interno bruto do país (Mwebaze *et al.*, 2010).

Sabendo que o turismo é hoje uma das fontes principais do produto interno bruto de Cabo Verde (INE, 2017) e que as receitas de Produto Interno Bruto (PIB) em São Vicente rondariam os 500 milhões de euros em 2019 dada a tendência crescente anual que se estima pós-pandemia, podemos estimar que se perderá um centésimo desse valor por falta de atractivos na ilha e arredores (ilha de Santa Luzia que passou a ser reserva parcial e potencialmente aberta ao turismo), o que corresponde a 5 milhões de euros. De facto, estudos científicos recentes (Holmes *et al.*, 2019) apontaram Santa Luzia como um dos locais mais importantes a nível mundial onde a irradicação de roedores deveria acontecer para beneficiar a conservação de espécies ameaçadas.

De igual modo, é provável que estes roedores causem impacto negativo nas plantas nativas e nos cultivos, como já se verificou noutras ilhas de forma desastrosa (Bunce *et al.*, 2009). Este impacto espera-se que seja maior nas ilhas mais vegetadas, com maior número de plantas endémicas e com maior produção agrícola, como Santiago e Santo Antão (Brochmann *et al.*, 1997; MAA, 2015).

Infelizmente, os danos causados por estes roedores nas culturas agrícolas, à semelhança de outros países como Portugal, não são quantificados em Cabo Verde (Isildo Gomes, comunicação pessoal), mas sabe-se que os prejuízos causados noutros locais do mundo são gigantescos. Por exemplo, na Tanzânia, uma outra espécie de roedor causa perdas anuais de 5 a 15% do milho produzido, o que corresponde a alimento suficiente para 2 milhões de pessoas e a um prejuízo no valor de 45 milhões de dólares (Leirs, 2003).

Sabendo que em Cabo Verde se produzem cerca de 6 mil toneladas de milho anualmente (MAA, 2015), estima-se então que só nesta produção poderá haver perdas entre 300 a 900 toneladas, o que equivale a cerca de 3 mil a 9 mil milhões de escudos cabo-verdianos por ano (considerando os preços médios trimestrais de 2018 para o milho local; ERIS, 2019). Na Austrália, o rato-doméstico, a mesma espécie-alvo deste projecto, pode causar prejuízos que afectam até 4% da produção nacional agrícola desse país, equivalendo a perdas de 40 milhões de dólares nas piores épocas (Singleton, 1997).

Como tal, são necessárias medidas optimizadas para controlar, monitorizar e erradicar os roedores invasores (Stenseth *et al.*, 2003). No entanto, os impactos destes micromamíferos nas plantas nativas e domésticas raramente são quantificados devido à falta de conhecimento taxonómico e morosidade na obtenção de dados usando métodos tradicionais de identificação morfológica dos itens predados. Assim, recorrendo a novos métodos genéticos de sequenciação de ADN em massa, como o *metabarcoding* dos conteúdos digestivos, é possível obter essas informações de forma rápida e com elevada precisão a nível taxonómico, sem necessidade de envolver muitos especialistas (Pompanon *et al.*, 2012).

Por último, promovendo estudos sobre os efeitos dos roedores na fauna nativa e planeando estratégias para a erradicação destes, Cabo Verde estaria no caminho certo para atingir alguns dos objectivos de Desenvolvimento do Milénio das Nações Unidas (UN, 2015), como prevenir a perda de biodiversidade (objectivo 15.5) e prevenir e reduzir o impacto das espécies invasoras nos sistemas aquáticos e terrestres (objectivo 15.8), bem como respeitar os acordos assinados a nível internacional, como o da Convenção sobre a Diversidade Biológica.

Deste modo, este estudo teve como objectivo explorar os conhecimentos mais críticos em falta no país:

- Determinar quais as plantas (domésticas e selvagens) e invertebrados predados pelo rato-doméstico e inferir qual o impacto deste invasor no ecossistema;
- Preparar as equipas da Universidade de Cabo Verde (UniCV) em São Vicente e do Instituto Nacional de Investigação e Desenvolvimento Agrário (INIDA) em Santiago nas metodologias adequadas para a monitorização das populações de rato-doméstico e do impacto sobre espécies nativas;
- Esboçar as primeiras estratégias de irradicação de rato-doméstico.

Metodologia

O trabalho de campo para amostragem dos micromamíferos decorreu entre os dias 11 e 27 de Novembro de 2019. Foram colocadas entre 40 a 70 armadilhas Sherman por local dependendo do tamanho dos campos de cultivo e da variedade de cultivos, em grupos de 10, com distâncias aproximadas de 5 m entre estas. Em Santiago foram amostrados dois locais, na Lage (15.06272, -23.59808) e em São Jorge dos Órgãos (15.04919, -23.61092), no concelho de São Lourenço dos Órgãos. Durante esse período, o técnico Jorge Tavares do INIDA foi formado nas técnicas de armadilhagem e recolha de amostras biológicas. Em Santo Antão e São Vicente foi amostrado um local, Furnalha (17.14589, -25.01865), no concelho do Paúl, e Ribeira de Vinha (16.86028, -25.00903), respectivamente. Durante esse período, a estudante da UniCV Lara Almeida foi formada nas técnicas acima mencionadas, e na dissecção e medição dos animais. Todos os animais capturados foram fotografados dorsal, ventral e lateralmente junto ao local de captura, sexados e classificados como adultos ou juvenis. O estado reprodutor dos adultos foi classificado como em não reprodução ou em reprodução, no caso das fêmeas apresentarem mamas visíveis ou ventres dilatados, ou os testículos no escroto, no caso dos machos. Por fim, foram ainda colhidas amostras de ADN de referência no Jardim Botânico, com a ajuda dos técnicos do INIDA das plantas endémicas de Cabo Verde, bem como de invertebrados, para comparar com as espécies predadas pelo rato-doméstico. Em Março e Maio foi dada toda a formação às ONGs Projecto Biodiversiadade, do Sal, e Projecto Vitó, do Fogo, co-responsáveis pela gestão das áreas protegidas no Sal, Fogo e Rombos, respectivamente.

Os indivíduos de rato-doméstico amostrados foram pesados com uma pesola, medidos com uma craveira para determinar a largura da cabeça, altura da orelha esquerda, comprimento do pé direito, da cauda e total do animal, do focinho até início da cauda. Posteriormente foram dissecados nos laboratórios do INIDA e UniCV para a recolha dos tubos digestivos com os conteúdos digestivos para posterior análise genética. A presença de fetos nas fêmeas foi assinalada. O procedimento metodológico laboratorial encontra-se resumido na **Figura 1**.

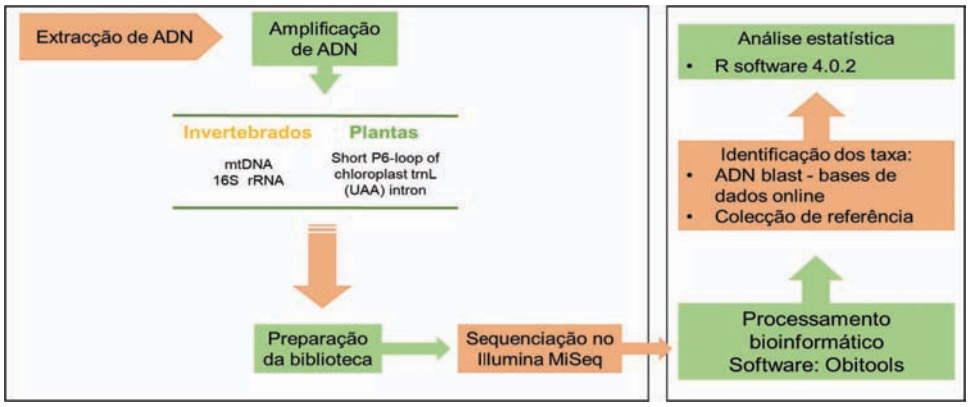

Figura 1. Resumo da metodologia laboratorial realizada neste estudo.

No laboratório do CIBIO/InBIO o tubo digestivo foi dividido em três secções (estômago, intestino delgado e intestino grosso), para deste modo possibilitar a extracção de todo o tubo que não cabia na coluna de extracção por inteiro. Foi extraído ADN dos conteúdos digestivos de 30 indivíduos por ilha, utilizando o kit de extracção "Stool DNA Isolation Kit (NorgenBiotek Corp., Canada)", seguindo as instruções do fabricante. Após a extracção das três porções, obtivemos um total de 296 amostras, cujo ADN foi amplificado com dois marcadores genéticos já validados em estudos anteriores: 16S para invertebrados e o loop curto P6 do intrão do TrnL de cloroplastos para plantas. Em seguida, foram preparadas bibliotecas seguindo o protocolo Illumina MiSeq "16S Metagenomic Sequencing Library Preparation". Após a sequenciação utilizando o kit de sequenciação "MiSeq Reagent Kit v2 (300-cycles)", as sequencias de ADN obtidas foram processadas ao nível bioinformático. Para tal, utilizamos ferramentas incorporadas no programa OBITools, que incluem o alinhamento das sequências obtidas e a eliminação de erros de sequenciação, para obter as unidades taxonómicas operacionais moleculares, MOTUs. Por fim, para identificar taxonomicamente os MOTUs presentes na dieta do rato-doméstico, os haplótipos obtidos foram comparados com as sequências de referência e ainda com as disponíveis em bancos de dados públicos (ex: GenBank, BOLD) considerando as listas bibliográficas publicadas para Cabo Verde.

Foram estimadas as frequências de ocorrência dos MOTUs de plantas e invertebrados nos conteúdos digestivos dos indivíduos das três ilhas. Utilizando o software R versão 4.0.2, foi realizada uma análise permutacional multivariada de variância (PERMANOVA) utilizando o pacote vegan (função ADONIS) com o objectivo de comparar a composição da dieta dos indivíduos entre as três ilhas (Oksanen *et al.*, 2019). Foi igualmente efectuado também um teste de homogeneidade de dispersão (PERMDISP), a fim de assegurar a importância do teste PERMANOVA, uma vez que pressupõe uma dispersão igual de valores entre os diferentes grupos.

Por fim, classificamos o impacto do rato-doméstico nos itens de dieta identificados nas amostras em três categorias. Foram considerados conjuntamente itens de flora (plantas silvestres e cultivadas, endémicas e exóticas) e invertebrados (endémicos e exóticos) de Cabo Verde. Assim, consideramos como impacto negativo se os itens consumidos fossem plantas ou invertebrados nativos ou endémicos, ou ainda plantas com importância económica (para consumo humano, para a alimentação de animais domésticos, medicinais, entre outras) ou invertebrados com funções ecológicas essenciais, como por exemplo polinizadores. Consideramos impacto positivo se os itens consumidos fossem plantas ou invertebrados exóticos que prejudicam a agricultura ou o ecossistema e impacto não identificado (NI) se os itens consumidos fossem identificados com uma resolução taxonómica baixa ou se o impacto não pudesse ser classificado.

Resultados e Discussão

Foram capturados nas armadilhas 41 ratos domésticos *Mus musculus domesticus* em Santiago, 31 em Santo Antão e 42 em São Vicente. Embora as armadilhas sejam específicas para rato-doméstico, foram ainda capturadas três ratazanas *Rattus* sp., duas em Santiago e uma em São Vicente (**Tabela S1**). Os ratos-domésticos capturados eram predominantemente adultos, representando os juvenis até cerca de um terço das amostragens em cada ilha (entre 19 e 29%). Os animais capturados rondavam entre os 10 e os 14 gramas de peso médio (mínimo 4 e máximo 25) e os 72.55 e 77.56 mm de comprimento total médio (mínimo 52 e máximo 90 mm), sendo que em Santiago se encontraram em média animais mais pesados, e também com comprimentos totais médios superiores aos das restantes ilhas (**Tabela S1**). Todas as fêmeas encontradas ou estavam a amamentar, visto terem mamilos visíveis, ou estavam grávidas (100% em Santiago e Santo Antão), apresentado as grávidas até 10 embriões por fêmea, o que atesta a grande capacidade invasora da espécie. Todos estes 117 animais encontram-se depositados no futuro Museu de Cabo Verde, Campus Ribeira de Julião, São Vicente, para que possam ser examinados e estudados pelos alunos das universidades nacionais, bem como por outros especialistas internacionais.

Nos indivíduos analisados das três ilhas identificamos um total de 131 itens de dieta, sendo 73 deles correspondentes a plantas e 58 a invertebrados. Estes itens pertencem a um total de 62 famílias diferentes, sendo 31 de plantas e 31 de invertebrados (**Tabela S2**).

Na ilha de Santo Antão foram identificados um total de 60 itens, em Santiago um 73 e em São Vicente 50 itens (**Figura 2**). Os indivíduos em Santo Antão parecem estar a ingerir uma maior variedade de plantas e menor variedade de invertebrados em relação às outras duas ilhas. Por outro lado, em Santiago o padrão parece ser inverso, com um maior número de itens de invertebrados, enquanto que em São Vicente as variedades dos dois grupos estão equilibradas na dieta (**Figura 2**).

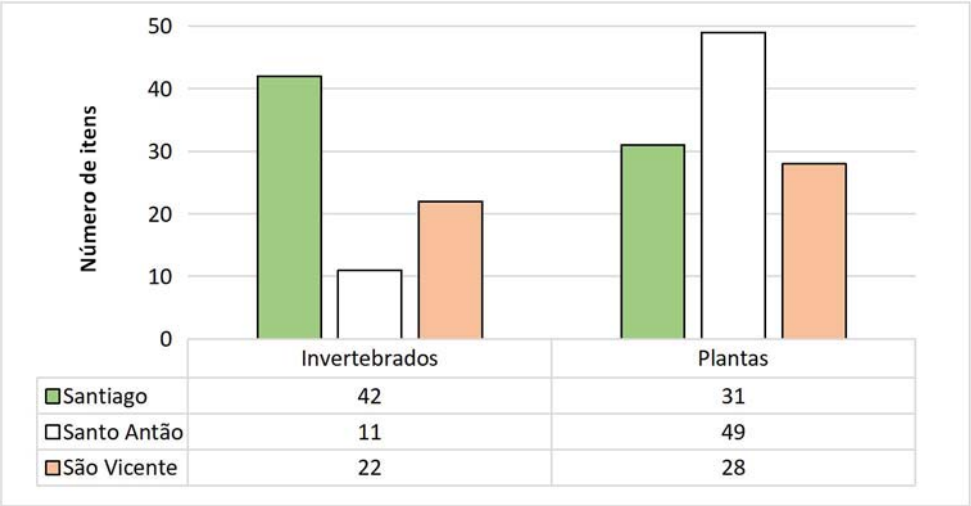

Figura 2. Número de itens de plantas e de invertebrados identificados na dieta do rato-doméstico em cada uma das três ilhas amostradas.

Verificámos que existem diferenças significativas na composição da dieta dos indivíduos das três ilhas (**Figura S1**). No entanto, tendo em conta o resultado não significativo do teste PERMDISP, esta diferença deve-se apenas a um efeito de localização. Isto é, existem itens de dieta que se encontraram apenas numa das ilhas possivelmente por não terem sido feitas réplicas em cada ilha. Podemos verificar, que vários itens de plantas foram apenas detectados nos estômagos de ratos da ilha de Santo Antão, como por exemplo a pega-bode, ou dá pega, *Mentzelia aspera* e papaia *Carica papaya*. Podemos também verificar que a baguinha de codorniz *Clitoria ternatea* e o travador *Euphorbia heterophylla* só foram identificados em Santiago, enquanto que a *Zaleya pentandra* e linhaxo *Leucaena leucocephala* só foram identificados em São Vicente. Estas espécies são também as que apresentam maior percentagem de ocorrência em cada ilha (**Figura 3**). No entanto, também existem itens de plantas que são comuns em todas as ilhas e apresentam elevada frequência nas amostras, como por exemplo a herbária do género *Setaria* e a jureminha ou anil-de-bode *Desmanthus virgatus* (**Figura 3 e Tabela S2**).

Isto acontece igualmente para os invertebrados, existindo também espécies comuns entre ilhas que apresentam uma frequência de ocorrência elevada sendo o caso da abelha-comum *Apis melífera* e do pulgão-do-algodão *Aphis gossypii* (**Figura 3 e Tabela S2**). Assim, estes resultados indicam que existem itens que estão amplamente dispersos sendo comuns na dieta dos indivíduos nas diferentes ilhas, enquanto que outros itens podem ser mais abundantes em determinadas ilhas.

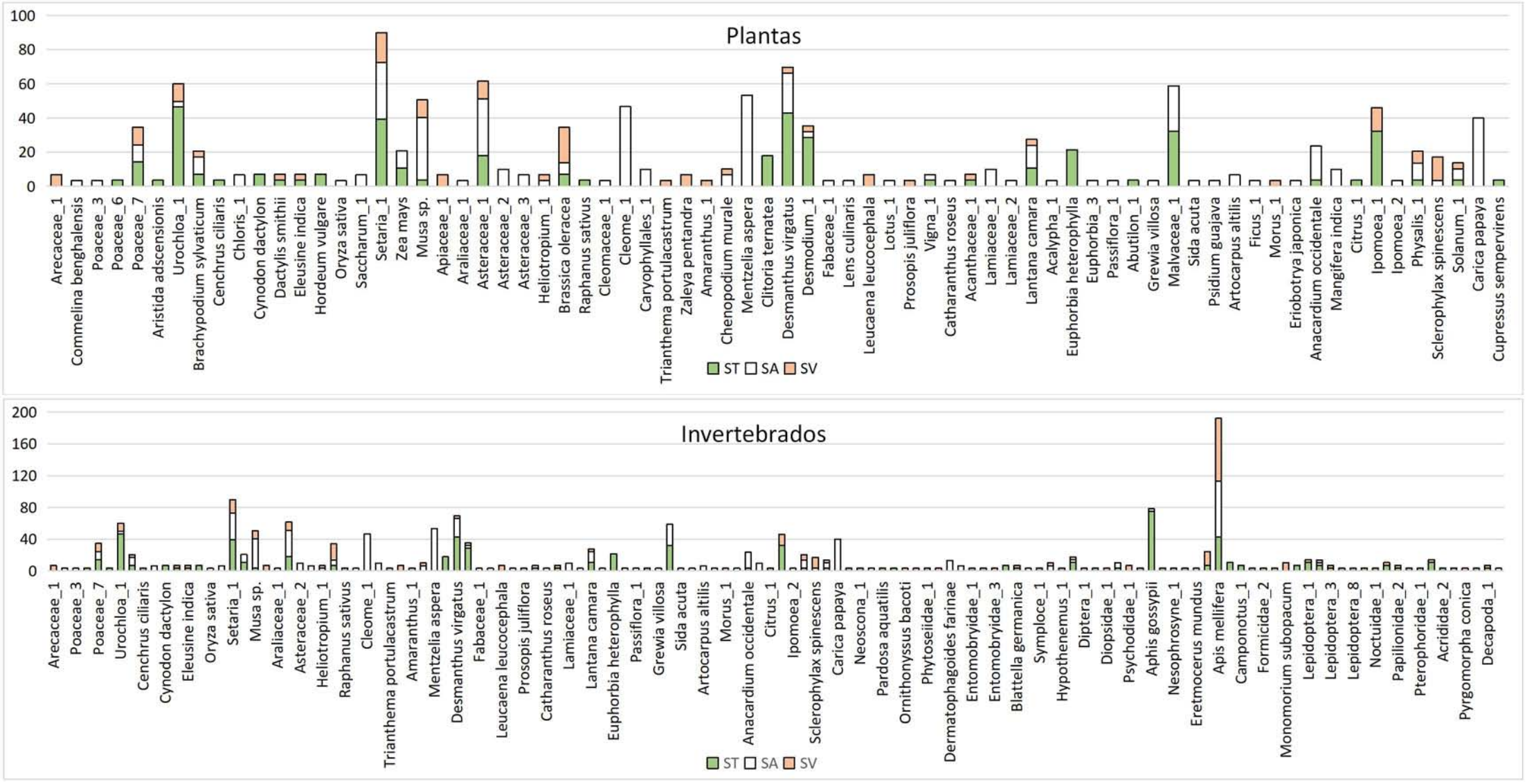

Figura 3. Percentagens de ocorrência dos diferentes itens de dieta (plantas e invertebrados) nas amostras fecais de rato-doméstico nas três ilhas amostradas (ST, Santiago; SA, Santo Antão, SV, São Vicente).

Dado que as colecções de referencia de sequências de ADN, principalmente para invertebrados, estão ainda incompletas, não foi possível identificar com elevada resolução taxonómica alguns dos itens consumidos. Deste modo, não foi possível avaliar o impacto de 39% da dieta de rato-doméstico. No entanto, podemos verificar claramente que o impacto deste invasor é predominantemente negativo, conforme já verificado em outros trabalhos em ilhas (**Figura 4**), e estimamos que o impacto indeterminado seria distribuído de forma semelhante ao determinado, aumentando ainda mais a proporção negativa do mesmo. O impacto seguiu o mesmo padrão nas três ilhas, sendo sempre predominantemente negativo.

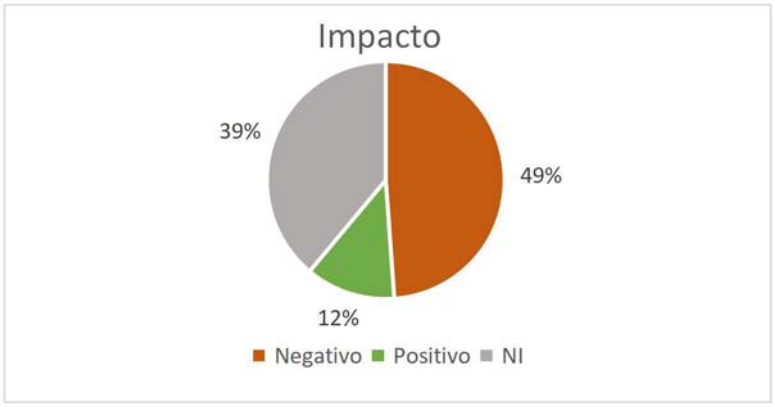

Figura 4. Avaliação do impacto da dieta do rato-doméstico nas espécies de flora nativa (plantas silvestres e cultivadas) e invertebrados de Cabo Verde. NI designa "Não identificado".

A dieta destes roedores tem impacto negativo em várias plantas cultivadas com importância humana e económica. Entre elas destacam-se o milho *Zea mays*, a banana *Musa sp.*, a couve *Brassica oleracea*, o rabanete *Raphanus sativus*, a goiaba *Psidium guajava*, a fruta-pão *Artocarpus altilis*, a manga *Mangifera indica*, a papaia *Carica papaya* e plantas do género *Solanum* que podem incluir tomates, batata, beringela entre outras hortícolas. A produção de milho representa um dos maiores cultivos de Cabo Verde, sendo produzido cerca de 6 mil toneladas de milho anualmente (MAA, 2015), deste modo o rato-doméstico representa uma grande ameaça à economia nacional. No entanto, também tem impacto positivo consumindo algumas herbáceas introduzidas e invasoras, como por exemplo a lantuna *Lantana camara,* uma das principais plantas invasoras em várias regiões tropicais, invadindo ecossistemas naturais e também agrícolas (Thomas & Ellison, 1999), e o travador *Euphorbia heterophylla,* uma espécie invasora de culturas de sequeiro e regadio, sendo que tem de ser retiradas manualmente na época do cultivo de cereais e outras culturas.

Em relação aos invertebrados, estes roedores têm um impacto também bastante negativo consumindo espécies nativas e endémicas entre as quais aranhas (ex: *Pardosa aquatilis*), formigas (ex:

Monomorium subopacum) e a abelha-comum *Apis melífera*. Estes invertebrados representam funções ecológicas de grande importância, principalmente a abelha-comum que tem um papel activo na polinização de várias culturas como por exemplo a couve e o feijão (Gill, 1990; Paudel *et al.*, 2015), e que se encontra em declínio a nível mundial (Paudel *et al.*, 2015). Contudo, o rato-doméstico também desempenha um papel no controlo de pragas domésticas como baratas (ex: *Blattella germanica* e *Periplaneta americana*) e o pulgão-do-algodão *Aphis gossypii*. Este último, é uma importante praga agrícola pois pode ter vários hospedeiros e transmite vários vírus importantes às culturas (Ebert & Cartwright, 1997). Os danos são directos através da alimentação que pode matar o hospedeiro, mas também pode reduzir a produtividade muito antes da morte das plantas (Andrews & Kitten, 1989). São exemplos de cultivos que podem ser hospedeiros alguns alimentos cultivados em Cabo Verde como por exemplo a papaia, o caju, a fruta-pão, a banana, entre outros (Ebert & Cartwright, 1997).

Em geral, podemos concluir que a utilização do método de *metabarcoding* neste estudo foi muito bem-sucedida possibilitando a identificação de uma grande variedade de itens de dieta do rato-doméstico. Esta técnica possibilitou a obtenção destes dados de uma forma muito mais rápida e simplificada em comparação com outras abordagens morfológicas, tal como em estudos anteriores (Gil *et al.*, 2020). Foi possível identificar as principais espécies de plantas e invertebrados predados por este invasor e assim inferir o impacto do mesmo em espécies endémicas e nativas, particularmente em plantas com importância económica. Deste modo, esta técnica pode ser uma mais valia para estudos de impacto de invasores podendo ser usada em outras ilhas para o mesmo efeito. Recomendamos que estudos desde género sejam realizados regularmente nas principais áreas protegidas do país, reservas naturais e ilhéus de acesso limitado, como nos Rombos e Santa Luzia, possibilitando assim de uma maneira confiável e rápida a avaliação periódica do impacto do rato-doméstico nestes locais de especial interesse para a conservação da biodiversidade.

Tendo em conta que já foram leccionados cursos no Sal e no Fogo com intuído da formação para a recolha das amostras usadas neste estudo, num futuro próximo será possível avaliar de igual modo qual o impacto do rato-doméstico nestas ilhas. No entanto, este género de estudo de sequenciação de nova-geração ainda não é exequível no arquipélago por falta de recursos materiais, nomeadamente um homogeneizador de amostras “Mixer Mill MM 400”e os elevados custos de sequenciação e transporte. Contudo, novas tecnologias como o sequenciador MinION podem tornar isto possível e todo o processo ainda mais rápido. A vantagem deste sequenciador é ser um dispositivo portátil que permite a sequenciação de DNA em tempo real, podendo assim ser usado até no campo, e ter kits associados que, em alguns casos, permitem que todo o processo seja realizado sem um laboratório especializado para o efeito (Johnson *et al.*, 2017).

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Material Suplementar

Tabela S1. Números de fêmeas (F), machos (M) e juvenis (J) e totais (T) de ratos-domésticos encontrados na ilha de Santiago, Santo Antão e São Vicente. Os valores médios (med) e desvio padrão (SD) do peso e medidas corporais dos ratos-domésticos por ilha são também indicados (CT, CP e CC, comprimento total, do pé e da cauda, respectivamente; LC, largura da cabeça; AO, altura da orelha).

Ilha	Classe				Peso		CT		CP		CC		LC		AO	
	F	M	J	T	med	SD	med	SD	med	SD	med	SD	med	SD	med	SD
Santiago	22	11	8	41	14.29	4.23	77.56	6.97	11.19	0.52	69.73	5.12	10.54	0.56	14.06	0.78
Santo Antão	10	12	9	31	10.36	3.68	71.93	9.36	11.92	0.52	68.21	7.65	10.42	0.82	13.88	0.77
São Vicente	14	19	9	42	10.10	2.08	75.45	5.50	11.91	0.70	70.12	5.38	10.50	0.46	13.61	0.72

Tabela S2. Identificação taxonómica e percentagem (%) de ocorrência dos itens de dieta de ratos-domésticos encontrados na ilha de Santiago (ST), Santo Antão (SA), São Vicente (SV) e no total (T). O impacto dos ratos domésticos sobre cada item é também apresentado (NI quando este não foi classificado).

Identificação Taxonómica					% de				Impacto
Filo	**Classe**	**Ordem**	**Família**	**Identificação Final**	**ST**	**SA**	**SV**	**T**	
Tracheophyt	**Liliopsida**	Arecales	**Arecaceae**	Arecaceae_1	0	0	7	2	negativ
		Commelinales	**Commelinaceae**	*Commelina benghalensis*	0	3	0	1	positivo
		Poales	**Poaceae**	Poaceae_3	0	3	0	1	NI
				Poaceae_6	4	0	0	1	NI
				Poaceae_7	14	10	10	11	NI
				Aristida adscensionis	4	0	0	1	negativ
				Brachiaria_1	46	3	10	20	negativ
				Brachypodium sylvaticum	7	10	3	7	positivo
				Cenchrus_2	4	0	0	1	negativ
				Chloris_1	0	7	0	2	NI
				Cynodon dactylon	7	0	0	2	negativ
				Dactylis smithii	4	0	3	2	negativ
				Eleusine indica	4	0	3	2	negativ
				Hordeum vulgare	7	0	0	2	negativ
				Oryza sativa	0	3	0	1	negativ
				Saccharum_1	0	7	0	2	NI
				Setaria_1	39	33	17	30	NI
				Zea mays	11	10	0	7	negativ
		Zingiberales	**Musaceae**	Musa sp.	4	37	10	17	negativ
	Magnoliopsid	Apiales	**Apiaceae**	Apiaceae_1	0	0	7	2	NI
			Araliaceae	Araliaceae_1	0	3	0	1	NI
		Asterales	**Asteraceae**	Asteraceae_1	18	33	10	21	NI
				Asteraceae_2	0	10	0	3	NI
				Asteraceae_3	0	7	0	2	NI
		Boraginales	**Heliotropiaceae**	Heliotropium_1	0	3	3	2	negativ
		Brassicales	**Brassicaceae**	*Brassica oleracea*	7	7	21	11	negativ
				Raphanus sativus	4	0	0	1	negativ
			Cleomaceae	Cleomaceae_1	0	3	0	1	NI
				Cleome_1	0	47	0	16	positivo
		Caryophyllales	**NI**	Caryophyllales_1	0	10	0	3	NI
			Aizoaceae	*Trianthema*	0	0	3	1	positivo
				Zaleya pentandra	0	0	7	2	negativ
			Amaranthaceae	Amaranthus_1	0	0	3	1	negativ
				Chenopodium murale	0	7	3	3	positivo
		Cornales	**Loasaceae**	*Mentzelia aspera*	0	53	0	18	positivo
		Fabales	**Fabaceae**	*Clitoria ternatea*	18	0	0	6	NI
				Desmanthus virgatus	43	23	3	23	negativ
				Desmodium_1	29	3	3	11	negativ
				Fabaceae_1	0	3	0	1	NI
				Lens culinaris	0	3	0	1	negativ
				Leucaena leucocephala	0	0	7	2	negativ
				Lotus_1	0	3	0	1	negativ
				Prosopis juliflora	0	0	3	1	negativ
				Vigna_1	4	3	0	2	negativ
		Gentianales	**Apocynaceae**	Catharanthus roseus	0	3	0	1	negativ
		Lamiales	**Acanthaceae**	Acanthaceae_1	4	0	3	2	NI
			Lamiaceae	Lamiaceae_1	0	10	0	3	NI
				Lamiaceae_2	0	3	0	1	NI
			Verbenaceae	Lantana camara	11	13	3	9	positivo
		Malpighiales	**Euphorbiaceae**	Acalypha_1	0	3	0	1	positivo
				Euphorbia heterophylla	21	0	0	7	positivo
				Euphorbia_3	0	3	0	1	NI
			Passifloraceae	Passiflora_1	0	3	0	1	NI
		Malvales	**Malvaceae**	Abutilon_1	4	0	0	1	NI
				Grewia villosa	0	3	0	1	negativ
				Malvaceae_1	32	27	0	20	NI
				Sida acuta	0	3	0	1	negativ
		Myrtales	**Myrtaceae**	Psidium guajava	0	3	0	1	negativ
		Rosales	**Moraceae**	Artocarpus altilis	0	7	0	2	negativ
				Ficus_1	0	3	0	1	negativ
				Morus_1	0	0	3	1	NI
			Rosaceae	Eriobotrya japonica	0	3	0	1	negativ

		Sapindales	**Anacardiaceae**	Anacardium occidentale	4	20	0	8	negativ
				Mangifera indica	0	10	0	3	negativ
			Rutaceae	Citrus_1	4	0	0	1	negativ
		Solanales	**Convolvulaceae**	Ipomoea_1	32	0	14	15	NI
				Ipomoea_2	0	3	0	1	NI
			Solanaceae	Physalis_1	4	10	7	7	negativ
				Sclerophylax spinescens	0	3	14	6	positivo
				Solanum_1	4	7	3	5	negativ
		Violales	**Caricaceae**	Carica papaya	0	40	0	14	negativ
	Pinopsida	Pinales	**Cupressaceae**	Cupressus sempervirens	4	0	0	1	positivo
Arthropoda	**Arachnida**	Araneae	**Araneidae**	Neoscona_1	4	0	0	1	negativ
			Gnaphosidae	Gnaphosidae_1	0	3	0	1	negativ
			Lycosidae	*Pardosa aquatilis*	4	0	0	1	negativ
			Theridiidae	*Theridion_1*	4	0	0	1	negativ
		Mesostigmata	**Macronyssidae**	*Ornithonyssus bacoti*	0	0	3	1	NI
			Phytoseiidae	*Euseius ovalis*	0	0	3	1	NI
				Phytoseiidae_1	4	0	0	1	NI
		Opiliones	**NI**	Opiliones_1	0	0	3	1	NI
		Sarcoptiforme	**Pyroglyphidae**	*Dermatophagoides farinae*	0	13	0	5	NI
	Diplopoda	NI	**NI**	*Diplopoda_1*	0	7	0	2	NI
	Entognatha	Collembola	**Entomobryidae**	Entomobryidae_1	4	0	0	1	negativ
				Entomobryidae_2	4	0	0	1	negativ
				Entomobryidae_3	0	0	3	1	negativ
	Insecta	NI	**NI**	Insecta_1	7	0	0	2	NI
		Blattodea	**Blattidae**	*Blattella germanica*	4	0	3	2	positivo
				Periplaneta americana	0	3	0	1	positivo
				Symploce_1	4	0	0	1	negativ
		Coleoptera	**NI**	Coleoptera_1	0	7	3	1	NI
			Curculionidae	Hypothenemus_1	4	0	0	3	negativ
		Dermaptera	**Anisolabididae**	*Euborellia annulipes*	11	3	3	6	positivo
		Diptera	**NI**	Diptera_1	0	0	3	1	NI
			Chironomidae	*Cricotopus_1*	4	0	0	1	NI
			Diopsidae	Diopsidae_1	0	0	3	1	NI
			Phoridae	Megaselia_1	4	7	0	3	negativ
			Psychodidae	Psychodidae_1	0	0	7	2	negativ
		Hemiptera	**Aphididae**	Aphididae_1	4	0	0	1	NI
				Aphis gossypii	75	3	0	25	positivo
			Cicadellidae	Cicadellidae_1	4	0	0	1	NI
				Nesophrosyne_1	4	0	0	1	negativ
		Hymenoptera	**NI**	Hymenoptera_2	4	0	0	1	NI
			Aphelinidae	*Eretmocerus mundus*	0	0	3	1	NI
			Apidae	Apidae_1	7	0	17	8	NI
				Apis mellifera	43	70	79	64	negativ
			Braconidae	*Diaeretiella rapae*	11	0	0	3	positivo
			Formicidae	Camponotus_1	7	0	0	2	negativ
				Cardiocondyla emeryi	4	0	0	1	NI
				Formicidae_2	4	0	0	1	NI
				Formicidae_3	4	0	0	1	NI
				Monomorium subopacum	0	0	10	3	negativ
			Thynninae	Neozeleboria_1	7	0	0	2	NI
		Lepidoptera	**NI**	Lepidoptera_1	11	0	3	5	NI
				Lepidoptera_2	7	3	3	5	NI
				Lepidoptera_3	4	0	3	2	NI
				Lepidoptera_6	4	0	0	1	NI
				Lepidoptera_8	4	0	0	1	NI
			Bombyx	*Bombyx_1*	4	0	0	1	NI
			Noctuidae	Noctuidae_1	4	0	0	1	negativ
			Papilionidae	Papilionidae_1	7	0	3	3	NI
				Papilionidae_2	4	0	3	2	NI
			Pieridae	*Colotis_1*	4	0	0	1	negativ
			Pterophoridae	Pterophoridae_1	4	0	0	1	NI
		Orthoptera	**Acrididae**	Acrididae_1	11	0	3	5	negativ
				Acrididae_2	4	0	0	1	negativ
			Gryllidae	*Gryllus_1*	4	0	0	1	negativ
			Pyrgomorphida	*Pyrgomorpha conica*	0	0	3	1	negativ
		Thysanoptera	**Phlaeothripidae**	Phlaeothripidae_1	4	0	0	1	negativ
	Malacostraca	Decapoda	**NI**	Decapoda_1	4	0	3	2	NI
				Decapoda_2	0	3	0	1	NI

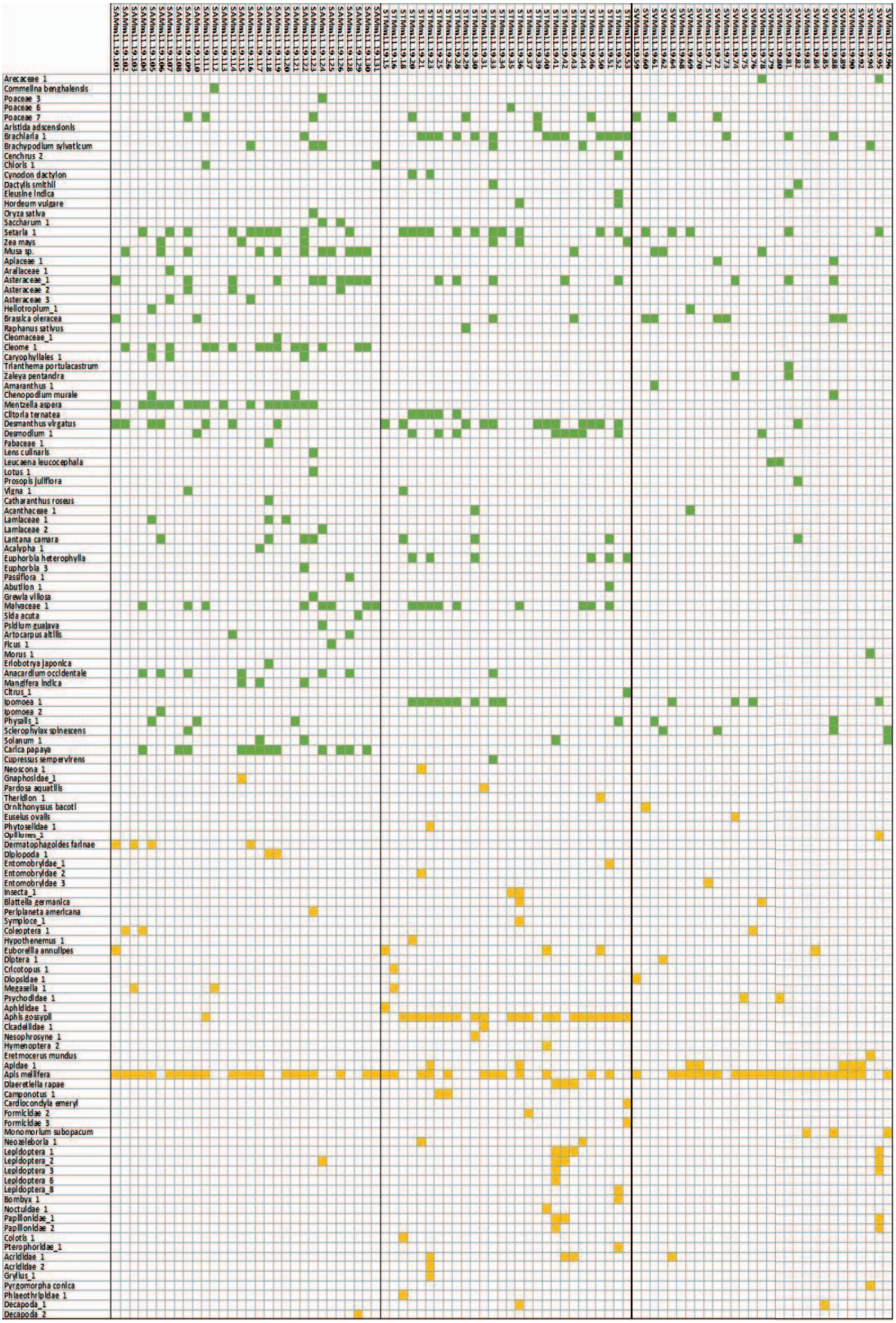

Figura S1. Representação da distribuição dos diferentes itens de dieta em cada amostras das três ilhas amostradas (ST, Santiago; SA, Santo Antão, SV, São Vicente).

MONITORING THE ENVIRONMENTAL IMPACT OF THE WIND FARM

OF SELADA DE FLAMENGO ON REPTILES OF THE INSULAR PLATE OF SÃO VICENTE – FINAL REPORT

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Abstract

The São Vicente wall gecko *Tarentola substituta* has been studied since 2009. However, no diet studies were conducted for this species yet. Knowledge of the diet composition of a species can reveal valuable information on its ecology and guide accurate management plans. Since 2013, *T. substituta* is classified as Least Concern by IUCN, thus it made sense to relocate studies to a broader reptile community. In the same insular plate as São Vicente, the Desertas Islands, inhabits the Near Threatened endemic Raso wall gecko *Tarentola raziana*. Almost nothing was done concerning this small gecko which is the most related species to *T. substituta*. Therefore, with this study, we intended to monitor the populations and assess the diet of both sister species to provide data for their conservation. This will allow us to understand how *T. substituta* may adapt to future drier conditions on São Vicente. Our results indicate that the populations of both species seem to be stable and that plants and invertebrate taxa have a significant role as diet items. We were able to identify the diet items to a higher taxonomic resolution than traditional methods and provide clues on adaptations of these endemic species to more arid circumstances. We also highlighted the importance of long-term studies to obtain an accurate representation of population dynamics and of involving local communities in the future conservation of Cabo Verde endemic species.

Keywords: Conservation genetics; Diet studies; Endangered species; Metabarcoding.

Introduction

The São Vicente wall gecko, *Tarentola substituta* Joger, 1984 has been studied since 2009 as mitigation of the direct impact suffered from the construction of the wind farm on São Vicente (Vasconcelos *et al.*, 2009). With the support of Cabeólica S.A., we have already conducted studies to know the pattern of activity and microhabitat preferences (Vasconcelos *et al.*, 2012a), density across the island (Vasconcelos *et al.*, 2017b), basic aspects of thermobiology and water biology (Carretero *et al.*, 2016) and behaviour (Pereira *et al.*, 2019) of this species. At the same time, we produced a documentary for RTP1 about the fauna and flora of Cabo Verde (Vasconcelos *et al.*, 2010b) lectures were given in all primary schools in São Vicente (Vasconcelos *et al.*, 2011), and the first record of an introduced species of reptile on the island was also discovered (Vasconcelos *et al.*, 2014). However, until now, no diet studies were conducted for this species, only anecdotal observations were reported (Schleich, 1987).

Dietary studies can reveal valuable information on how species exploit the available food resources and intervene in ecological processes (Siegenthaler *et al.*, 2019). Several methods can be implemented to characterize diets, such as direct observation, morphological identification, and stable isotopes analysis (Margalida *et al.*, 2005; Martín *et al.*, 2017; Symondson, 2002). However, with the recent generalization of high throughput sequencing methodologies, the identification of prey items was further improved (Pompanon *et al.*, 2012). DNA-metabarcoding, combined with the power of next-generation sequencing (NGS) technologies (Shendure & Ji, 2008), became widely used to characterize species diets (Taberlet *et al.*, 2012). DNA-based methodologies allow the identification of prey material even when hard parts cannot be recovered after digestion, contrary to other methods. These provide comprehensive taxonomic identification of diet items within highly diverse diets, relying less on taxonomic expertise, and can be applied to non-invasive or degraded samples (Pompanon *et al.*, 2012).

As since 2013, the conservation status of this species was updated from Data Deficient in the national red list (Schleich, 1996) to Least Concern in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Vasconcelos, 2013a), it makes sense to refocus the target of the studies to a broader reptile community. Near São Vicente Island, in the same insular plate (Fig 1), endemic geckos of the same genus are classified as Near Threatened and Endangered by IUCN (Vasconcelos *et al.*, 2013), and Rare and Endangered in the Nacional Red List (Schleich, 1996). The Raso wall gecko, *Tarentola raziana* Schleich 1984, and the giant wall gecko, *Tarentola gigas* (Bocage, 1875), are currently restricted to the Branco and Raso islets and Santa Luzia Island (Schleich, 1982) of the Desertas Island group, which formed, in the past, a joint island with São Vincente (Fig. 1). We already had the opportunity to study the sister species *T. gigas*, since we found that it was at greater risk of extinction (Lopes *et al.*, 2019; Pinho *et al.*, 2018) and Cabeólica previously supported a filedtrip to Raso Vasconcelos *et al.*, 2012a). However, almost or nothing was done concerning the smallest species of this genus, *T. raziana*, which is the species most related to *T. substituta* (Vasconcelos *et al.*, 2012a). These two share part of mitochondrial DNA, and are the only species of reptiles from Cabo Verde where this was registered (Vasconcelos *et al.*, 2010a). They even were, before 2012, designated as belonging to the same species *Tarentola caboverdiana* (Vasconcelos *et al.*, 2012b). This DNA sharing shows that both species must have been in contact in the past (Vasconcelos *et al.*, 2012b). By assessing the diet of the Near Threatened gecko population of *T. raziana* we wil try to understand how *T. substituta* may have evolved in a different direction from this sister species and how it may adapt in the near future when conditions on São Vicente will be similar to the present day on Desertas Islands, that is, more arid.

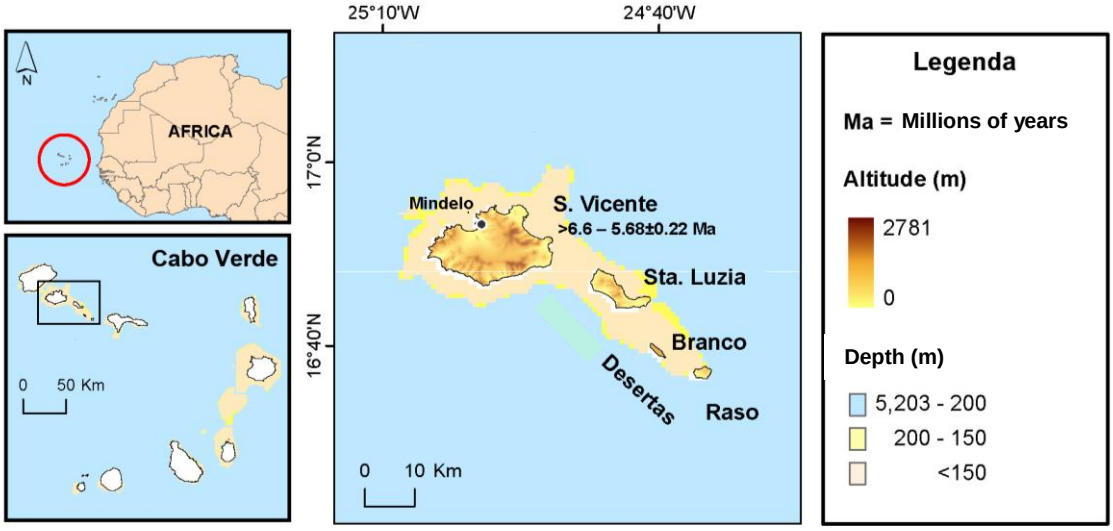

Figure 1. Map of the Cabo Verde Archipelago evidencing the geographical location, elevations and bathymetry of the island formed by Desertas and S. Vicente (Geographic Coordinate System, Datum WGS84) during the sea level falls in the Pleistocene (beige). The estimated geological age of S. Vicente is also given.

Climate change is a big concern for reptile species, especially to these ones (Vasconcelos, 2013a, 2013b). Recent models suggest that extremely dry and extremely humid years may be increasingly frequent in the next century (Huntingford *et al.*, 2005). Since the Cabo Verde Archipelago is already highly prone to drought, the islands of São Vicente and Desertas in particular, an increase in drought severity may represent additional pressure for the populations *of T. raziana* (Vasconcelos, 2013b) and, to a lesser extent, *for T. substituta*. Consequently, studies on the diet of these species are essential to infer the impact these extreme changes may have on them and for the subsequent development of suitable conservation strategies. Studying the diet of *T. raziana* will thus serve as a portal to the future, when São Vicente becomes more arid due to the anticipated effects of climate change, to foresee how *T. subsituta* may adapt. If the diet of geckos consists of a low variety of items, the likelihood of adapting to the increased severity of drought will be slimmer. It is necessary to assess whether these species include items adapted to arid climates in their diets, such as succulent plants, seeds and chitinous invertebrates, which may increase the probability of acclimatization to climate

changes. These data will be important to predict the adaptation of these species to climate change, but it will also be essential to understand the relationships of *T. raziana* with other species that inhabit the insular plate of which São Vicente belongs. In particular, it will allow us to understand if there are differences in the diet of the population of Santa Luzia and the population that inhabits the Raso Islet together with *Tarentola gigas*. With the possible reintroduction of this giant gecko on Santa Luzia, this will allow us to understand if these two species compete in trophic terms or not, which is extremely important for the conservation of both species.

Additionally, the smallest species *T. raziana* is threatened by other factors, one of them the introduction of mammal species, in particular rodents and cats (Vasconcelos, 2013b). By evaluating the diet of this gecko and comparing it with the diet of introduced mammals (a developing study supported by the Ministry of Education of Cape Verde), we can estimate how the invader is competing with this and better plan strategies for conservation, both for *T. raziana* and for sister species *T. substituta*.

Finally, it is important to note that, according to the IUCN (Vasconcelos, 2013b, 2013c), it is necessary to raise awareness for the importance of the endemic reptile species of Cabo Verde and their conservation. Geckos are feared due to traditional beliefs, and there may even be occasional persecution. Therefore, education is essential to clarify to the population that these animals are harmless to humans and inform them about the threats they face.

Objectives

Monitoring *Tarentola substituta* population at the wind farm of Selada de Flamengo

We intend to proceed with the population study on this species by measuring the sex ratio, the size, and the number of individuals, in order to compare with the previous recovered values. We also intend to collect faecal samples to explore the species of plants and invertebrates that constitute the diet of this species.

Evaluating the diet of the sister species *Tarentola raziana* on the Desertas Islands

We intend to carry out a preliminary study on the diet and morphology of the Near Threatened population that inhabits Santa Luzia Island, the same insular plate as São Vicente in order to better understand the evolution of both species and contribute to their conservation.

Promoting awareness about the gecko species of the insular plate São Vicente – Desertas

We intended to give lectures open to the general public to disseminate information on the importance of the Cabo Verde endemic gecko species, the threats to which they are subject and the actions that are important for their conservation

Methodology

Study species

The São Vicente wall gecko, *Tarentola substituta* Joger, 1984 occurs on the islands of São Vicente. The Raso wall gecko, *Tarentola raziana* Schleich 1984, is the smallest Cabo Verdean wall gecko and is classified as Near Threatened by the IUCN Red List of Threatened Species, occurring in both Raso and Santa Luzia (Vasconcelos, 2013b).

Study area

This study took place on São Vicente and Santa Luzia, both located in the same insular plate on the northwest alignment of the Cabo Verde Archipelago (Fig. 1). São Vicente has a total area of 227 km^2 (Fig. 1) and its landscape is composed of stony plains, sandy dunes, and barren hills, as it is very dry with scarce vegetation (Duarte *et al.*, 2008). Santa Luzia Islands presents an area of 35 km^2 and is a very arid island, yet there are more humid zones close to the river line, with hills, rocky plains, and dunes being the main landscapes (Freitas *et al.*, 2015).

Sampling

Sampling on Santa Luzia, in Chã de Tope de Àgua Doce, took place between 4 and 7 of May 2021 and on São Vicente, at the wind farm of Selada de Flamengo, between 10 and 14 of May 2021. We captured 38 individuals of *T. raziana* from Santa Luzia and 35 of *T. substituta* from São Vicente by hand and performed a belly massage in order to release the fresh faecal pellets, which were preserved in tubes with 96% ethanol. We also sexed the individuals based on the absence or presence of cloacal pouches (Vasconcelos *et al.*, 2012b), and photographed them to confirm the data in case of uncertainties. We collected morphological measures of both species to the nearest 0.1 mm using digital callipers and then released the animals. Particularly, we measured snout-vent length

(SVL), trunk length (TL), head length (HL), head width (HW), head height (HH), Eye–snout distance (ES), Ear–snout distance (ES2), Eye–Ear distance (EE) and Eye diameter (ED).

We collected samples of invertebrates and plants from Santa Luzia and São Vicente to build a DNA reference collection of possible food items. For the collection of invertebrates, we placed 20 pitfalls on each island. We set replicas on two locations on each island to gather a representative sample of the island invertebrate biodiversity. Then, we separated the specimens into different high-level taxonomic groups based on morphological identification by photographing them with a camera assembled on a magnifying lens. Our expert collaborators then performed morphological identification with a higher taxonomical resolution. For the plant collection, we collected samples of leaves and flowers across the islands and took pictures in the field to allow morphological taxonomic assessment by experts.

DNA Extraction and Sequencing

We extracted the DNA from the faecal pellets collected, including blanks, using the Stool DNA Isolation Kit (Norgen Biotek Corp., Canada), following the manufacturer's instructions.

We amplified the DNA from the pellets of the two species with three genetic markers already validated in previous studies (Pinho *et al.*, 2018). For invertebrates we used a modified version of the IN16STK-1F/IN16STK-1R primers, targeting the mitochondrial 16S rRNA (Kartzinel & Pringle, 2015). For plants, we used the g/h primers targeting the short P6-loop of chloroplast trnL (UAA) (Taberlet *et al.*, 2007), and for vertebrates the 12sv5F and 12Ssv5R primers targeting the V5-loop fragment of the mitochondrial 12S gene (Riaz *et al.*, 2011). We performed all PCRs, including blanks, as described in Pinho *et al.* (2018). Then, we prepared the libraries on Freedom EVO® NGS workstation following the Illumina MiSeq protocol ‘16S Metagenomic Sequencing Library Preparation’ (Illumina, 2013). Finally, we sequenced the samples in the MiSeq sequencer (Illumina) using the MiSeq Reagent Micro Kit v2 (Illumina, San Diego, CA, USA) for an expected average of 12,000 paired-end reads per sample.

We processed the obtained DNA sequences at the bioinformatics level using tools incorporated in the software package OBITtools (http://metabarcoding.org/obitools), which include the alignment of the sequences and the filtering of sequencing errors, to obtain the molecular operational taxonomic units, MOTUs (Pinho *et al.*, 2018). In the final dataset, we removed all the samples with less than 500 reads and within kept samples, we also excluded haplotypes representing less than 1% of the total number of reads of that sample (Mata *et al.*, 2016). Finally, to taxonomically identify the MOTUs present in the diets, we compared the obtained haplotypes with our reference database and with those available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). We classified only to the class level the sequences that had less than 90% of similarity with known species, to the family level the ones with values between 90% to 95%, and the remaining sequences with similarity values above 95% to the genus or species level. When a haplotype matched more than one species or genus with similar probabilities, we considered only species or genera known to occur on our sampled sites, or the surrounding islands of the archipelago. The haplotypes identified as contaminations were removed (e.g., human DNA).

Results

Monitoring *Tarentola substituta* population at the wind farm of Selada de Flamengo

We captured a total of 35 individuals, 16 females and 19 males. This reflects a sampling effort of six geckos per person per day. The average SVL of the individuals was 5.3 ± 0.5 cm (Table 1). Mean values of all the collected morphological measures are represented in Table 1. We were able to collect faecal pellets of 30 individuals.

Overall, we identified 17 prey items of four taxonomic classes, 11 orders and 15 families. Plant and invertebrate items were identified in 67% and 47% of the samples, respectively (**Figure 2**). The eight plants MOTUs identified were distributed among two classes, six orders, and six families (Fabaceae occurred more frequently, **Figure 3**). The nine invertebrate MOTUs were identified from two classes, four orders, and nine families, with higher frequencies of Theridiidae and Cheiracanthiidae families (**Figure 3**). The most frequent plant MOTU was from the genus Indigofera, which was also the most frequent MOTU in the overall diet, present in 40% of the samples. The most frequent invertebrate MOTUs were spiders from the genus *Cheiracanthium* and *Theridion*, both present on approximately 14% of the samples. No vertebrate items were identified as part of the diet of this species.

Table 1. Mean values and respective standard deviations of the morphological measures for *Tarentola substituta* and *Tarentola raziana*. Snout-vent length (SVL), trunk length (TL), head length (HL), head width (HW), head height (HH), Eye–snout distance (ES), Ear–snout distance (ES2), Eye–Ear distance (EE) and Eye diameter (ED) are given for female, male and all individuals of each species (in cm).

	Tarentola substituta			***Tarentola raziana***		
	Female	**Male**	**All**	**Female**	**Male**	**All**
SVL	5.1 ± 0.50	5.4 ± 0.50	5.3 ± 0.50	4.8 ± 0.30	5.0 ± 0.30	4.8 ± 0.30
TL	2.2 ± 0.30	2.4 ± 0.30	2.3 ± 0.30	2.0 ± 0.20	2.1 ± 0.20	2.1 ± 0.20
HL	1.6 ± 0.20	1.9 ± 0.20	1.8 ± 0.20	1.7 ± 0.09	1.8 ± 0.09	1.7 ± 0.09
HW	1.0 ± 0.10	1.1 ± 0.10	1.1 ± 0.10	0.9 ± 0.08	1.0 ± 0.08	0.9 ± 0.07
HH	0.7 ± 0.08	0.7 ± 0.08	0.7 ± 0.08	0.6 ± 0.05	0.7 ± 0.05	0.6 ± 0.05
EE	0.5 ± 0.05	0.5 ± 0.05	0.5 ± 0.05	0.5 ± 0.04	0.5 ± 0.04	0.5 ± 0.04
ES	1.1 ± 0.09	1.1 ± 0.08	1.1 ± 0.08	1.1 ± 0.04	1.1 ± 0.04	1.1 ± 0.04
ES_2	1.4 ± 0.10	1.5 ± 0.10	1.5 ± 0.10	1.4 ± 0.07	1.4 ± 0.07	1.4 ± 0.07
ED	0.4 ± 0.04	0.4 ± 0.04	0.4 ± 0.04	0.4 ± 0.03	0.4 ± 0.03	0.4 ± 0.03

Evaluating the state of the sister species *Tarentola raziana* on the Desertas Islands

We captured a total of 38 individuals, 19 females, 16 males and three juveniles. The average SVL of the adult individuals was 4.8 ± 0.3 cm (Table 1). Mean values of all collected morphological measures are represented in Table 1. Morphological measures of juveniles were not collected. We were able to collect faecal pellets of 30 individuals, including juveniles.

Overall, we identified 32 prey items of four taxonomic classes, 15 orders and 19 families. Plant and invertebrate items were identified in 100% and 35% of the samples, respectively (**Figure 2**). The 19 plant MOTUs identified were distributed among two classes, nine orders, and nine families, with a higher frequency of Zygophyllaceae family. The 13 invertebrate MOTUs identified were distributed among two classes, six orders, and 10 families, with a higher frequency of the Trogiidae family. The most frequent plant MOTU was *Tetraena simplex* (L.), which was also the most frequent MOTU in the

overall diet, as it was present in 65% of the samples. The most frequent invertebrate MOTU was from the genus *Cerobasis*, present on approximately 15% of the samples. No vertebrate items were identified as part of the diet of this species.

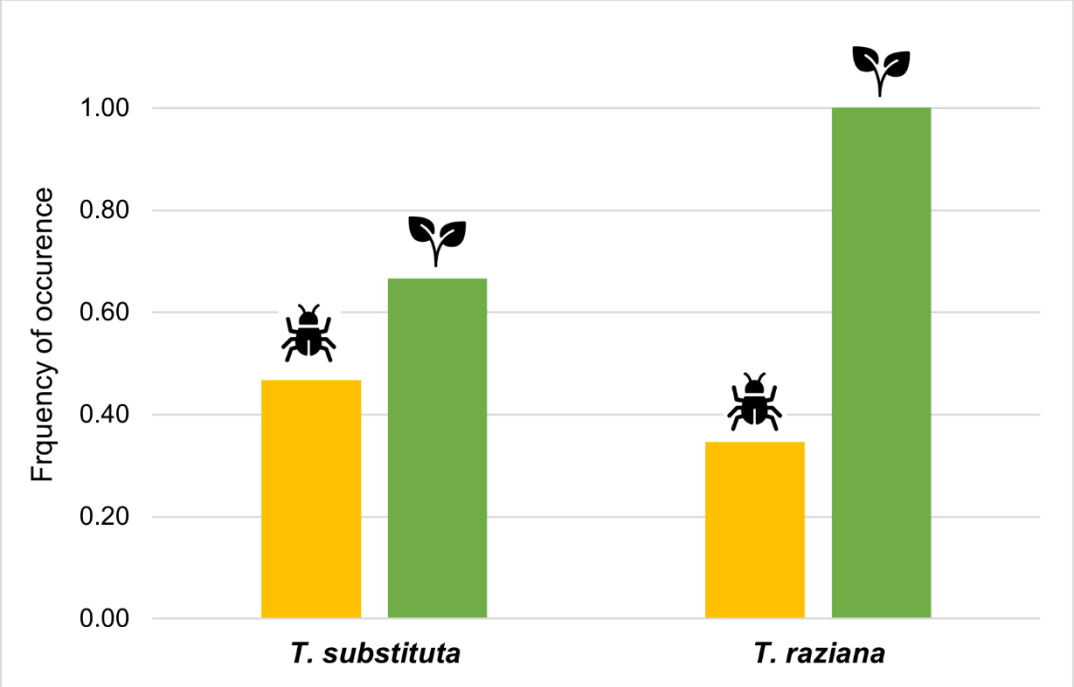

Figure 2. Frequencies of occurrence of plants (in green) and invertebrates (in yellow) in the diet of the two *Tarentola* species.

Comparing the diet of the two *Tarentola* sister species

We obtained significant differences in the occurrence of the two taxonomic groups (plants and invertebrates) in the diet of both *Tarentola* species ($X^2_{(1)} = 6.5526$, $p = 0.01$), with *T. raziana* presenting a higher frequency of plants and *T. substituta* of invertebrates (**Figure 2**). Four plant families were common in the diet of the two species (Poaceae, Asteraceae, Fabaceae and Zygophyllaceae; **Figure 3**), whereas only one invertebrate family was common in both diets (Trogiidae; **Figure 3**). Several diet items were exclusively present in the diet of one of the species (**Figure 3**).

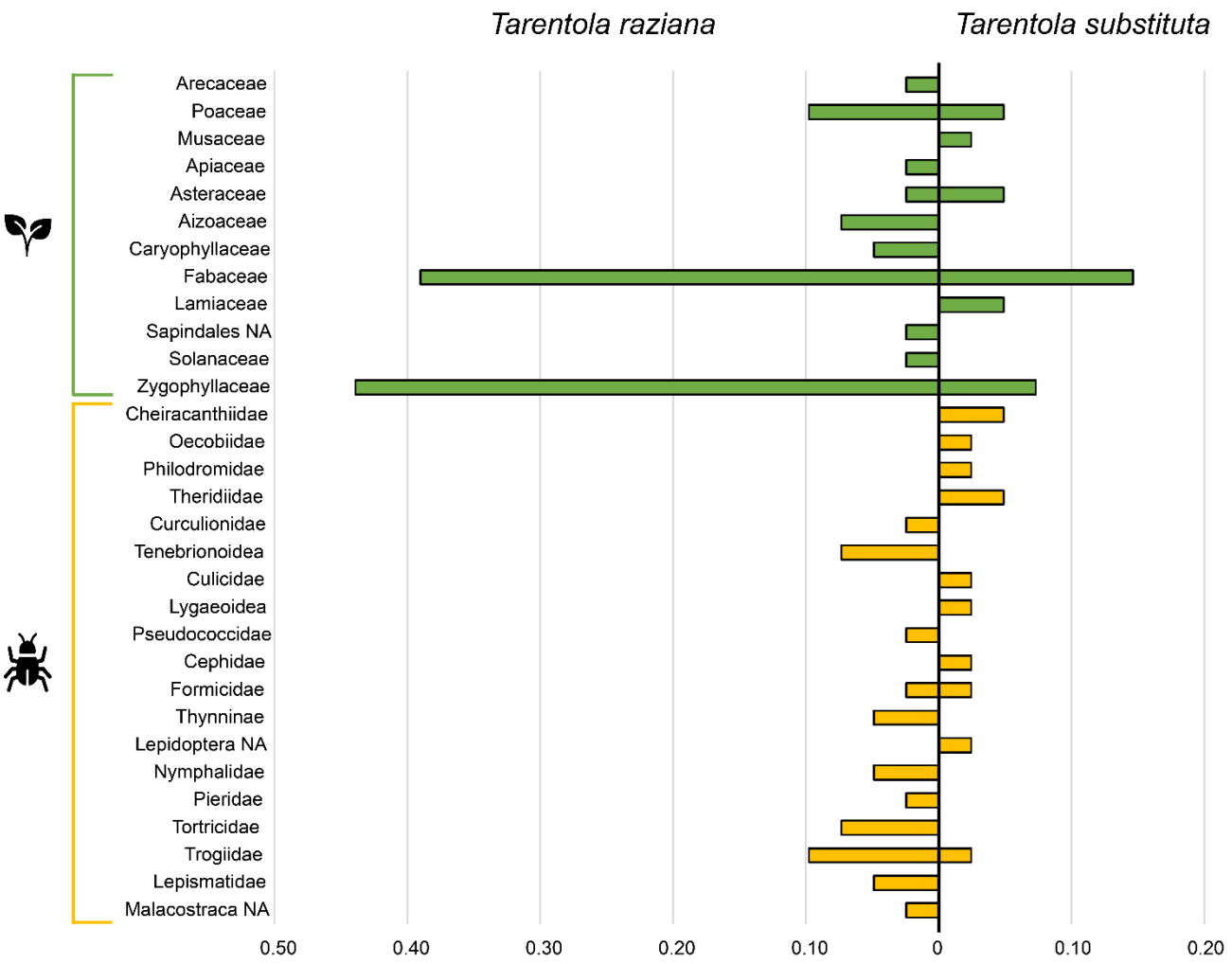

Figure 3. Frequency of occurrence of the families of plants (green) and invertebrates (yellow) in the diet of the two *Tarentola* species.

Promoting awareness about the gecko species of the insular plate São Vicente – Desertas

With the support of Prof. Evandro Lopes and Prof. Rui Freitas, we have presented two oral communications at Universidade Técnica do Atlântico (UTA), Mindelo, São Vicente to the Marine Biology class about the PhD project of the student Catarina J. Pinho that included the research work developed in 2012 with Cabeólica's support (**Figure 4**). We consider very important local community to be involved in the study in order to motivate people to the future conservation of Cabo Verde endemic species and to allow local students to learn scientific methodologies directed to herpetological studies.

Figure 4. Oral communications at Universidade Técnica do Atlântico, Mindelo, São Vicente.

Discussion

Monitoring of *Tarentola substituta* at the wind farm of Selada de Flamengo revealed that the populations are stable, and densities are coherent with the previously recorded values. However, sampling took place during the dry season and has always been done at this time for this species. Therefore, to better understand the population dynamics of this species it would be necessary to perform long term population studies that also include sampling during the wet season (White, 2018). Short term studies are likely not reliable to detect population trends and can lead to inaccurate management decisions (White, 2018). Consequently, density studies must take into account all the possible factors influencing abundance in order to lead to viable conservation actions. The sex ratio for the São Vicente wall gecko corresponded with the expected based on previous monitoring studies of this species (Vasconcelos *et al.*, 2012a; Vasconcelos *et al.*, 2017a). Additionally, all morphological measures corresponded to the mean values reported for this species (Vasconcelos *et al.*, 2012b; Vasconcelos *et al.*, 2017a). Moreover, we observed four couples occupying the same rock, emphasizing previous reports on the high aggregation patterns in *T. substituta* (Vasconcelos *et al.*, 2017b). Male-female aggregations seem to be common for this species and our observations highlight the relevance of these patterns.

Regarding the sister species, *Tarentola raziana* population on Santa Luzia, we consider that the population seems to be stable, as the number of individuals found corresponded to our expectations. The sex ratio differed from *T. substituta* as more females were found for this sister species. Similarly to *T. substituta,* all morphological measures corresponded to the mean values reported for this species (Vasconcelos *et al.*, 2012b). Moreover, we also observed three couples occupying the same rocks. This emphasizes the need for further studies on this thematic in order to understand the importance of aggregation patterns for the wellbeing of *Tarentola* populations in general.

This study provides the first DNA-based data on the diet of *Tarentola substituta*. Previously to this study, all data on the diet of *T. substituta* was based on anecdotal observations and morphological

examinations of faeces (Schleich, 1987). For this species only invertebrate items had been reported as part of the diet (Schleich, 1987). However, in our results, we were able to identify different invertebrate items and reveal the importance of plant items in the diet of this species. This was expected, as with morphological assessments, there is a tendency to underestimate prey incidence, taking into account that only partially digested or items with non-digestible parts can be detected, whereas with metabarcoding it is possible to detect small, soft, and invisible items (Pompanon *et al.*, 2012; Gil *et al.*, 2020). The diet of *Tarentola raziana* on Raso Islet was previously analysed using DNA-based methods (Seguro, 2017), however, the diet of the population inhabiting Santa Luzia was unknown. For *T. raziana* on Raso, Schleich (1987) reported the presence of invertebrates and a plant mass in the faeces, however he failed to identify which plant species this mass corresponded to. Seguro (2017) was able to give a more comprehensive view of the diet of *T. raziana*, identifying both invertebrate and plant items. However, our study is the first to describe the diet for this population of Santa Luzia.

Hence, this study is the first to report the generalist diet of *T. substituta* and highlights the importance of plant items on the diet of both species. Our results show that plant items had a higher incidence on the samples than invertebrates on both species that can be related to the lower availability of arthropod preys during drier periods (Pérez-Cembranos *et al.*, 2016), leading to a higher intake of plant items. Thus, long term monitoring of these species would be vital to identify seasonal fluctuations of the diet and better understand the adaptations of these geckos to foreseen increasingly dry or extremely humid years (Huntingford *et al.*, 2005). Nevertheless, our results already give clues on the adaptations to arid climatic conditions as, for instance, the most frequent item in the diet of *T. raziana* was the succulent plant. Only four diet items were common in the diet of both species, however, this was expected due to the distinct availability of food items in the two islands analysed in this study.

Finally, during our expedition to Santa Luzia Island, we observed high densities of mice, some in direct contact with the geckos occupying the same rock. Additionally, in the metabarcoding analysis, we found mouse DNA. As the geckos are not actively ingesting mice, this indicates a close

coexistence and involuntarily ingestion of mice residues by geckos. This may pose a threat to the already threatened reptile endemic species. In that way, it is essential to measure the predation intensity of these introduced mammals on the endemic reptiles and to attenuate its most likely negative impact.

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