Metabarcoding analysis of endemic lizards' diet for guiding reserve management in Macaronesia Islands

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Todas as correcções determinadas pelo júri, e só essas, foram efectuadas.

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O Presidente do Júri,

MSC

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Abstract

Islands are considered natural laboratories as they represent simplified models for a wide range of studies and hold a higher number of endemic species when compared with the mainland. However, these endemics are more threatened on these systems, therefore studying their ecological networks is of high importance for the development of accurate conservation plans. Very interesting study models for evolutionary and ecological studies are the reptiles of the Macaronesian islands, especially the ones inhabiting remote areas. Some of them present uncommon patterns of colonization and diversification, and most have simplified trophic nets and still remain poorly studied.

The diet of the most widespread continental *Tarentola* species is already widely studied using classical methods. However, using next-generation sequencing (NGS) techniques only one known study was performed for this genus and very few for reptiles in general. In this thesis, the main objective was to assess diet composition of two endemic geckos from Macaronesia, the emblematic giant wall gecko of Cabo Verde *Tarentola gigas*, and the Selvagens gecko *Tarentola (boettgeri) bischoffi*, in order to provide valuable information to the conservation of these threatened species. Little was known on both their ecology and dietary habits. In the first study, we aimed to compare the diet of the two subspecies of *T. gigas* to guide its reintroduction on an island where it went Extinct. In the second study, we compared morphological and DNA metabarcoding techniques associated with very different sampling efforts to check the impacts on the representation of *T. (boettgeri) bischoffi* diet. Results have revealed that both species are generalist eaters, feeding on plants, invertebrates and even vertebrates. Plants revealed to have a significant role as prey items, which was previously unspotted using traditional methods. Using metabarcoding, we were able to identify a higher diversity of dietary items and with generally higher taxonomic resolution. In the first study, we were able to discuss the options regarding the reintroduction of *T. gigas* and on the second the advantages and limitations of metabarcoding.

Overall, with this thesis, we were able to reveal a fresh range of prey items that formerly went unnoticed in these *Tarentola* diets with a reasonable taxonomic resolution. The information revealed by these ecological networks is important for the development of conservation plans on these protected areas and reinforce the important and commonly neglected role of reptiles on island systems.

Keywords

Tarentola gigas; *Tarentola (boettgeri) bischoffi*; Conservation genetics; Phyllodactylidae; Protected Areas, Remote areas.

Resumo

As ilhas são consideradas laboratórios naturais, pois representam modelos simplificados para uma ampla gama de estudos, e possuem um maior número de espécies endémicas quando comparado com o continente. No entanto, essas espécies endémicas são mais ameaçadas nestes sistemas, sendo o estudo das redes ecológicas das mesmas de grande importância para o desenvolvimento de planos de conservação precisos. Modelos de estudo muito interessantes para estudos evolutivos e ecológicos são os répteis das ilhas da Macaronésia, especialmente os que habitam áreas remotas. Alguns apresentam padrões incomuns de colonização e diversificação e a maioria exibe redes tróficas simplificadas, no entanto permanecem pouco estudados.

A dieta das espécies mais difundidas de *Tarentola* continentais já é amplamente estudada usando métodos clássicos. No entanto, usando técnicas de sequenciação de nova geração (NGS) apenas um estudo conhecido foi realizado para este género e muito poucos para répteis em geral. Nesta tese, o propósito principal foi avaliar a composição da dieta de duas osgas endémicas da Macaronésia, a emblemática osga gigante de Cabo Verde *Tarentola gigas*, e a osga das Selvagens *Tarentola (boettgeri) bischoffi*, a fim de fornecer informações valiosas para a conservação destas espécies ameaçadas. Pouco se sabia sobre a ecologia e hábitos alimentares das mesmas. No primeiro estudo, tínhamos como objectivo comparar a dieta das duas subespécies de *T. gigas* para guiar a reintrodução na ilha onde se extinguiu. No segundo estudo, comparamos técnicas morfológicas e de metabarcoding associadas a esforços de amostragem muito diferentes para verificar os impactos na representação da dieta de *T. (boettgeri) bischoffi*. Os resultados revelaram que ambas as espécies têm uma dieta generalista, alimentando-se de plantas, invertebrados e até vertebrados. As plantas revelaram ter um papel significativo como itens de dieta, o que anteriormente passou despercebido usando métodos tradicionais. Usando metabarcoding, fomos capazes de identificar uma maior diversidade de itens de dieta e com uma resolução taxonómica geralmente maior. No primeiro estudo, pudemos discutir as opções em relação à reintrodução de *T. gigas* e, no segundo, as vantagens e limitações da técnica de metabarcoding.

No geral, com esta tese, pudemos revelar uma nova gama de presas que anteriormente passaram despercebidas nestas dietas de *Tarentola* com uma resolução taxonómica razoavelmente maior. As informações reveladas por essas redes ecológicas são importantes para o desenvolvimento de planos de conservação nessas áreas protegidas e reforçam o importante e comumente negligenciado papel dos répteis nos sistemas insulares.

Palavras-chave

Tarentola gigas; *Tarentola (boettgeri) bischoffi;* Genética da Conservação; Phyllodactylidae; Áreas protegidas; Áreas remotas.

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List of Abbreviations

BSA - Bovine serum albumin

COI - Cytochrome c oxidase I

DNA - Deoxyribonucleic Acid

IUCN - International Union for Conservation of Nature

m.a.s.l - Meters above sea level

MOTU - Molecular Operational Taxonomic Units

NCBI - National Center of Biotechnology Information

NGS - Next Generation Sequencing

OTU - Operational Taxonomic Unit

PCR - Polymerase Chain Reaction

PERMDISP - Permutational analysis of multivariate dispersions

PERMANOVA - Permutational Multivariate Analysis of Variance

rRNA - Ribosomal Ribonucleic Acid

SVL - Snout-Vent Length

1. General Introduction

1.1. Islands and Macaronesia

Islands are commonly considered natural laboratories as they are isolated, well-defined geographically and have distinct boundaries which led to a microcosmal nature and exclusively evolved biota (Whittaker *et al.*, 2017). As islands represent the greatest concentration of both biodiversity and species extinctions, their study is especially important also from a conservation point of view. Oceanic islands, such as Galapagos, Canary, Madeira, Selvagens and Cabo Verde, arouse special interest as they have no continental origin and are frequently formed as the result of volcanic action. Hence, are colonized initially by species that dispersed from elsewhere and then enriched by speciation. The scarcity of gene flow between islands leads to geographical isolation and subsequent population differentiation. Even though islands normally have a small number of species, comparing to mainland systems, the number of endemics is usually high, especially in remote islands (Whittaker & Fernández-Palacios, 2007). However, those are also more prone to extinction than the mainland species due to the synergy of genetic and demographic factors (Frankham, 1997). The number of terrestrial species varies depending on the interchange of isolation, islands area and shape, habitat diversity, distance to other islands and the mainland, taxon biology and human influence (Triantis *et al.*, 2003; Whittaker *et al.*, 2008). Moreover, islands commonly have more simplified ecological networks, as they present a disharmonic biota leading to a decrease in the number of taxonomic groups, principally in more remote islands (Frankham, 1997). In this way, islands represent simplified models, ideal for studying ecological networks, as the species inhabit more confined areas, being possible to sample in a more complete manner. These studies are very important for the accurate development of conservation measures (Frankham, 1997).

The Macaronesia biogeographical region is located in the North Atlantic Ocean off the European and African coasts (Figure 1). Composed by five archipelagos of volcanic origin and thought to be the product of several geological hotspots (Whittaker & Fernández-Palacios, 2007), comprises Azores, Madeira, Selvagens, the Canary Islands, and the Cabo Verde Islands. Macaronesia climate ranges from the maritime subtropical climate in Azores to the Cabo Verde oceanic tropical-arid climate, including the Mediterranean climates of Madeira and Canaries in the middle (Fernández-Palacios & Dias, 2001). Islands of this region differ from other oceanic islands in means of being close to possible mainland source areas (Carine *et al.*, 2004), presenting links of variable strength between each other and to diverse continental regions (Whittaker & Fernández-Palacios, 2007). Moreover, Macaronesia represents a very interesting study model as it presents several uncommon patterns of colonization and diversification, simplified trophic nets and still little is known about these topics.

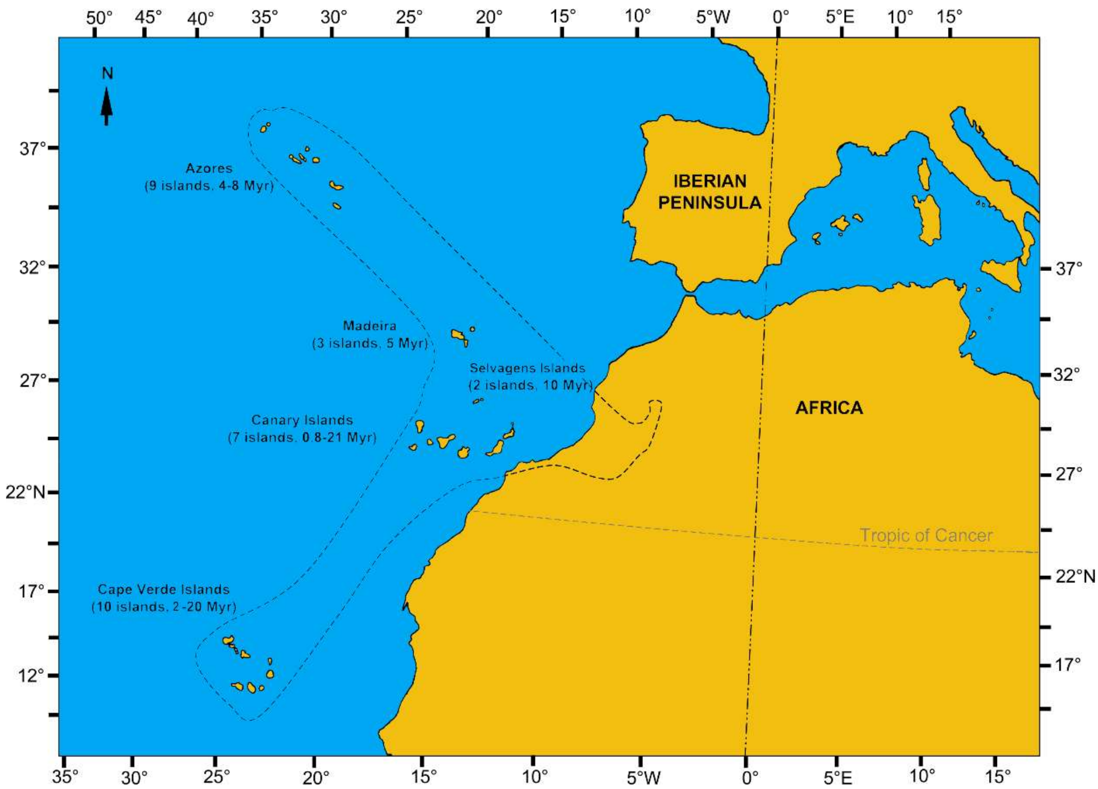

Figure 1. Map of the biogeographical region of Macaronesia, comprising the five volcanic archipelagos (Azores, Madeira, Selvagens Islands, Canary Islands, and Cabo Verde Islands). Adapted from Kim *et al.* (2008).

Cabo Verde is an oceanic archipelago situated about 500 km off the West African coast (Figure 1). This archipelago has a volcanic origin and it was never connected to the mainland (Mitchell-Thomé, 1976). It embraces ten main islands and several islets, arranged in a horseshoe shape, with ages between 2 and 26 million years (Ma), and roughly the youngest ones are situated on the farther west ends of the arc (Stillman *et al.*, 1982; Mitchell *et al.*, 1983; Ancochea *et al.*, 2015). These can be categorized into three main groups according to their ages, characteristics and locations (Holm *et al.*, 2008). The southern group that includes Maio, Santiago, Fogo and Brava islands. The eastern group is composed by Boavista and Sal islands. Lastly, the northern group is constituted by São Nicolau, São Vicente and Santo Antão islands, Raso and Branco islets and the island of Santa Luzia. The latter three compose the Desertas group. Some islands of the last group and probably Boavista and Maio, were most probably linked during the sea-level oscillations during the Pleistocene (Holm *et al.*, 2008). The areas and landscapes of the islands of this archipelago diverge markedly: Santiago is the largest (around 1000 km^2) and Raso islet (<6 km^2) among the smallest; Fogo is the highest (about 2800 m a.s.l.) and Raso islet one of the flattest areas.

The remote Selvagens Archipelago is also a group of oceanic islands of volcanic origin, shaped over oceanic plates. Located around 250 kilometres south of the Madeira Island and 165 kilometres north to the Canary Islands, this archipelago consists of two main islands, Selvagem Grande (about 5 km^2), and Selvagem Pequena (20 ha), and the Ilhéu de Fora (8 ha) and some other islets (Figure

1). These islands can be divided into two groups according to their volcanic basis: the Northeast group composed by Selvagem Grande and the islets Palheiro da Terra and Palheiro do Mar; and the Southwest group comprising Selvagem Pequena, Ilhéu de Fora and other small adjacent islets (Alves *et al.*, 2010). The Selvagens islands were designated as a natural reserve in 1971, as they represent an important nesting point for plentiful bird species, as for example Cory's shearwater (Granadeiro *et al.*, 2006). Nowadays, they are part of Madeira Nature Park and only inhabited by a permanent team of staff members, policeman, and occasionally by researchers and members of the Zino's family (previous owners of the rights to hunt in the area and known as 'the guardians of the Selvagens').

1.2. Reptiles and islands

Reptiles are widely distributed worldwide and are present in all continents, apart from Antarctica. In this way, this is the group of terrestrial vertebrates with higher diversity, about 10,793 species (Uetz *et al.*, 2018), even though is still poorly studied. This group is also, after birds, the vertebrate group more capable of colonizing islands, due to their low metabolic rates and great resistance to dryness and even salinity in some groups like geckos (Carranza *et al.*, 2000). Consequently, reptiles are commonly predominant on islands, representing a remarkable model for evolutionary and ecological studies in those systems (Pincheira-Donoso *et al.*, 2013). Lizards, in particular, play important roles in the ecosystem as seed dispersers, pollinators (Borzí, 1911; Elvers, 1977; Whitaker, 1987; Nyhagen *et al.*, 2001) and in few cases even as top predators (Alcover & McMinn, 1994; Miranda, 2017), more often than in mainland systems. Moreover, reptiles inhabiting remote oceanic islands frequently present peculiar ecological adaptations. For instance, gigantism or dwarfism and lack of appropriate defensive mechanisms are common characteristics in insular reptiles (Zunino & Zullini, 1995).

The genus *Tarentola* belongs to the Phyllodactylidae family, includes about 33 distinct species (Joger, 1984a, 1984b; Schleich, 1984; Carranza *et al.*, 2002) generally named wall geckos. These geckos have their distribution across the Mediterranean Islands, southern Europe, and North Africa. The subgenera Makariogecko can be found in several islands of the Macaronesia region, specifically Madeira, Selvagens, Canary Islands and Cabo Verde Islands, but some species also occur in West India (Carranza *et al.*, 2000; Vasconcelos *et al.*, 2010). Individuals of this genus are typically more active by night, even though they often can be found during the day. They generally inhabit relatively dry areas with rocky surfaces, presenting often climbing habits, but can be as well found in non-natural habitats (Arnold & Ovenden, 2002). In more widespread species, as *Tarentola mauritanica* (Linnaeus, 1758), the dietary composition is already fairly studied (Gil *et al.*, 1994; Hódar *et al.*, 2006). However, in species with more restricted distribution ranges, these studies are not common and sometimes even difficult to accomplish, especially those inhabiting remote islands. In these cases, diet analysis studies are even more essential to expand our understanding of community assembly and population dynamics, revealing food web structures and the ecosystem functioning as a whole (Valentini *et al.*, 2009; Kartzinel *et al.*, 2015). Thus, those studies are important in the understanding of animal ecology, evolution, and conservation needs (Symondson, 2002; Krahn *et al.*, 2007; McDonald-Madden *et al.*, 2016). Additionally, reptiles represent good models for dietary studies since they are usually locally abundant, easy to manipulate and to collect non-invasive samples.

The *Tarentola* from Cabo Verde are particularly interesting, as they reached these islands about 7.7 million years ago (Ma) (Vasconcelos *et al.*, 2010) originated in a single colonization event from the western Canary Islands (Carranza *et al.*, 2000). From this event, 12 endemic species and several subspecies were originated: *T. boavistensis* (Joger, 1993), *T. bocagei* (Vasconcelos, Perera, Geniez, Harris & Carranza, 2012), *T. fogoensis* Vasconcelos, (Perera, Geniez, Harris & Carranza, 2012), *T. darwini* (Joger, 1984b), *T. caboverdiana* (Schleich 1984), *T. substituta* (Joger, 1984), *T. raziana* (Schleich, 1984), *T. nicolauensis* (Schleich, 1984), *T. maioensis* (Schleich, 1984), *T. rudis* (Boulenger, 1906), *T. protogigas* (Joger, 198), and *T. gigas* (Bocage, 1875) (Vasconcelos *et al.* 2012). Nevertheless, scarce information is available on the ecology of these geckos, including diet apart from some occasional observations by Schleich (1987) and Mateo *et al.* (2016).

The Selvagens gecko *Tarentola (boettgeri) bischoffi* (Joger, 1984) is endemic to the remote Selvagens Archipelago, occurring in three isolated subpopulations, which correspond to Selvagem Grande, Selvagem Pequena and Ilhéus de Fora (Rebelo, 2010). The taxonomy and systematics of this taxa are still under discussion; however it is accepted that this gecko belongs to a group of the *Tarentola* genera, which besides being found in Selvagens, also occur in two islands of the Canary Archipelago – Gran Canaria, *Tarentola boettgeri boettgeri* (Steindachner, 1891), and El Hierro, *Tarentola boettgeri hierrensis* (Joger & Bischoff 1983). The group is related with *Tarentola mauritanica* populations from North Africa, from which were separated about 17.5 Ma as a result of an ancient Macaronesian colonization (Carranza *et al.*, 2000; Carranza *et al.*, 2002). There is some information on their ecology (Penado *et al.*, 2015) and diet (Gil, 2011), however, the latter is only based on classical methods.

1.3. Diet assessment and DNA metabarcoding

Several methods can be implemented to analyse the diet of reptiles, such as direct observation, morphological identification, enzyme electrophoresis, immunological assays, stable isotopes analysis and, more recently, DNA-based methods (Symondson, 2002). Direct observations is a simple approach that depends on limited equipment, but presents several limitations, as possible disturbance of the natural behaviour of the predator and the potential preys by the presence of the researcher (Litvaitis, 2000). Furthermore, direct observations can be unsuitable to perform when studying nocturnal, burrowing species (Pompanon *et al.*, 2012), as is the case. When using morphological analysis by microscopic inspection of gut or faeces contents, there is a tendency to underestimate the frequency occurrence of prey (Brown *et al.*, 2012), especially when only soft tissue has been ingested or when the prey is completely soft-bodied. Also, this technique requires knowledge of several experts to correctly identify the prey of different taxonomic groups, demanding a large expenditure of time (Brown *et al.*, 2014). Analysis that involve stomach contents of reptiles generally implies the sacrifice of the animal or an important decrease of its fitness, especially ectotherms inhabiting arid environments with low food-availability (Litvaitis, 2000). Stable isotopes can offer a non-invasive alternative, providing information on trophic interactions, habitat use, migration and diet composition (Najera-Hillman *et al.*, 2009). This method uses stable isotopes ratios, as carbon and nitrogen, to determine the type of diet and measure the relative proportions of each prey assimilated. Nevertheless, this approach lacks resolution on prey items due to the overlap of isotopic values in some cases (Layman *et al.*, 2007; Caut, 2013). In addition, a broad knowledge of the prey isotopic signatures is required, which can be difficult to obtain (Corse *et al.*, 2010), especially regarding generalist species.

Hence, DNA-based methods are the most recent approach used in dietary studies. With the recent generalization of high throughput sequencing methodologies, and even more recent development of DNA metabarcoding techniques, the identification of prey items was further improved. Using DNA-based methodologies it is possible to identify prey material even when the hard parts do not survive the digestive process, which most times cannot be achieved with other methods. The DNA metabarcoding Next Generation Sequencing (NGS) is a technique that allows the identification of multiple food items in a species diet through sequencing standardized DNA fragments (Pompanon *et al.*, 2012). This technique is based on the mass-amplification of DNA using general or group-specific primers, followed by the cloning and sequencing of amplicons to identify individual taxa. DNA metabarcoding is a very advantageous methodology for diet studies of species difficult to observe in the act of eating or whose prey is difficult to identify visually (Kartzinel & Pringle, 2015), as it can be applied to non-invasive samples. It provides comprehensive taxonomic identification of food items within highly diverse diets, relenting less on taxonomic expertise and using non-invasive or degraded samples (Pompanon *et al.*, 2012). This precision is possible due to the capacity of this technique to maximize resolution, detect rare events, and detect soft, small and invisible prey items, and ultimately correct biases in ecological models (Taberlet *et al.*, 2012). Furthermore, accessing diet composition from faecal DNA is particularly advantageous because samples can be obtained with minimum impact to the animals (Pompanon *et al.*, 2012; De Barba *et al.*, 2014).

Despite the potential of this technique, it has some methodological implications. The first to consider before starting a metabarcoding study is the marker choice, the primers should be able to amplify the range of expected prey without amplifying other taxa that may be present in the samples, also the taxonomic resolution of the marker region should be considered when selecting a primer set (Pompanon *et al.*, 2012; Deagle *et al.*, 2014). It is also needed to have in consideration that this method only provides the species present in the samples and not their relative abundances (Piñol *et al.*, 2015). The number of reads resulting from the NGS does not correspond to the number of food items ingested, due to some biological aspects as prey digestibility and size (Jarman *et al.*, 2013), different tissue cell densities and variation in gene copy number (Pompanon *et al.*, 2012). The obtained data can also be biased during DNA extraction, PCR pooling, sequencing and bioinformatic processing (Pompanon *et al.*, 2012). In dietary studies using faecal pellets, another implication rises as the DNA extracted is much degraded; therefore, DNA from the predator is highly dominant comparatively with the prey DNA (Vestheim & Jarman, 2008). Oligonucleotides are 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). These blocking primers compete with the amplicon-specific primers, binding to predator DNA by preference, also they are modified with a 3-carbon spacer (C3-spacer) at the 3'-end which blocks further amplification (Vestheim & Jarman, 2008).

In conclusion, NGS studies represent a revolutionary tool for conservation research and management being more and more used for a variety of cases (Allendorf & Luikart, 2009). Metabarcoding methods are particularly important as they can deliver rapid and holistic results on species composition, diversity, ecological networks, among others, at relatively low costs (Taylor & Gemmell, 2016). Moreover, metabarcoding can provide a high amount of data in a short time being of great help to raise the success of biodiversity conservation actions by responsible institutions (Ji *et al.*, 2013), especially in areas that are of difficult access and that require urgent actions as it is the case of the biodiversity hotspots (Taylor & Harris, 2012; Thomsen & Willerslev, 2015).

2. General objectives

This thesis is divided in a general introduction, two manuscripts, general conclusions and supplementary material. Both manuscripts used DNA metabarcoding approaches to study the diet of Macaronesian geckos. The first one focuses on the diet of the giant wall gecko *Tarentola gigas* present in the Desertas group of the Cabo Verde Archipelago and aims to be a comparison between the diet of its two subspecies, one from Branco Islet and the other from Raso Islet. This information can be applied in a guidance plan for the future reintroduction of the species on Santa Luzia Island, where the species previously occurred but no longer exists due to human pressure. This is my main manuscript as I processed all the samples from the Branco Islet and part of the samples from Raso.

The second manuscript focuses on the Selvagens gecko *Tarentola (boettgeri) bischoffi* of the Selvagens Archipelago. The main objective was to compare the efficacy between the metabarcoding approach and classic methods in remote areas in recovering the diet diversity of top predators. Since it is a zone of difficult access, rapid surveys using metabarcoding analysis could provide results with better resolution and diversity of preys with less field and processing effort. For this manuscript, I did all the metabarcoding analysis, whose results were compared with data previously obtained during two expeditions of several weeks, analysed using traditional methods.

Manuscript I

What is the Giant Wall Gecko having for dinner? Conservation genetics for guiding reserve management in Cabo Verde.

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Abstract

Knowledge on diet composition of a species is an important step to unveil its ecology and guide conservation actions. This is especially important for species that inhabit remote areas within biodiversity hotspots, with little information about their ecological roles. The emblematic giant wall gecko of Cabo Verde, *Tarentola gigas*, is restricted to the uninhabited Branco and Raso islets, and presents two subspecies. It is classified as Endangered, and locally Extinct on Santa Luzia Island; however, little information is known about its diet and behaviour. In this study, we identified the main plant, arthropods, and vertebrates consumed by both gecko subspecies using next generation sequencing (NGS) (metabarcoding of faecal pellets), and compared them with the species known to occur on Santa Luzia. Results showed that plants have a significant role as diet items and identified vertebrate and invertebrate taxa with higher taxonomic resolution than traditional methods. With this study, we now have data on the diet of both subspecies for evaluating the reintroduction of this threatened gecko on Santa Luzia as potentially successful, considering the generalist character of both populations. The information revealed by these ecological networks is important for the development of conservation plans by governmental authorities, and reinforces the essential and commonly neglected role of reptiles on island systems.

Keywords: Desertas Islands; conservation; diet; metabarcoding; protected areas; *Tarentola gigas*

1. Introduction

Biodiversity is supported by an entangled network of interactions, and recognising this is crucial to guarantee the persistence of endemic and restricted-range taxa. The existing literature shows a clear bias towards certain taxonomic groups (e.g. for birds; see [1]) with reptiles typically receiving poor attention. Even though perceived as negligible, reptile species are important for several trophic ecological processes, especially on island ecosystems [2]. Gathering quantitative research on the subject is one of the solutions suggested to change this misleading paradigm [3].

The investigation of diet composition is one of the first steps to unveil species ecology and collect reliable data to guide conservation actions. Since detailed methods for faecal analysis were described, this technique has been widely used for determining the diet of many species, including reptiles [4]. This resulted in a large amount of information on biotic interactions of great importance to learn about their functional roles for the management of their habitats, which is especially valuable for threatened species. These entangled networks can only begin to be understood by a quantitative analysis of diets. However, several disadvantages of faecal analysis based on morphological identification of prey have also been reported, as in these methods there is a tendency to underestimate the frequency occurrence of prey [5], especially when only soft tissue has been ingested or when the prey is completely soft-bodied. Also, this technique requires the knowledge of several experts to correctly identify the prey of different taxonomic groups, thus demanding a large expenditure of time [6].

To maximize the allocation of scarce resources for conservation efforts in face of extensive anthropogenic threats, climate change, and accelerating extinction rates, the use of new and faster tools is needed [7]. This is especially relevant especially in developing countries within biodiversity hotspot areas. Next Generation Sequencing (NGS) metabarcoding is an advantageous alternative to classic approaches in the analysis of faecal pellets. This technique maximizes resolution, detection of rare events, and detection of soft, small and invisible prey items. It can ultimately correct biases in ecological models, and it is less reliant on taxonomic expertise [8]. Moreover, this method facilitates diet characterization for species that are difficult to observe in the act of eating, such as nocturnal reptile species [9], and that inhabit remote areas with an urgent need for conservation actions. Little is known about the dietary composition of geckos and their functional roles on island ecosystems. Some of the few existing studies were conducted based on direct observations and using classic techniques to analyse faecal contents of the most widespread species, such as the moorish gecko *Tarentola mauritanica* (Linnaeus, 1758) [10,11] and the white-spotted gecko *Tarentola annularis* (Saint-Hilaire, 1827) [12,13].

The Cabo Verde Islands belong to the biogeographical region of Macaronesia and is a young developing country within the Mediterranean biodiversity hotspot. This archipelago is located in the Atlantic Ocean, approximately 500 km off the African coast and comprises ten main islands and several islets, formed by a volcanic hotspot and never connected to the mainland [14]. Some of these islands and islets are presently uninhabited, such as Santa Luzia Island, Branco and Raso islets, and so are named the Desertas Islands. These are important breeding grounds for birds classified as Integral Nature Reserves since 1990 [15] and as a Marine Protected Area since 2003 [16].

This uninhabited tropical dry island group holds important endemic and highly-threatened species, some of them occurring exclusively on these areas [17], yet are poorly studied due to their remoteness and harsh logistical constrains, such as the lack of potable water and need of special permits. These islands hold seven seabird and 11 terrestrial breeding bird species, and four threatened species of reptiles [18]. After birds, reptiles are the most important and the only terrestrial vertebrate group, and knowledge about their diet composition is also an indirect way of learning about the whole trophic network and the richness in biodiversity of these under-sampled areas.

An emblematic Cabo Verdean reptile is the Endangered giant wall gecko *Tarentola gigas* (Bocage, 1875), which is presently one of the largest geckonids in the world. This endemic species is predominantly nocturnal [19], oviparous [20] and actually restricted to Branco and Raso islets [21]. Subfossil evidence from owl pellets and other remains indicate that the species inhabited once São Vicente and Santa Luzia islands, but disappeared from the diet of predators following human settlement and the introduction of mice and cats [22,23]. Little information is known about their population size (expected to fluctuate strongly due to fluctuating pressures on the populations of its prey species related with rainfall), diet and behaviour in the two islets. Morphological analysis of the gecko's faecal pellets and gut contents has already shown the presence of plants, invertebrates, fish scales, and seabird feathers [23]. Nevertheless, more information is needed on the ecology of the species; therefore, research is recommended for its conservation [24] and to investigate the possibility of its reintroduction on Santa Luzia [25]. Just recently, a restoration action plan was proposed that includes the on-going removal of introduced mammal predators of that island [26], enabling this conservation strategy to be set in action.

Concerning the functional role of *T. gigas,* the species is supposed to show strong trophic links with birds due to the scarcity of insects and other small prey on the islets [27]. The two subspecies were described as commensal of seabirds, as they normally inhabit their cliff-holes and burrows near the coast, although they can also be found under rocks inland [27]. The subspecies present on Raso *T. gigas gigas* (Bocage, 1875) usually feed on regurgitated food from several seabird colonies, but also on broken eggs and possibly the young of nesting birds [20,28]. It is probably the major natural predator of eggs of the Raso lark *Alauda razae* (Alexander, 1898), a Critically Endangered bird now restricted to Raso Islet [29], although that was not confirmed in the recent study based on morphological analyses of faecal pellets [30]. In this study, fishes were the most frequent item, followed by arthropods and plants. On Branco, where Raso lark is absent, the subspecies *T. gigas brancoensis* (Schleich, 1984) presumably relies primarily on colonies of the Near Threatened endemic Cabo Verde Shearwater *Calonectris edwardsii* (Oustalet, 1883), though little information is available. Since the reintroduction of the Raso lark on Santa Luzia Island started in 2018 (with the release of around 25 birds and the first nestlings born in August), it is necessary to confirm and access the importance of the predation of this gecko on this bird species. Metabarcoding analysis can be really useful in this situation, as it provides, with less effort and time, a large and reliable amount of data, identifying multiple food items [8].

With this study, we intend to quantify the trophic interactions of the two subspecies, *T. gigas gigas* and *T. gigas brancoensis*, so that authorities can use this data to evaluate the reintroduction of this threatened gecko on Santa Luzia. For that, the main objective of this study is to identify the main plant, arthropod and bird species consumed by both subspecies of *T. gigas* using NGS methods (metabarcoding of faecal pellets) and compare them with the species known to occur on Santa Luzia. The information revealed by these networks is of great importance to evidence the ecological role of reptiles in ecosystems, especially in islands where little is known, and to help in the development of conservation plans on these protected areas.

2. Methodology

2.1. Study Area

This study took place on the Desertas islands, composed by Santa Luzia Island and Branco and Raso islets, situated on the northwest alignment of the Cabo Verde Archipelago (16°48'N, 24°47'W and 16°36'N, 24°34'W; Figure 1A), flanked by the islands of São Vicente to the West and São Nicolau to the East (Figure 1B). The three islands have a total of 43.3 km^2 of land area and present quite low elevations compared to the other islands of the archipelago. This group of islands is located at the border of the North African arid and semi-arid climatic regions, presenting a climate defined as dry tropical Sahelian, predominantly represented by flat, very arid lowlands (Figure 1C), followed by very arid medium elevation areas, then beaches, dunes and sandy areas, streams and floodplains [31]. By means of the low elevation, the annual precipitation is among the lowest in Cabo Verde, which should be the primary limiting factor of the distribution of terrestrial biodiversity in the islands, leading to a low diversity of plant and insects in the area, mainly on Raso [18].

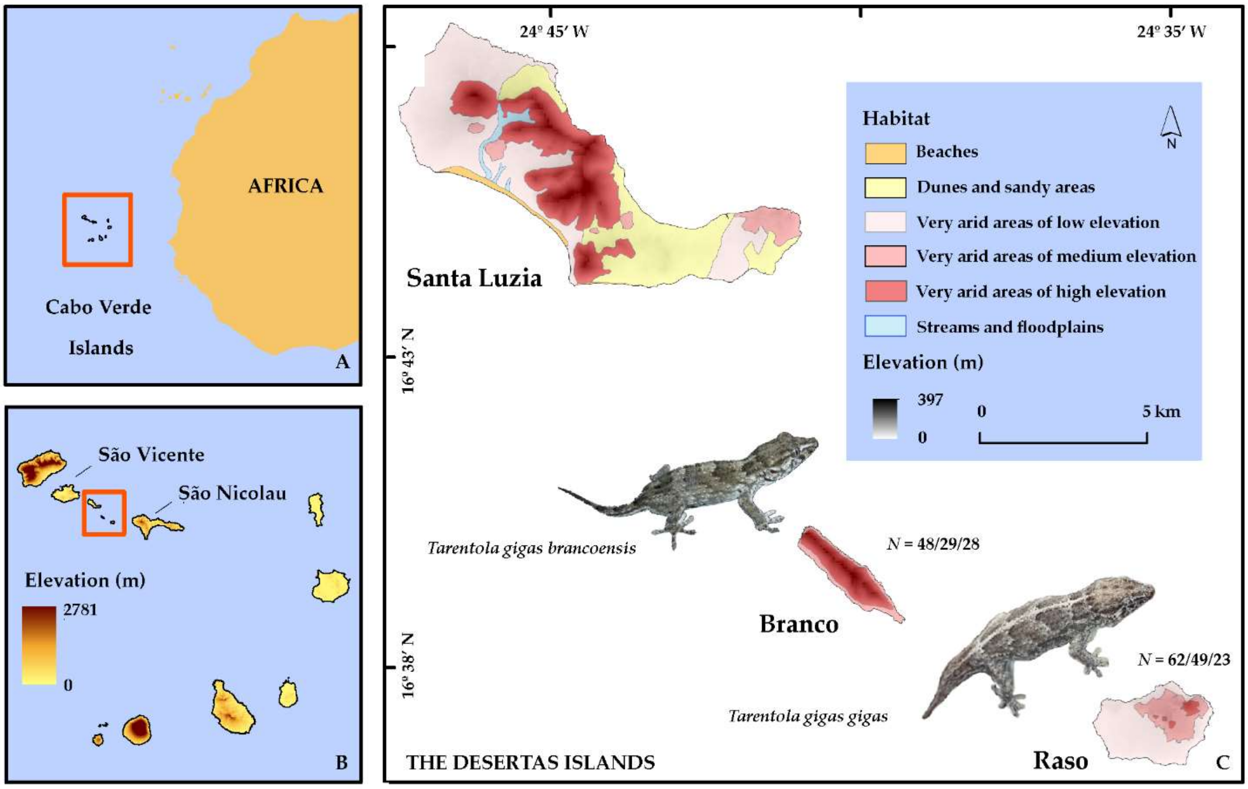

Figure 1. Studied area and taxa. Map of the Cabo Verde Islands, showing the geographic location (**A**), elevation (**B**), and focusing on the Desertas group (**C**). The habitat types and the two studied subspecies are also represented, as well as the number of samples (*N*) collected, extracted, and used in the analyses, respectively (Geographic Coordinate System, Datum WGS84)

Santa Luzia presents a land area of approximately 35 km^2, and has the highest elevation of the group, reaching 397 m. This island is very arid, yet there are more humid zones close to the river line, with hills, rocky plains and sand dunes being the main landscapes. Branco Islet is the smallest of the group with a land area of approximately 3 km^2. Mountainous (2 km^2) and medium-elevation (1 km^2) arid areas dominate the islet's landscape [32]. The islet is of difficult access due to the roughness of the sea, lack of safe natural ports, and steepness (there is only a minor area of plane ground of about 400 x 200 m). Raso Islet has a land area less than 6 km^2 and, in contrast with Branco, is almost flat in all its extent. This islet is fundamentally characterized by plains and low-altitude arid zones (Figure 1C) with patches of grassy vegetation.

2.2. Study species

The Cabo Verde giant wall gecko *T. gigas* is the largest gecko in Cabo Verde, reaching a maximum of 155 mm snout to vent [21]. It was classified as Endangered in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species mainly, due to its reduced distribution and exploitation of its prey species [24]. The subspecies *T. gigas gigas* inhabits the Raso islet and differs morphologically from the subspecies of Branco islet, *T. gigas brancoensis*, by its longer snout, larger number of scales at mid-body and by the proportion between the width and length of the fourth toe, normally lower than 1:5 [21,33,34]. As mentioned above, both subspecies are nocturnal, oviparous, most ground-dwelling, and use rocks and seabird's burrows as diurnal refuges [21,24,33,34].

2.3. Sampling

Sampling on Raso took place from September to December 2016 and on Branco during September 2017. Based on precipitation data from São Pedro (São Vicente) weather station (https://cv.freemeteo.com), these months belonged to the wet season. Several different sites of the islets were sampled with the purpose of embracing all possible habitats that could provide different food resources for the reptiles, overlapping with occurrence sites of several bird species. We collected 62 specimens in Raso and 48 in Branco (Figure 1C). All of them were captured by hand and received a belly massage in order to release the fresh pellets, which were preserved in tubes with 96% ethanol. The individuals were also sexed based on the absence or presence cloacal pouches [21], measured (snout-vent length (SVL) to the nearest mm), and a sample of the tip of the tail was collected before releasing each animal. Each sample was geolocated using a GPS device and photos were taken to confirm the data in case of uncertainties (e.g., sexing). Sampling and protocols were approved by "Direção Geral do Ambiente" (DNA), Cabo Verde (no 58/2017).

Samples of invertebrates, vertebrates and plants were collected from Santa Luzia, Raso and Branco in order to build a DNA reference collection of possible food items. For the collection of invertebrates, pitfalls were placed on each island in two areas of the islands (sandy and compact soil). Replicas were set on a different location with the same soil type on each island to gather a representative sample of the island invertebrate biodiversity. Specimens were separated in different high-level taxonomic groups based on morphological identification, and photographed with a camera assembled on a magnifying lens for morphological identification at higher taxonomical resolution by experts. Vertebrate samples were collected in the field from dead animals or traces of presence such as feathers and eggshells. Samples of leaves and flowers were collected across the islands, and pictures were also taken in the field to allow morphological taxonomic assessment by experts.

2.4. DNA Extraction and amplification

The collected *T. gigas* pellets were completely dehydrated in an incubator at 50ºC in order to remove all traces of ethanol. Then, DNA was extracted using the Stool DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada), following the manufacturer's instructions. Two DNA elutions were obtained in a total volume of 50 µL each and were frozen at -20 ºC until DNA amplification.

The plant reference library was constructed by extracting DNA from leaves and flowers collected in the islands, using DNeasy Plant Mini Kit (Qiagen, Crawley, UK) following some alterations according to [35]. Invertebrate DNA extraction was carried out from a leg or wing sample of each different Operational Taxonomic Unit (OTU) identified by the experts using saline extraction methods [36]. This protocol was also used for the DNA extraction of vertebrates.

Three different DNA fragments were chosen to identify the distinct prey groups (plants, invertebrates, and vertebrates) that presumably compose the diet of the study species. For plants was used the g/h primers targeting the short P6-loop of chloroplast trnL (UAA) intron (see Supplementary Table S1, [37]) and for invertebrates a modified version of the IN16STK-1F/IN16STK-1R primers was used, targeting the mitochondrial 16S rRNA (Supplementary Table S1). The 16S primers were specifically designed to amplify the insect diet of lizards while avoiding the amplification of the lizards, while the trnL has been extensively used in both environmental DNA (eDNA) assessments and diet studies. For the amplification of vertebrate DNA, the 12sv5F and 12Ssv5R primers targeting the V5-loop fragment of the mitochondrial 12S gene (Supplementary Table S1) were used. This marker has been shown to have great resolution power for genus and species identification across numerous vertebrate taxa [38]. To avoid the amplification of *T. gigas* DNA, a blocking primer (5'-CCCCACTATGCTCAACCGTTAACAAAG (C3 spacer)-3') was used. This primer was designed by building an alignment with available 12S sequences of this species, as well as of birds and fishes known to occur in Cabo Verde or of taxonomically related species. We further modified the all primers in order to contain Illumina adaptors and a 5 bp individual identification barcode to allow individual identification of each sample.

For the trnL and 16S markers, PCR reactions were carried-out in volumes of 25 µL, comprising 10.4 µL of QIAGEN Multiplex PCR Master Mix (Quiagen, Crawley, UK), 0.4 µL of each 10µM primer, 10.8 µL of ultra-pure water, and 3 µL of DNA extract. 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 and 52°C, respectively, for 30s and extension at 72 °C for 30s, with a final extension at 72 °C for 10 min. For the 12S marker, PCR reactions were carried-out in volumes of 25 µL, comprising 10.4 µL of QIAGEN Multiplex PCR Master Mix, 0.4 µL of each 10µM primer, 8µL of 10µM blocking primer, 2.8 µL of ultra-pure water, and 3 µL of DNA extract. Cycling conditions used an initial denaturing at 95 °C for 15 min, followed by 39 cycles of denaturing at 95 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min.

Reference collection plant samples were amplified for the chloroplast trnL (UAA) using primer 'e' and 'f' [39]. PCR reactions were carried-out in volumes of 25 µL, comprising 4 µL of QIAGEN Multiplex PCR Master Mix, 1 µL of each 10µM primer, 16.4 µL of ultra-pure water, 0.5 µL of bovine serum albumin (BSA 20mg/ml) and 3 µL of DNA extract. Cycling conditions used an initial denaturing at 94 °C for 10 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 50°C for 3 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 8 min. Invertebrate and vertebrate DNA for the reference collection was amplified for the same markers stated before for this groups to allow the matching with the dietary sequences. The DNA from Invertebrates was amplified for 16S using the same PCR conditions; however, these samples were also sequenced for the standard cytochrome oxidase I (COI) barcode fragment using LCO1490/HC02198 following PCR conditions as described in [40], allowing, in this way, to confirm dubious taxonomic assignations. PCR reactions for vertebrate DNA were carried-out in volumes of 25 µL, comprising 10.4 µL of QIAGEN Multiplex PCR Master Mix, 0.4 µL of each 10µM primer, 11.8 µL of ultra-pure water, and 2 µL of DNA extract, following the same cycling conditions referenced before for this marker.

2.5. Library preparation

Succeeding amplification, library preparation was carried out following the Illumina MiSeq protocol 16S Metagenomic Sequencing Library Preparation [41]. Before sequencing, PCR products were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) to remove free primers and primer dimers, following two cleaning steps with ethanol and a final dilution using 10nM Tris. The purified products were quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and subsequently normalized to 10 ng/µL. Samples amplified with different barcodes were pooled together. Afterwards, an indexing PCR was performed for the incorporation of the Illumina-compatible indexing primers to each pool, using the Nextera XT Kit (Illumina, San Diego, CA, USA), allowing individual identification of each amplified product. The PCR reactions and cycling conditions were similar to the ones of the first PCR except that only eight cycles of denaturing, annealing and extension were done, with annealing at 55 ºC. The indexed PCR products were again cleaned, quantified and pooled at equimolar concentrations (15 nM). The final pool was quantified by qPCR (KAPA Library Quant Kit qPCR Mix, Bio-Rad iCycler, Hercules, CA, USA), diluted to 4 nM, and run in a MiSeq sequencer (Illumina) using a 2x150 bp MiSeq Reagent Kit for an expected average of 12,000 paired-end reads per sample.

The reference collection samples amplified for COI and trnL markers were sequenced using Sanger sequencing.

2.6. Bioinformatics

The software package Obitools (https://git.metabarcoding.org/obitools/obitools) was used for general sequence processing (as described in [42]). Forward and reverse sequences were aligned (command illuminapairedend) and discarded if the overlapping quality was less than 40. Reads were then assigned to samples and primers and barcodes were removed (command ngsfilter), this allowed a total of four mismatches to the expected primer sequence. Lastly, the reads were collapsed into unique haplotypes. Singletons (haplotypes with only one read) and the potentially erroneous haplotypes resultant from PCR errors were deleted (command obiclean), by removing haplotypes that differ by 1 bp from most abundant haplotypes. This way any 'A' haplotype differing one base-pair from a 'B' haplotype, with an absolute read count lower than 'B', and that was not found without the presence of 'B' in any sample, was removed. After that step, the samples with less than 100 reads in total were considered to have failed and removed. For the remaining ones, haplotypes representing less than 1% of the total number were removed from each sample [42].

Haplotypes were identified by comparing the final set against the GenBank online database (https://www.ncbi.nlm.nih.gov/), as well as the obtained reference samples. The sequences with less than 90% of similarity between known species were classified only to class level, the ones with similarity between 90-95% were classified to the family level, and sequences presenting more than 95% of similarity between known species were classified to species or genus level. The obtained results were also compared with Cabo Verde databases referred to in the literature [17] and other databases for birds (https://avibase.bsc-eoc.org), marine species (http://www.marinespecies.org/), and the encyclopedia of life (http://eol.org). When the same haplotype matched more than one species or genus with similar probabilities, there were only considered species or genera known to occur in Cabo Verde. After identifying all the haplotypes, the ones with a high probability of arising from lab contaminations were discarded.

2.7. Data analysis

Frequencies of occurrence of plants, invertebrates and vertebrates (fishes, reptiles and birds) were estimated for both diets. Overlap on the occurrence of the plants, invertebrates and vertebrates was visualized with Euler proportional elliptic diagrams, using the Euler command from the package eulerr [43] of the statistical environment R 3.4.1., to check the possibility of secondary consumption and the generalist/specialist character of the individuals. Differences of frequencies of each group between diets were compared using chi-square tests in the same statistical environment. They were also compared with previous published results [30].

In order to assess if there were differences in prey species richness between the two islets, measured in Molecular Operational Taxonomic Units (MOTUs) we compared MOTU richness using a chi-square comparison and calculated asymptotic MOTU richness and 95% confidence intervals with an endpoint of 1000 samples, using command iNEXT from the INEXT package [44], using R 3.4.1.

A permutational multivariate analysis of variance (PERMANOVA) was carried out using the vegan package (function ADONIS) with the aim of comparing diet composition between the two subspecies of each islet [45]. A matrix of the presence of each MOTU in all samples was made. For the invertebrates, due to the lack of taxonomic resolution, MOTUs were grouped into orders. A dissimilarity matrix was calculated using the Jaccard measure, due to the binary (presence/absence) nature of our data. A homogeneity of dispersion test (PERMDISP) was also carried out in order to assure the significance of the PERMANOVA test, as it assumes an equal dispersion of values across the different groups. Afterwards, a similarity percentage analysis was performed, also using the vegan package (function simper), to infer the contribution of each prey to the differentiation between diets. Also, the Czekanowski niche overlap index was calculated to understand the niche overlap between the two subspecies diet using command czekanowski from EcoSimR package [46], using R 3.4.1.

3. Results

A total of 110 faecal samples were collected (Raso = 62; Branco = 48) of which 78 samples showed clear signs of amplification and were therefore sequenced (Raso = 49; Branco = 29). After all the analytical and bioinformatics procedures, our final dataset comprised 51 samples (Raso = 23; Branco = 28).

Overall, we identified 139 prey items of 11 taxonomic classes, from plants to birds (Supplementary Table S2). Plants were distributed among three classes, 17 orders, and 21 families (Zygophyllaceae occurred more frequently). Invertebrates from five classes, 13 orders, and 42 families were detected, with higher frequencies of Noctuidae (Lepidoptera) and Culicidae (Diptera). Vertebrates were identified from three classes, seven orders, and 12 families. Some families were exclusively detected in one subspecies diet. For example, Tenebrionidae invertebrates were only present in the *T. gigas brancoensis* subspecies, while Aizoaceae plants were only found in the *T. gigas gigas* subspecies.

The occurrence of the three taxonomic groups (plants, invertebrates, and vertebrates) in both diets was very similar, with a high overlap of groups and very few samples with just one taxonomic group detected (Figure 2A). The overlap was higher between plants and invertebrates (plants and invertebrates: Raso = 54%, Branco = 35%) than between the other combinations of groups. The number of samples with an overlap between plants and invertebrates was also higher than the number of samples with an overlap of all three groups (plants, invertebrates, and vertebrates: Raso = 37%, Branco = 25%). In both diets, plants and invertebrates were the most frequent groups, followed by birds and reptiles (Figure 2B). On Raso, the frequency of all groups was higher (plants: X^2 (1) = 1.751, p = 0.186; invertebrates: X^2 (1) = 3.328, p = 0.068, birds: X^2 (1) = 0.000, p = 0.990; reptiles: X^2 (1) = 3.59 x e^{-31}, p = 1.000) with the exception of the fishes, that were more frequent in Branco samples (X^2 (1) = 0.628, p = 0.428), yet all these differences were not significant. In comparison with previously published results [30], the number of occurrences was always higher for all taxonomic groups, with the exception of fishes. Although plants had a higher occurrence, invertebrates showed a higher diversity of MOTUs (Supplementary Table S2).

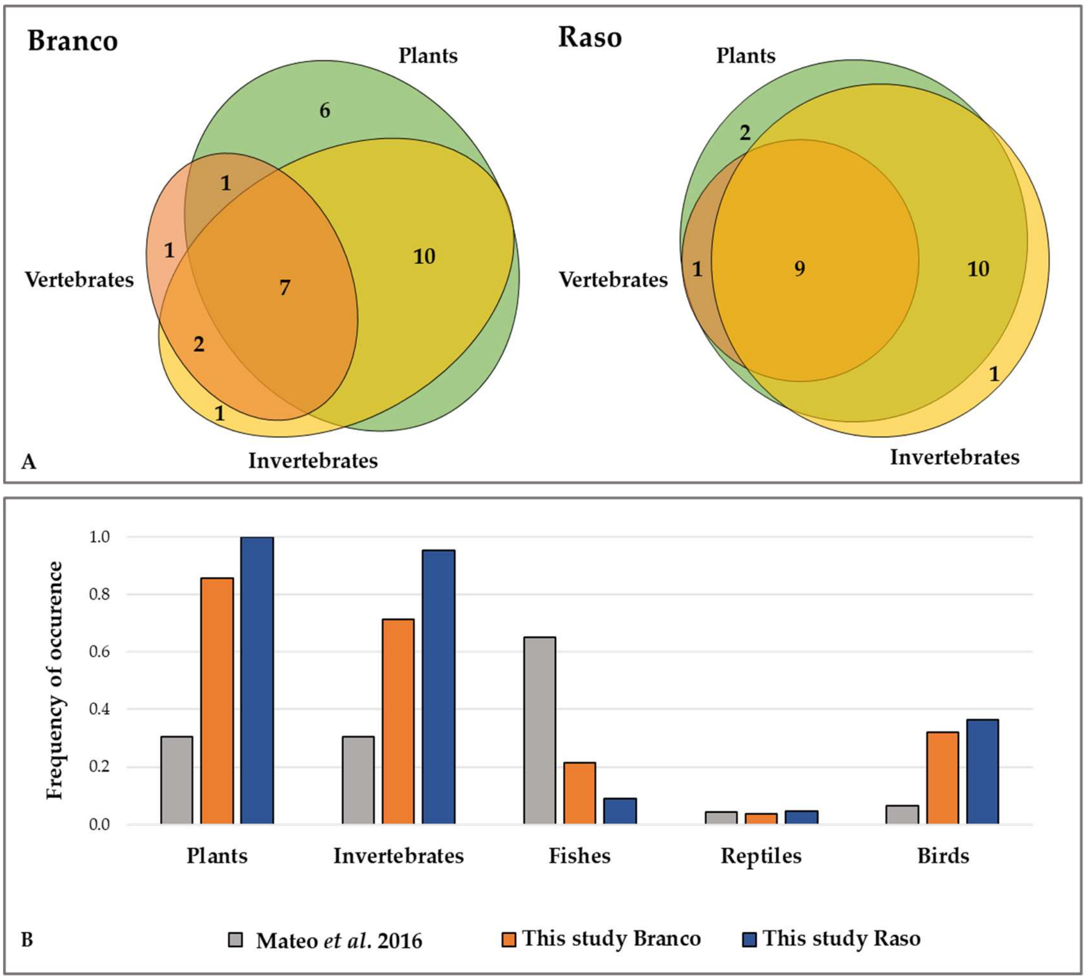

Figure 2. Metabarcoding results for each subspecies and comparison with classic methods. (**A**) Euler diagrams showing the occurrence and overlap of the three main prey groups (plants in green, invertebrates in yellow, and vertebrates in orange) in the faecal samples from the Branco and Raso islets. (**B**) Frequencies of occurrence of plants, invertebrates and vertebrates (fishes, reptiles, and birds) in the faecal samples from the Branco (orange) and Raso islets (blue). The results from a previous study [30] are also shown for comparison (grey).

Species richness was similar between diets (Raso = 84, Branco = 95, X^2 (1) = 0.032, p = 0.932). The extrapolated species richness in Branco was higher, but with high overlap on the lower limit of the 95% confidence interval (Raso = 208 ± 53.8 (139 – 364); Branco = 249 ± 60.7 (168 - 419)). There were significant differences in the MOTU composition between diets (Supplementary Table S3) and no effect of data dispersion on the results (Supplementary Table S4). This was corroborated by the low overlap between diet MOTUs (Czekanowski index = 0.31). Hemiptera and Lepidoptera were the MOTUs that contributed the most for the differences between diets, followed by a diverse set of MOTUs belonging to plants and birds (Figure 3).

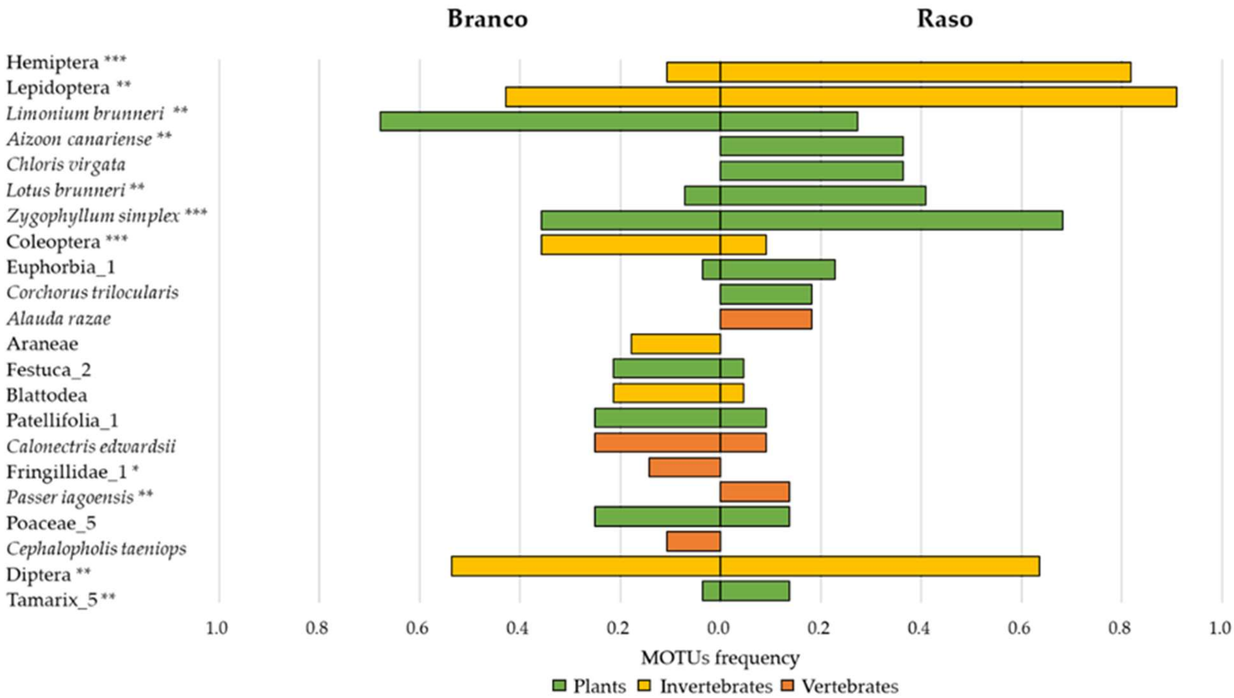

Figure 3. Results of the similarity percentage analysis. Frequency of occurrence of Molecular Operational Taxonomic Units (MOTUs) with the highest contribution to differences between the diets of *Tarentola gigas* in both islets. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05.

4. Discussion

Our study reveals the first DNA-based data on the diet of the two subspecies of the Endangered and endemic Cabo Verdean *T. gigas*. In our study, plants and invertebrates were the most frequent groups, followed by birds. Even though our results cannot be compared in a straightforward way to the ones based purely on morphological examination previously published [30], since their sampling took place in the dry season and our sampling in the wet season, both confirm that the study species has a generalist diet, feeding on plants, invertebrates and vertebrates. However, in our study, the number of occurrences is always higher for all taxonomic groups, apart from fishes, where the detection was higher in the classic study, and reptiles that were detected in similar proportions

(Figure 2B). The differences in the incidence of fishes can be justified by a sampling bias in the previous works [30], considering that all their samples were collected near seabird colonies where the fish remains are common, whereas our sampling on Raso was more widespread.

Plants and invertebrates presented the highest differences between the two studies (Figure 2B). This was already expected, as with morphological examination there is a tendency to underestimate prey incidence, considering that only partially digested or items with non-digestible parts can be detected [47,48], whereas with metabarcoding it is possible to detect small, soft and invisible items. Moreover, the samples used in the previous work were possibly dated from 1999 [30] and could be in a more degraded state, making prey identification difficult. Nonetheless, plant items occurred in higher frequency in the diet of *T. gigas* than what was previously thought. In a recent study on the diet of *Tarentola raziana*, similar results were revealed to this syntopic species [49]. We found 20 plant families consumed by this gecko, while the previous report only stated the presence of Poacea, and occasional observations of Schleich [20,50] did not mentioned plants at all. Additionally, we could reach a higher taxonomic resolution than earlier studies. In all three taxonomic groups, we were able to identify some items to the species level (Supplementary Table S2), whereas in the previous work, only *Sula leucogaster* and *Calonectris edwardsii* were identified at the same taxonomical resolution [30]. For invertebrates, only Coleoptera, Diptera, Orthoptera, Hymenoptera and Mantodea orders were formerly detected [20,30,50]. Even though we were unable to detect Mantodea, we identified nine additional orders formerly undescribed for *T. gigas* diet. Also, previous authors reported one case of cannibalism and one ingestion of *Tarentola raziana* [30], which could not be confirmed by our study as we used a blocking primer to prevent DNA amplification of *Tarentola*. However, we identified the occurrence of *Chioninia stangeri* (Gray, 1845) which can be an indicator of the predation on other reptiles (dead or alive). There is previous evidence of predation by larger species of lizards, as is the case of Lehrs' lizard, *Gallotia caesaris* (Lehrs, 1914), on smaller ones [51], so this is expected to be even more common on other islands' systems where the resources are more limited. Apart from the impossibility of detecting cannibalism, metabarcoding dietary studies are somehow affected by the deficiency of DNA reference sequences and the Linnean shortfall [8]. We started the construction of a reference database for collections of the flora and fauna for our system that was very helpful in correctly identifying some diet items; however, higher resolution of taxa identification would be possible if there were up-to-date species checklists and more sequenced taxa of these poorly studied islands. Some other limitations of this approach is the possibility of false inferences due to contaminations, therefore a careful interpretation of doubtful taxa was carried out, probably discarding true positives. Finally, we needed to have in consideration that this method only provides the taxa occurrences in the samples and not their relative abundances [8]. Nevertheless, as the technological procedures evolve in accelerated rates, these and other metabarcoding issues discussed above are being solved. It is expected that these tools will continue to improve, reducing the costs and providing high-quality data to better guide conservation planning of excellency [7].

The Euler diagrams show high overlap on the detection of taxonomic groups (plants, invertebrates and vertebrates) for both populations, with very few samples with just one taxonomic group detected, reinforcing the generalist diet of the species at the individual level. The overlap between plants and invertebrates was also higher than the number of samples with an overlap of all three groups. This may be explained by the secondary consumption of plants when invertebrates are consumed [52], and if this is the case, we may be detecting plant DNA consumed by the arthropod species, leading to an overestimation of the plant items consumed. On the other hand, ten of the samples contained only plants. In many cases, islands lizards have fewer terrestrial predators and are capable of reaching higher densities. This, along with a lower availability of insects for preying and the arid conditions of the study area, may have favoured the importance of plant items in their diet. Additionally, a higher plant matter ingestion is associated with species with a large body and gut sizes [53], characteristics owned by *T. gigas* specimens (personal observation). Herbivory in lizards is also associated with arid warmer areas and ecosystems with few competitors and predators [54], such as this one. Indirectly, insular lizards may then have an important role in seed dispersal and pollination in these systems [55], which could be the case of our study species. In fact, we found several geckos with their snouts covered with pollen during sampling on Branco, and so this deserves further study. Finally, both previously published works [20,30] found small stones in the pellets, known to be swallowed to help plant digestion [56].

We found a higher observed and extrapolated MOTU richness in Branco. However, the difference between both diets has a small ecological magnitude may be due to the reduced sample size, since the confidence intervals partially overlap. Despite the more homogeneous sampling effort, as the Branco samples were collected only in five days of the same month and in a restricted area due to the roughness of the ground, we may argue that we could find a higher diversity in Branco pellets if we sampled during a longer period of time and in a wider range of habitats. This would be expected taking into account that Branco presents a higher altitudinal gradient than Raso, which is nearly flat in its extent. Branco consequently presents higher humidity levels, thus embracing a greater variety of niches that can hold higher diversity of plants and invertebrates.

More explicitly focusing on the MOTUs that revealed a higher contribution for the differences between the diets of the two gecko populations, some invertebrates contributed to the higher differences in the two diets, namely Hemiptera and Lepidoptera species. Unfortunately, most MOTUs were not possible to be identified to the highest taxonomic resolution, providing little information to infer whether one of the two populations particularly relies on a certain arthropod group. Barely any information also exists on the status and distribution of invertebrates on these islands; therefore, we were not able to compare which population would be better for reintroduction, based solely on the incidence of this group. It is necessary to improve reference collections and catalogues of the diverse species of arthropods which inhabit the Desertas islands to make further conclusions.

Several plants also appear to be important, - some are exotic, such as *Chloris virgate* Swartz, while others are native, such as *Zygophyllum simplex* L., and still, others are endemic of Cabo Verde Archipelago, such as *Limonium brunneri* (Webb) Kuntze which is consumed more frequently by the Branco population. This may happen due to a higher availability of this taxon on Branco, or a preference for *T. g. brancoensis*. Given the generalist character of this gecko diet, the first hypothesis is more likely. This species occurs both on Branco and Raso, as well as on Santa Luzia. It is classified as Critically Endangered due to its restricted distribution, and as its population seems to be decreasing on Santa Luzia reserve [57]. The reintroduction of *T. gigas* on Santa Luzia could favour pollination and/or the dispersal of seeds, depending on the parts of the plant that are ingested. It is necessary to improve data on this to understand know the extent of this service to make further conclusions. Concerning vertebrate MOTUs, we found that some bird species are important for Raso subspecies diet, but not for Branco one. This is the case of the Endangered species *A. razae* and other Passeriformes with low populations sizes, such as *Passer iagoensis* (Gould, 1837). This is an expected result, since these prey items do not occur in Branco (*A. razae*) or their breeding is unknown (*P. iagoensis*). However, we confirmed that the Branco population preys with more frequency on the Near Threatened *C. edwardsii*. A strong commensal link of *T. g. brancoensis* with these seabirds could also explain the higher frequencies of fish found on Branco.

Considering the overall obtained data, the most important fact is that the diet of this gecko, in both islands is rather a generalist. This means that differences in the diet between sites may be more due to species availability rather than population differences in trophic ecology. In the perspective of the reintroduction of this gecko on Santa Luzia our data needs to be interpreted as valuable for the integration with other kinds of data. Concerning the survival of reintroduced geckos due to diet requirements, we consider that both populations could be reintroduced on Santa Luzia. The Branco population seems to have a wider range of diet items, and their acclimatisation on Santa Luzia could be easier than for the Raso population, which, due to the more homogenous range of habitats, seems to have a less diverse diet. Also, *T. g. brancoensis* is probably the subspecies more genetically closer to the extinct population of Santa Luzia (based on geographic distances [21] and the age of the islands [58]). Branco is also the geographically closest to Santa Luzia Island. This would be economically more advantageous and safer, due to the roughness of the sea. On the other hand, disembark on Raso is relatively easier and fieldworkers have more temporary conditions to perform fieldwork, despite being more distant. Concerning the success of the ongoing translocation of *A. razae*, and depending on the overlap of the distributions of both species, the introduction of a known predator could have a negative impact on the survival of the bird nestlings. Since the population of

Branco is not used to preying on Raso larks, it could be naïve to prey on this bird for a first stage, and a better choice for successful acclimatization of the birds. However, due to the generalist character of both diets, this is just one scenario among many, and it is probably advisable to obtain more data to model the impact of another predator on the viability and growth of this new *A. razae* population before any action is taken. Overall, an evaluation of the best population source would benefit from the inclusion of data about the genetic diversity and similarity between subspecies (ongoing), the densities of each population, and a careful analysis of the cost–benefits of each option.

5. Conclusions

Our results revealed that *T. gigas* has a generalist diet that encompasses most of the diversity of resources found in both islands, from plants to birds. In the future, it would be interesting to understand the importance of this gecko in connecting the marine and terrestrial ecosystems by recycling nutrients (e.g., ingestion of regurgitated fishes by birds on Branco), whether they have a significant phytosanitary effect that keeps bird populations free of diseases, or to what extent the species provides ecological services to the maintenance of threatened plant species. All these hypotheses require future research, and for that we need to expand our knowledge on the plants and invertebrates present on Santa Luzia, by completing our reference collection and improving the list of described species for the island. This would allow us to analyse and obtain more insights to understand the ecological interaction of *T. gigas* with plant and invertebrate species.

6. References

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7. Supplementary material

Table S1. Primer sequences forward and reverse used in this study.

Primer Name	Fragment length (bp)	Primer F	Primer R	Reference
12sv5	73-110	12sv5F TAGAACAGGCTCCTCTAG	12sv5R TTAGATACCCCACTATGC	(Riaz *et al.*, 2011)
IN16STK-mod	~ 110	IN16STK-1F-mod TRAACTCAGATCATGTAA	IN16STK-1R-mod TTAGGGATAACAGCGTWA	This manuscript (based on Kartzinel and Pringle, 2015)
g/h	10-143	g_F GGGCAATCCTGAGCCAA	h_R CCATTGAGTCTCTGCACCTATC	(Taberlet *et al.*, 2007)

Table S2. List of the identified MOTUs to the maximum resolution obtained, with the respective occurrence in the samples of *Tarentola gigas brancoensis* (T.g.b) and *Tarentola gigas gigas* (T.g.g).

Phyllum	Order	Family	Final ID	*T.g.b*	*T.g.g*
Tracheophyta					
Brassicales	Capparales	Brassicaceae	Lobularia_1	6	3
Liliopsida	Poales	Poaceae	*Chloris virgata*	0	8
			Festuca_1	0	1
			Festuca_2	6	1
			Poaceae_2	2	2
			Poaceae_5	7	3
			Poaceae_7	0	2
Magnoliopsida	Apiales	Apiaceae	Apiaceae_2	2	1
	Aquifoliales	Aquifoliaceae	*Ilex_1*	1	0

	Asterales	Asteraceae	Asteraceae_1	0	1
			Asteraceae_2	1	0
	Caryophyllales	Aizoaceae	*Aizoon canariense*	0	8
		Plumbaginaceae	*Limonium brunneri*	20	6
		Tamaricaceae	*Tamarix_5*	1	3
		Amaranthaceae	*Chenopodium murale*	5	2
			Chenopodium_2	1	0
			Patellifolia_1	7	2
			Patellifolia_2	1	0
	Cucurbitales	Cucurbitaceae	Cucurbitaceae_1	1	1
	Fabales	Fabaceae	Fabaceae_3	1	0
			Fabaceae_4	1	0
			Fabaceae_5	1	0
			Lotus brunneri	2	9
	Lamiales	Lamiacea	*Lavandula coronopifolia*	0	1
	Laurales	Lauraceae	Lauraceae_1	2	0
	Malpighiales	Euphorbiaceae	*Euphorbia_1*	1	5
	Malvales	Malvaceae	*Corchorus trilocularis*	0	4
	Myrtales	Lythraceae	Lythraceae_1	1	0
	Rosales	Rosaceae	Rosaceae_3	1	0
	Sapindales	Anacardiaceae	*Rhus_1*	1	0
	Solanales	Convolvulaceae	Convolvulaceae_1	1	1
		Solanaceae	Solanaceae_3	1	0
	Zygophyllales	Zygophyllaceae	*Tribulus cistoides*	4	3
			Zygophyllum simplex	10	15
Arthropoda					
Arachnida	Araneae	Gnaphosidae	*Drassodes_2*	1	0
		Oecobiidae	*Uroctea_1*	1	0
		Philodromidae	*Thanatus vulgaris*	1	0
		Selenopidae	Selenopidae_1	2	0
Chilopoda	UNK	UNK	Chilopoda_2	0	1
Collembola	UNK	UNK	Collembola_1	0	1
Insecta	UNK	UNK	Insecta_16	0	1
	Blattodea	UNK	Blattodea_4	1	1
			Blattodea_5	1	0
			Blattodea_6	6	0
	Coleoptera	UNK	Coleoptera_10	1	0
		Carabidae	Carabidae_3	0	1
		Cerambycidae	Cerambycidae_1	0	1
		Coccinellidae	Coccinellidae_1	1	0
		Dermestidae	*Dermestes_1*	1	0
		Scarabaeidae	Scarabaeidae_1	1	0
		Staphylinidae	Staphylinidae_4	3	0
		Tenebrionidae	Tenebrionidae_3	1	0
			Tenebrionidae_4	7	0
		Tetratomidae	Tetratomidae_1	1	0
	Diptera	UNK	Diptera_6	3	6
		Agromyzidae	Agromyzidae_1	0	1
		Cecidomyiidae	*Mayetiola destructor*	1	0
			Mayetiola_1	1	0
		Ceratopogonidae	*Culicoides_1*	1	0
		Chironomidae	*Chironomidae_1*	0	1
			Chironomus tepperi	3	3
			Cricotopus_1	6	1
		Culicidae	*Aedes_1*	0	1
			Culicidae_3	10	6
		Drosophilidae	Drosophilidae_1	2	0
		Psychodidae	Psychodidae_1	3	0
		Sarcophagidae	Sarcophagidae_1	1	0

			Wohlfahrtia_1	1	1
		Sciaridae	Sciaridae_1	2	1
	Hemiptera	UNK	Hemiptera_2	1	3
		Acanthosomatidae	Acanthosomatidae_1	0	1
		Aphididae	*Aphis_1*	1	0
		Cicadellidae	Cicadellidae_1	0	5
			Orosius_1	1	1
		Lygaeidae	Lygaeidae_1	0	1
			Nysius_1	0	1
			Nysius_2	0	1
		Nabidae	Nabidae_2	0	14
		Pentatomidae	*Halyomorpha halys*	0	1
			Pentatomidae_1	0	1
			Pentatomidae_3	0	6
		Pteromalidae	Pteromalidae_2	0	1
		Apidae	Apidae_3	1	0
	Hymenoptera	Formicidae	*Solenopsis_1*	1	1
		Thynnidae	Tiphiidae_2	1	0
		Tiphiidae	Tiphiidae_3	2	0
	Lepidoptera	UNK	Lepidoptera_12	0	1
			Lepidoptera_17	0	1
			Lepidoptera_10	1	0
			Lepidoptera_16	1	0
			Lepidoptera_23	0	1
			Lepidoptera_24	4	1
			Lepidoptera_26	0	1
			Lepidoptera_30	1	0
			Lepidoptera_4	1	0
		Crambidae	Crambidae_1	0	1
			Crambidae_2	0	2
			Nomophila noctuella	0	1
			Tegostoma_1	1	0
			Tegostoma_2	1	0
			Tegostoma_3	2	1
		Noctuidae	*Acraprex_1*	0	1
			Agrotis_1	2	14
			Agrotis_2	0	6
			Agrotis_4	3	2
			Noctuidae_2	1	1
			Noctuidae_3	0	1
			Noctuidae_5	0	1
			Noctuidae_6	0	14
		Pieridae	Pieridae	0	2
	Neuroptera	UNK	Neuroptera_1	0	1
		Myrmeleontidae	Myrmeleontidae_1	2	0
	Odonata	Calopterygidae	Calopterygidae_1	2	0
		Coenagrionidae	Coenagrionidae_1	1	0
		Libellulidae	Libellulidae_1	0	1
	Orthoptera	Acrididae	Acrididae_2	1	0
			Acrididae_5	6	2
			Schistocerca_1	0	2
		Gryllidae	Gryllidae_1	1	0
	Zygentoma	UNK	Zygentoma_1	0	1
		Lepismatidae	*Heterolepisma_10*	1	0
			Heterolepisma_11	1	0
			Heterolepisma_3	1	0
			Lepismatidae_8	0	1
			Thermobia domestica	1	0
Malacostraca	Decapoda	UNK	Decapoda_1	1	1
	Isopoda	UNK	Isopoda_1	0	1

Chordata					
Actinopterygii	Beloniformes	Belonidae	*Tylosurus _1*	0	1
		Hemiramphidae	*Hemiramphus*	1	0
	Carangiformes	Carangidae	*Trachinotus ovatus*	0	1
	Perciformes	Acanthuridae	*Acanthurus_1*	1	0
		Epinephelidae	*Cephalopholis taeniops*	3	0
		Scaridae	*Sparisoma_1*	1	0
	Salmoniformes	Salmonidae	*Oncorhynchus_1*	1	0
Aves	Passeriformes	Alaudidae	*Alauda razae*	0	4
		Fringillidae	Fringillidae_1	4	0
		Passeridae	*Passer iagoensis*	0	3
	Procellariiformes	Procellariidae	*Calonectris edwardsii*	7	2
Reptilia	Squamata	Scincidae	*Chioninia stangeri*	1	1

Table S3. PerMANOVA results of island effect in the diet of the two subspecies of *Tarentola gigas*. d.f. stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

Variable	d.f.	SS	MS	F.Model	R2	Pr (>F)
Islet	1	1.8438	1.84383	5.5849	0.10423	**0.001**
Residuals	48	15.8470	0.33015		0.89577	
Total	49	17.6908			1.00000	

Table S4. PerMADISP test results. d.f. stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

	d.f.	SS	MS	F.Model	Pr (>F)
Groups	1	0.11085	0.11085	11.186	**0.001606**
Residuals	48	0.47568	0.00991		

Manuscript II

More haste, less speed? Classic versus metabarcoding approaches for the diet study of a remote island endemic gecko

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Abstract

Dietary studies can reveal valuable information on how species exploit their habitats and are of particular importance for insular endemics conservation as they present a higher risk of extinction. Reptiles are often neglected in islands systems, principally the ones inhabiting remote areas, therefore little is known on their ecological networks. The diet of the most widespread continental *Tarentola* species is already widely studied using classical methods. However, using next-generation sequencing (NGS) techniques only one known study was performed for this genus and very few for reptiles in general. The Selvagens gecko *Tarentola (boettgeri) bischoffi*, endemic to the remote and integral reserves of Selvagens Archipelago, is classified as Vulnerable. Little is known on their ecology and dietary habits, supposed to be exclusively insectivorous. Considering the lack of information on its diet and its conservation interest, we used morphological and DNA metabarcoding approaches to characterize it. We also compared the traditional method of morphological identification of prey remains in faecal pellets collected over a long period with metabarcoding methods associated with rapid sampling surveys. Molecular results revealed that this species is a generalist eater, feeding on invertebrate, plant and even vertebrate items, even though the morphological approaches were unable to detect the latter two. This method identified a higher diversity of dietary items and with generally higher taxonomic resolution. On the other hand, with the traditional method, it was possible to calculate relative abundances and biomasses of the ingested arthropods, a parameter that was not possible to measure with metabarcoding. Results of this study are useful to show the applicability of rapid surveys on remote islands of difficult access around the world.

Keywords: *Tarentola (boettgeri) bischoffi*, gekkonidae, Macaronesia, conservation, Selvagens Archipelago, seasonality.

1. Introduction

Knowing the feeding habits of a species increases the knowledge about the way it exploits its environment. Therefore, dietary studies represent an important topic in herpetology (Brock *et al.*, 2014) and conservation, continuing to be essential for species for which there is little information (Pérez-Mellado *et al.*, 2011). Insular reptiles often differ markedly in their diets, when compared to continental congeners (Brock *et al.*, 2014; Sagonas *et al.*, 2015). Some studies show a generalization of the diet in islands (Sagonas *et al.*, 2015), while others reveal marked changes in the trophic niche as an adaptation to the different insular prey communities (Carretero & Lo Cascio, 2010; Briggs *et al.*, 2012).

Islands are normally considered natural laboratories as they are geographically isolated and hold exclusively evolved biota (Whittaker *et al.*, 2017). Even though islands generally present a small number of species, in relation to mainland systems, the number of endemics is usually high, principally in remote islands (Whittaker & Fernández-Palacios, 2007). However, these endemic species are more prone to extinction due to the synergy of genetic and demographic factors (Frankham, 1997). Therefore, the study of these systems is very important as they represent simplified models, ideal for studying ecological networks, as the species inhabit more confined areas being possible to sample more thoroughly. These studies serve therefore as a baseline for the accurate development of conservation measures (Frankham, 1997; Caujapé-Castells *et al.*, 2010).

Geckos comprise the largest lizard family, with about 2000 species worldwide, inhabiting mainly on warm climate regions, including several iconic examples of island colonization in the three main oceans (Vitt & Caldwell, 2013). Within this family, the genus *Tarentola* is the most widespread in the Western Mediterranean and includes several island endemics in Macaronesia (Vasconcelos *et al.*, 2010; Rato *et al.*, 2012). Several classic studies, based on morphological identification of prey, were performed on the diet of the most widespread species, such as *Tarentola annularis* (Geoffroy-St-Hilaire, 1827) and *Tarentola mauritanica* (Linnaeus, 1758). The diet of the first one, in northern Egypt mainly consists in flying arthropods with some vestiges of plant material also being reported (Ibrahim, 2004), however, their predation on small mammals was also referenced (Crochet & Renoult, 2008). The diet of the second, in the Iberian Peninsula, consists almost exclusively of ground-dwelling arthropods (Gil *et al.*, 1994; Hódar & Pleguezuelos, 1999; Hódar *et al.*, 2006), while in the historical centre of Rome, Italy, it is composed mainly of flying arthropods, such as Diptera and adult Lepidoptera (Capula & Luiselli, 1994).

Few studies were performed using next-generation sequencing (NGS) techniques to study the diet of reptiles, and only one (to our best knowledge) on *Tarentola* geckos (Seguro, 2017). Metabarcoding is a non-invasive technique that allows the identification of multiple food items in a species diet through sequencing standardized DNA fragments (Pompanon *et al.*, 2012). This method is based on the mass-amplification of DNA using general or group-specific primers, followed by the cloning and sequencing of amplicons to individual taxa identification. Metabarcoding can be advantageous for diet studies, mainly for insular species from remote areas, as with less effort and time it's possible to obtain a large amount of data. Additionally, in relation to classic methods, this technique maximizes resolution, detection of rare events, and detection of soft, small and invisible prey items and ultimately can correct biases in ecological models, and it is less reliant on taxonomic expertise (Pompanon *et al.*, 2012).

The Selvagens gecko *Tarentola (boettgeri) bischoffi* (Joger, 1984) is endemic to the remote Selvagens Archipelago (Figure 1), about 250 km of Madeira Island (Cabral *et al.*, 2005), occurring in three isolated subpopulations, which correspond to the three largest islands of the archipelago (Rebelo, 2010). It is a protected species, considered Vulnerable by the Portuguese Red Data Book (Cabral *et al.*, 2005). The closest relatives of this gecko live in two islands of the Canary Archipelago – Gran Canaria, *Tarentola boettgeri boettgeri* (Steindachner, 1891), and El Hierro, *Tarentola boettgeri hierrensis* (Joger & Bischoff 1983). The group is related with *T. mauritanica* populations from North Africa, from which were separated about 17.5 million years ago as a result of an ancient Macaronesian colonization (Carranza *et al.*, 2000).

It is known that the Selvagens gecko will eat insects (Olivera *et al.*, 2010), but there is still a complete lack of studies on its feeding habits in nature. Considering the lack of information on its diet and its plasticity, in this study we used two approaches to characterize it. We compared the traditional method of morphological identification of prey remains in faecal pellets collected over a long period with metabarcoding methods associated with rapid sampling surveys. Results of this study will be useful to evaluate the applicability of rapid surveys on remote islands of difficult access around the world.

2. Materials and Methods

2.1. Study area

Sampling was carried out in the largest island of the archipelago, Selvagem Grande (Figure 1), in 2010, 2011 and 2017. Selvagem Grande is a plateau approx. 120 m a.s.l. surrounded by steep cliffs. The climate is semi-arid, as their low altitudes do not favour precipitation (below 200 mm), but there are occasional winter torrential floods.

The flora of Selvagens Islands is composed by approximately 75 taxa, with seven of them exclusive endemics, and the majority classified as threatened (Borges *et al.*, 2008a). In Selvagem Grande, since the successful eradication of house mouse and rabbit in 2005 (Olivera *et al.*, 2010), the scarce vegetation is steadily recovering and is mainly composed of Shrubby sea-blite *Suaeda vera* Forssk. ex J. F. Gmel with some individuals of the Macaronesian endemic *Schizogyne sericea* (L.f.) DC. (Penado *et al.*, 2015).

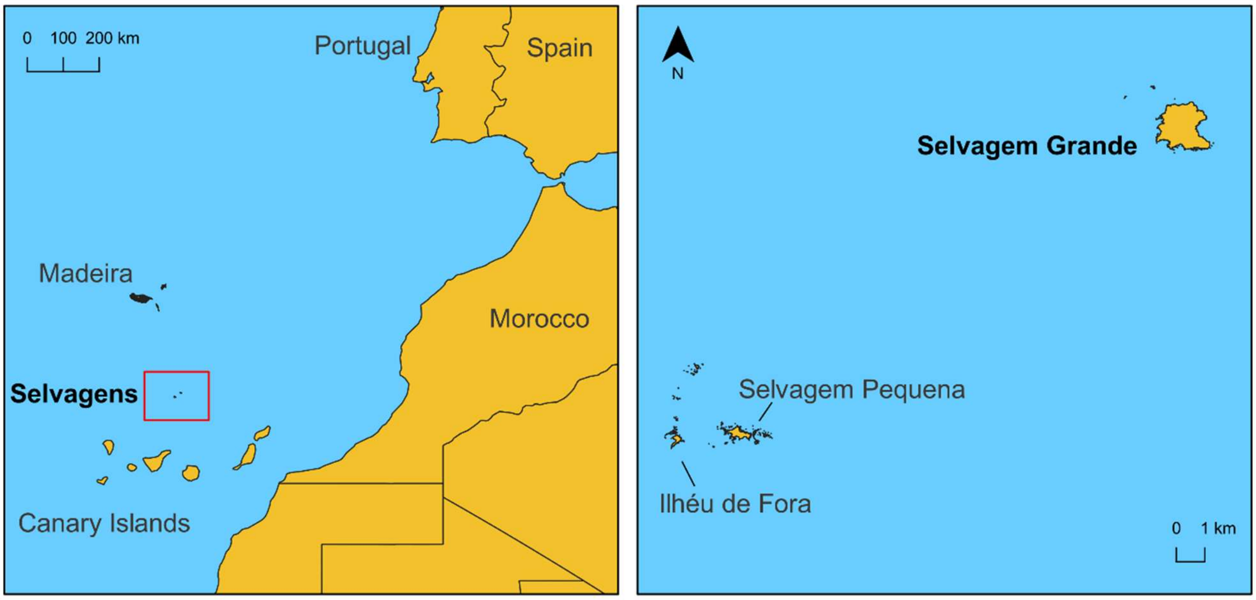

Figure 1. Detail of the East Atlantic coast with the location of Selvagem Grande.

The Selvagens Archipelago is one of the most important breeding areas for seabirds in Macaronesia, classified as Important Bird Area (IBA) by Birdlife International. Nine breeding species occur, for which these islands embody one of the last sanctuaries in the world. More specifically, these islands play a key role in the protection of Corry's shearwater *Calonectris borealis* (Cory, 1881), as they shelter one of the largest breeding colony in the world (Granadeiro *et al.*, 2006). There are also two endemic reptiles – the mainly diurnal *Teira dugesii* (Milne-Edwards, 1829) and the strictly nocturnal *T. bischoffi*. The terrestrial arthropod community in the Island is diverse, including 201 *taxa* (Borges *et al.*, 2008b).

2.2. Data collection and analysis

The study was carried out in two seasons: the end of summer of two different years, from 6 to 15 of September 2010 and from 10 to 11 of September 2017 (corresponding to 24h); and late spring of just one year, from 9 to 30 of May 2011. Samples for metabarcoding analysis were only collected in September 2017.

The soil arthropod community was sampled with 50 mL pitfall traps which were left open for 12 hours on two occasions per season in 2010/2011 and in one occasion in 2017. In 2010 and 2011, five traps containing water, alcohol and detergent were placed in each of four 1 ha squares scattered along the island plateau and left open overnight. In September 2017, eight traps were placed on two areas of the island (sandy and fine-grained soil), for the DNA reference collection of arthropods. These pitfall traps did not contained detergent to prevent DNA degradation. All arthropods were photographed with a camera assembled on a magnifying lens and identified to the family level whenever possible. Specimens collected in 2010 and 2011 were also weighted to obtain an estimate of the average body mass of each taxonomic category. A leg or wing sample was used, from the specimens collected in 2017, of each different Operational Taxonomic Unit (OTU) identified by the experts to perform DNA extraction.

Arthropod DNA was extracted using saline extraction methods (Carranza *et al.*, 1999) and amplified using both IN16STK-1F/IN16STK-1R primers (see Appendix I) targeting the mitochondrial 16S rRNA (Kartzinel & Pringle, 2015) to allow the match with the diet sequences, and standard COI barcode fragment using LCO1490/HC02198 following PCR conditions described in Folmer *et al.*, 1994, allowing to confirm dubious taxonomic assignations by comparison with sequences available in BOLD database (http://boldsystems.org/).

Plant and vertebrate samples were collected to build a DNA reference collection. Vertebrate samples were extracted using saline methods and amplified for the V5-loop fragment of the mitochondrial 12S gene using 12sv5F and 12Ssv5R primers (see Appendix I). Plants were photographed and identified by experts, and DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Crawley, UK) following some alterations according to Romeiras *et al.* (2015), and amplified using primer 'e' and 'f' (Taberlet *et al.*, 1991) targeting the chloroplast trnL (UAA) (see Appendix I). DNA from all reference samples was sequenced using Sanger sequencing.

Gecko faecal pellets (N = 16 in September 2010; N = 66 in May 2011, and N = 27 in September 2017) were obtained by gently pressing adult individuals (> 45 mm SVL; Penado *et al.*, 2015) that were caught by lifting rocks on the plateau during the day. For the 2010 and 2011 samples, pellets were stored dry in plastic tubes, and later dispersed in water and examined with a binocular magnifying glass. The numbers of each prey item in each pellet were estimated from the cephalic capsules, wings (including elytra) and legs, following the minimum numbers criterion. For the metabarcoding analyses, pellets were stored in tubes with 96% ethanol, for DNA preservation, labelled with the respective animal code. The collected pellets were then completely dehydrated in an incubator at 50ºC in order to remove all traces of ethanol. Then, DNA was extracted using the Stool DNA Isolation Kit (Norgen Biotek Corp.Canada), following the manufacturer's instructions. Three different DNA fragments were chosen to identify the distinct prey groups presumably preyed by the study species: for plants the g/h primers were used targeting the short P6-loop of chloroplast trnL (UAA) intron (Taberlet *et al.*, 2007), for invertebrates a modified version of the IN16STK-1F/IN16STK-1R primers were used, targeting the mitochondrial 16S rRNA (Kartzinel & Pringle, 2015) and for the amplification of vertebrate DNA, the 12sv5F and 12Ssv5R primers targeting the V5-loop fragment of the mitochondrial 12S gene (Riaz *et al.*, 2011) were used. To avoid the amplification of *T. (boettgeri) bischoffi* DNA, a blocking primer (5'-CTCCTCTAGGTTGGTTTGGGACACCGTC (C3 spacer)-3') was used in the latter case. We further modified all primers in order to contain Illumina adaptors and a 5-bp individual identification barcode to allow individual identification of each sample. Succeeding amplification, a library preparation was carried out following Illumina MiSeq protocol 16S Metagenomic Sequencing Library Preparation (Illumina, 2013) (see Appendix I for details). The sequences were processed using the software package Obitools (https://git.metabarcoding.org/obitools/obitools) and the taxa were assigned using GenBank (https://www.ncbi.nlm.nih.gov/), and lists of species occurring on Selvagens Islands (Borges *et al.*, 2008a).

Using both morphological and molecular methods, prey items were identified to the lowest possible taxonomic level. Accumulation curves were built for all three sampling moments considering the family level. The Shannon-Wiener diversity Index was calculated to characterize the gecko's diet in 2010 and 2011 samples and the diversity indices were compared between seasons and methods with t-tests (Zar, 2010). This approach was not used for the 2017 sample as it is not possible to estimate the number of individuals of each prey item using metabarcoding methods (see discussion).

Diet composition was expressed in terms of frequency of occurrence (% FO), for both methods, and numerical frequency (% N) and percentage of biomass (% B), for the morphological identification only. As the main prey items identified were adult holometabolous insects (see below), for the estimation of the ingested biomass we used the average weight of the exemplars collected in the pitfalls. A relative importance index (RII) was assigned to each taxonomic category from the above metrics as RII =% FO * (% N +% B) (Pinkas, 1971). This calculus was possible only for those species for which we had biomass values.

3. Results

A total of twelve, ten and eleven arthropod families were identified in the samples collected in the pitfalls in September 2010, May 2011 and September 2017, respectively. The relative abundance or presence of each type of arthropod prey in each of the two sampling seasons and methodologies in the reference collections is shown in Table 1. Ants (Formicidae) were the most numerous, with similar abundance in both seasons. All the other families were relatively rare, with the exceptions of

Psyllipsocidae in September and a single species of Diptera in May. For plants and vertebrates, we collected in 2017 a total of nine and two species, respectively (Table 1).

Table 1. Number of preys collected in the two sampling seasons (September 2010 and May 2011), and the in the reference collections sorted according to class, order and family whenever possible. N stands for number of items. The presence of each prey item in our DNA reference collection is also signalled (•). NI stands for non-identified preys.

	N		DNA
Taxonomic category	**09/10**	**05/11**	**09/17**
Arachnida			
Acari NI	0	2	
Pseudoscorpiones			
Cheliferidae	1	1	
Araneae			•
Gnaphosidae	7	2	
Salticidae	-	-	•
Insecta			
Coleoptera			
Carabidae	5	2	•
Sp. A	1	0	
Tenebrionidae			
Hegeter latebricola	-	-	•
Diptera			
Dolichopodidae	2	3	
Hybotidae	2	0	
Limoniidae	1	0	
Sp. C	6	18	
Hemiptera			
Aphididae	0	1	
Cicadellidae	-	-	•
Hymenoptera			
Formicidae	213	147	
Porcellionidae	-	-	•
Lepidoptera			
Cosmopterigidae	-	-	•
Pyralidae	-	-	•
Psocoptera			
Ectopsodidae	1	0	
Psyllipsocidae	32	1	
Zygentoma			
Lepismatidae	2	0	•
Chilopoda			
Scutigeromorpha			
Scutigeridae			
Scutigera coleoptrata	-	-	•

	DNA
Taxonomic category	**09/17**
Magnoliopsida	
Apiales	
Apiaceae	
Astydamia latifolia	•
Asterales	
Asteraceae	
Senecio incrassatus	•
Caryophyllales	
Aizoaceae	
Aizoon canariensis	•
Mesembryanthemum nodiflorum	•
Amaranthaceae	
Chenopodium coronopus	•
Fabales	
Fabaceae	
Lotus glaucus	•
Solanales	
Solanaceae	
Lycopersicon esculentum	•
Solanum nigrum	•
Gentianales	
Apocynaceae	
Periploca laevigata	•
Aves	
Procellariiformes	
Procellariidae	
Bulweria bulwerii	•
Calonectris borealis	•

Using classical methods, a total of 324 specimens from seven orders and 16 different arthropod families were retrieved and identified from the pellets (11 families in September 2010 and 10 families in May 2011). Ants (mainly the common species *Monomorium subopacum* Smith, 1858) were the most numerous preys in both seasons (Table 2); however, their frequency and relative importance were strikingly lower in May than in September. This shift was due to a higher consumption of the much heavier carabids (mainly the common species *Hegeter latebricola* Wollaston, 1854) in spring. Other beetle species were also more frequently consumed in May, as well as Diptera (Table 2). Ants were the most frequent prey in September, having been found in 81.25% of the pellets, whereas in May the most frequent prey was Carabidae (39.4% of the pellets). Considering the percentage of biomass, Carabidae were the most important in both seasons, as the biomass of a single carabid is roughly 500 times that of an ant. The higher values of the relative importance index belong to

Formicidae in September and to Carabidae in May. No plants or vertebrate OTUs were retrieved from pellets using the classical method (Table 2).

Table 2. Composition of the diet of *Tarentola bischoffi* during the three sampling periods (September 2010 and 2017 and May 2011), classified according to class, order and family, whenever possible. % FO - Frequency of occurrence; % B – Percentage of biomass; %N – percentage number; RII - Relative importance index; NI, not identified.

Taxonomic category	%N		%B		RII		%FO		
	09/10	05/11	09/10	05/11	09/10	05/11	09/10	05/11	09/17
Arthropoda									
Arachnida									
Araneae									
Philodromidae	-	-	-	-	-	-	-	-	3.70
NI (Acari)	0.72	0	0.03	0	4.69	0	6.25	0	-
Pseudoscorpiones									0.00
Cheliferidae	1.44	1.08	0.06	0.01	18.77	3.3	12.5	3.03	-
Insecta									
Blattodea NI	-	-	-	-	-	-	-	-	3.70
Coleoptera									
Anobidae	2.16	0.54	-	-	-	-	18.75	1.52	-
Carabidae	3.6	16.76	95.67	98.98	3102.18	4559.1	31.25	39.39	29.63
Chrysomelidae	-	-	-	-	-	-	-	-	3.70
Coccinelidae	0.72	0	-	-	-	-	6.25	0	-
Curculionidae	0.72	0	-	-	-	-	6.25	0	-
Lycidae	-	-	-	-	-	-	-	-	11.11
Sp. A	0	14.59	0	0.14	0	401.64	0	27.27	-
Sp. B	5.04	11.89	-	-	-	-	43.75	24.24	-
Scarabaeidae	-	-	-	-	-	-	-	-	7.41
Staphylinidae	0.72	0	-	-	-	-	6.25	0	3.70
Tenebrionidae	1.44	0	-	-	-	-	12.5	0	11.11
NI	-	-	-	-	-	-	-	-	11.11
Diptera									
Cecidomyiidae	-	-	-	-	-	-	-	-	3.70
Chironomidae	-	-	-	-	-	-	-	-	18.52
Culicidae	-	-	-	-	-	-	-	-	33.33
Limoniidae	-	-	-	-	-	-	-	-	3.70
Muscidae	-	-	-	-	-	-	-	-	11.11
Psychodidae	-	-	-	-	-	-	-	-	3.70
Sciaridae	-	-	-	-	-	-	-	-	7.41
NI	0	9.73	0	0.49	0	263.21	0	25.75	3.70
Hemiptera									
Acanthosomatidae	-	-	-	-	-	-	-	-	7.41
Aphididae	0	1.08	0	0.01	0	3.3	0	3.03	3.70
Cicadellidae	-	-	-	-	-	-	-	-	7.41
Flatidae	-	-	-	-	-	-	-	-	14.81
Lygaeidae	-	-	-	-	-	-	-	-	25.93
Miridae	-	-	-	-	-	-	-	-	3.70
Pteromalidae	-	-	-	-	-	-	-	-	3.70
NI (Homoptera)	1.44	0	-	-	-	-	12.5	0	-
NI	-	-	-	-	-	-	-	-	7.41
Lepidoptera									
Lycaenidae	-	-	-	-	-	-	-	-	3.70
Noctuidae	-	-	-	-	-	-	-	-	11.11
Tortricidae	-	-	-	-	-	-	-	-	3.70
NI	-	-	-	-	-	-	-	-	44.44
Hymenoptera									
Formicidae	82.01	29.73	4.24	0.34	7007.66	774.63	81.25	25.76	7.41
Pteromalidae	-	-	-	-	-	-	-	-	3.70
Psocoptera									
Psyllipsocidae	0	2.7	0	0.03	0	8.26	0	3.03	-
Trogiidae	-	-	-	-	-	-	-	-	25.93
NI	0	11.89	-	-	-	-	0	24.24	3.70
Zygentoma									
Lepismatidae	-	-	-	-	-	-	-	-	25.93
NI									7.41
Malacostraca									
NI	-	-	-	-	-	-	-	-	7.41
Decapoda									
NI	-	-	-	-	-	-	-	-	7.41
Isopoda									
Porcellionidae	-	-	-	-	-	-	-	-	7.41
NI	-	-	-	-	-	-	-	-	7.41

Taxonomic category	%FO		
	09/10	05/11	09/17
Tracheophyta			
Capparales			
Brassicaceae			
Lobularia	-	-	29.63
Liliopsida			
Poales			
Poaceae	-	-	40.74
Magnoliopsida			
Apiales			
Apiaceae	-	-	14.81
Asterales			
Asteraceae	-	-	29.63
Caryophyllales			
Aizoaceae	-	-	33.33
Plumbaginaceae	-	-	44.44
Amaranthaceae	-	-	40.74
Cucurbitales			
Cucurbitaceae	-	-	3.70
Ericales			
Ericaceae	-	-	3.70
Theaceae	-	-	3.70
Actinidiaceae	-	-	11.11
Fabales			
Fabaceae	-	-	14.81
Lamiales			
Oleaceae	-	-	3.70
Plantaginaceae	-	-	3.70
Malvales			
Malvaceae	-	-	3.70
Rosales			
Moraceae	-	-	3.70
Rosaceae	-	-	7.41
Sapindales			
Anacardiaceae	-	-	3.70
Solanales			
Convolvulaceae	-	-	3.70
Solanaceae	-	-	14.81
Zygophyllales			
Zygophyllaceae	-	-	3.70
Chordata			
Actinopterygii			
Perciformes			
Scombridae	-	-	3.70
Syngnathiformes			
Centriscidae	-	-	3.70
Cypriniformes			
Cyprinidae	-	-	7.41
Aves			
Charadriiformes	-	-	3.70
Procellariiformes			
Procellariidae	-	-	11.11
Reptilia			
Testudines			
Cheloniidae	-	-	3.70

The metabarcoding results revealed invertebrate and plant items presenting almost the same proportion in the samples (77.7% and 74.1% frequency of occurrence, respectively). Vertebrates were also detected in 33% of the samples. With this method, a total of 106 diet items, 62 corresponding to arthropods, 37 to plants, and seven to vertebrates were identified. For arthropods, a total of 12 orders and 29 families were identified. For plants, we were able to identify 16 orders and 21 families, and six orders and six families for vertebrates. The plant family Plumbaginaceae (specifically *Limonium papillatum* Webb & Berthel, 1891) had the higher frequency of occurrence of the group, and in the general diet considering all taxonomic groups. For the arthropods, even though, non-identified items of Lepidoptera order were more frequent, Culicidae was the arthropod family with a higher incidence in the samples. Regarding vertebrates, Procellariidae (specifically *Calonectris borealis*) was the family with higher frequency of occurrence.

Comparing the two methods, with metabarcoding we were able to identify plants and vertebrates whereas with classical methods it was not possible. In addition, with metabarcoding, we have recovered 13 more families of arthropods than using classical methods. Diet composition in each of the two sampling seasons and for both methods is expressed in Table 2.

Taxa accumulation curves for the classic method (Figure 2) very quickly reached a plateau (after 5 pellets in both seasons), indicating that even the reduced September sampling effort is probably sufficient to characterize the species' diet. However, using metabarcoding we could not reach that plateau (Figure 2).

The diversity in the pellets was higher in May than in September (H' 09/10 = 0.84; H 05/11 = 1.90; t_{188} = -8,192; P < 0.0001).

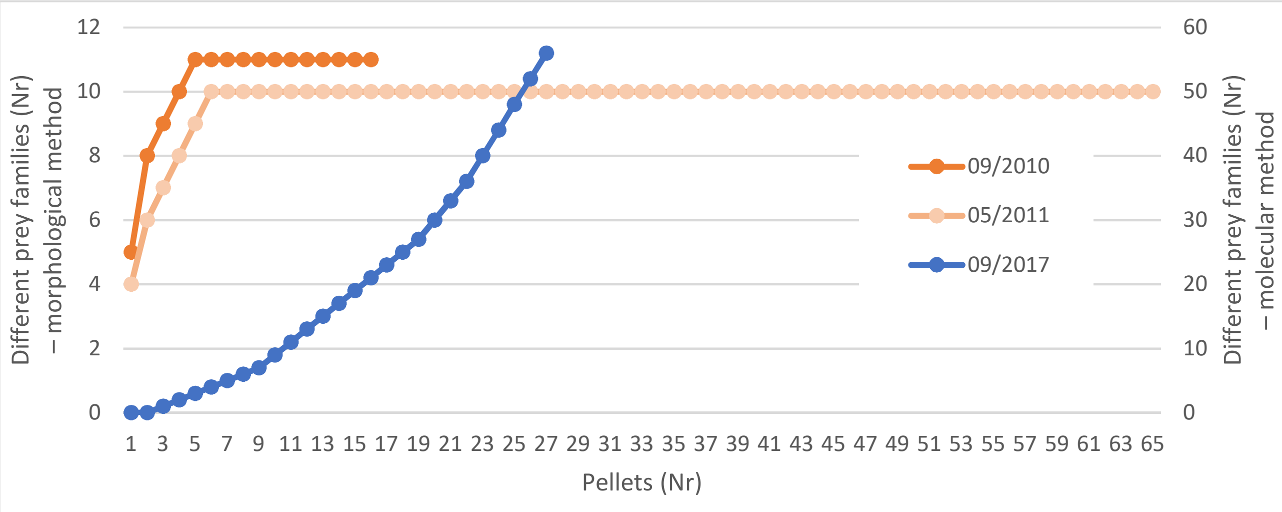

Figure 2. Species accumulation curves, for September 2010 and 2017 and May 2011.

4. Discussion

These are the first data on the diet of endemic and threatened *T. (boettgeri) bischoffi*. Looking exclusively into the morphological results, the Selvagens gecko appears to be mostly myrmecophagous at the end of the dry season, shifting to larger prey (especially carabids) during spring. In fact, ants were consistently the most numerous preys in both seasons, and the much heavier beetles provided the highest biomass consumed also in both seasons. In May, although

Formicides continued to dominate the diet of the species, its occurrence was much lower than in September, with an increase of Carabidae, Diptera and other Coleoptera.

By these results agree with Carretero & Cascio (2010), who showed that even geckos belonging to a predominantly myrmecophagous genus do not consume ants indiscriminately, adding the metabarcoding results a different picture emerges: that *T. (boettgeri) bischoffi* does not rely exclusively on arthropods and probably has a more generalist diet, consuming also plant and vertebrate items. Moreover, plants seem to be as important as arthropods in the diet, occurring barely with the same proportions in the samples. It could be the case that plant DNA might have been detected due to the consumption of phytophagous arthropods, but in our case, we found at least six pellets with plants and no invertebrates, showing that in fact, plants are a primary food item for these geckos.

This species' diet is then very different from the continental congeners. In an arid zone of south-east Iberian Peninsula, the main groups present in the diet of *T. mauritanica* were Araneae, Homoptera, Lepidoptera and Carabidae larvae, and Formicidae. Considering prey biomass, the larvae of Lepidoptera and Carabidae dominated the diet, being followed by non-Araneae Arachnida, Araneae and Onyscidae and plants were not very important diet items (Hódar and Pleguezuelos, 1999; similar in Hódar *et al.*, 2006). Similarly, in Central Iberian Peninsula, the most frequent prey were Araneae, Coleoptera, Homoptera, Diptera and Formicidae (Gil *et al.*, 1994), while in an anthropic environment (historical centre of Rome, Italy) there was a clear predominance of flying groups like Diptera and Lepidoptera (Capula & Luiselli, 1994), clearly captured using a sit-and-wait strategy near artificial light. However, none of the previous studies used metabarcoding. Here, we showed the importance of plants and, to a lesser degree, of vertebrates for this insular species. It is common for insular reptile species to develop this type of behaviour, as due to the low number of terrestrial predators they can reach high densities facing higher competition for food (Pérez-Mellado & Corti, 1993). Moreover, associating this the low arthropod availability in these arid systems force reptiles to expand their dietary range of options. In this way, an increase in the consumption of plants by island reptiles is usually detected, such as the Mediterranean *Podarcis* (Pérez-Mellado & Corti, 1993). On islands, reptiles may even play a significant role in seed dispersal and pollination as the amount of pollinators is low. This includes some geckos, such as the diurnal *Phelsumas* and the nocturnal *Hoplodactylus* (Godínez-Álvarez, 2004). Reptiles can even become top predators (Miranda, 2017) and prey on seabirds ingesting their eggs or juveniles, or simply their regurgitations, a behaviour was previously observed in other *Tarentola* species (Schleich, 1984; Alcover & McMinn, 1994; Mateo *et al.*, 2016). As we could show with metabarcoding that our study species is somehow linked to Cory's Shearwater, *Calonectris borealis*, it would be important to study how the gecko interacts with this bird.

The natural diet of other Macaronesian *Tarentola* endemics that resulted from the colonization of the Canaries and Cabo Verde archipelagos is still poorly known. With the remarkable exception of the Cabo-Verdean *Tarentola gigas*, all island endemics are somewhat smaller than continental *T. mauritanica* (Pleguezuelos *et al.*, 2004; Vasconcelos *et al.*, 2012). In Raso islet, Cabo Verde, Mateo *et al.* (2016), studied the diet of small-sized *T. raziana* and *T. gigas* and found that the first consisted mainly on insects and other ground arthropods and the latter on vertebrates, but also included arthropods and plants. The main taxa found in *T. raziana*'s diet, that is more similar in size with our studied species, was Coleoptera, followed by Heminoptera and Aranae. These preys are, excepting the latter, also main preys found in our results. Only in the case of *T. raziana*, Coleoptera was actually the most frequent prey in the diet, instead of Himenoptera. This could be explained by their study having only been performed in June, still prior to the dry season, or the more arid conditions of that archipelago. The metabarcoding study of Seguro (2017) on *T. raziana* revealed, similarly to our results, the importance of plants and arthropods on this species diet and also the presence of vertebrate items. Classic studies on the diet of the sympatric to our study species *Teira dugesii* found that it mainly consists in Coleoptera and Formicidae species, being also reported the ingestion of plants and feather which indicated predation on seabird juveniles (Aguilar, 2016; Rund, 2016). These results are also consistent with ours, however, we could not infer the type of predation of our gecko on seabirds.

Regarding seasonal variation, the Formicidae family was the most common prey, especially in September. However, proportion wise, the difference in ant availability between the two seasons was almost inexistent, and higher consumption of beetles in May is not explained by a variation in ant availability, but either by an increase in the availability of other prey or by the selection of more nutritious prey by the geckos. The increase in consumption of other arthropods, such as Carabidae (which were also the most frequent), some other Coleoptera and Diptera from September to May coincided with the decrease in ant consumption. Since Carabidae were also the most important prey in terms of biomass in both seasons and presented the higher values for the relative importance index in May, the species seems to show a dietary pattern similar to that described by Hódar and Pleguezuelos, 1999 for *T. mauritanica*, showing that between April and July, Lepidoptera and Carabidae larvae and Araneae where the main groups consumed by *T. mauritanica*, whereas from July to September their presence in the diet decreased with an increase in Homoptera, other Coleoptera and Formicidae. This pattern is related with the preference for less sclerotized, highly profitable groups such as larvae in spring and a shift to prey species adapted to drought and food scarcity in summer.

Thus, although quite probable, it was not possible to prove that the differences in the consumption of prey were due to variations in the food supply. On the other hand, the higher consumption of non-ant arthropods in May coincided with a longer rainy season in the previous months, which agrees with Greenville and Dickman (2005) who showed that the flexibility of feeding strategies can be expected in arid environments with a large variation in precipitation. In the study of James (1991) of *Ctenotus species* in Australia, also stated that, as with the ants in this study, the proportion of termites in the diet was higher during the drier periods of the study, concluding that termites constitute a good source of food during drought conditions. *T. (boettgeri) bischoffi* seems to be a seasonal ant specialist since this item has always been the most consumed, but a possible increase in the availability of other prey already leads to adopt a more varied diet and therefore, a more generalist food regime. This supports the theory that many reptiles maintain a flexible diet by opportunistically exploiting diverse food resources when available (Murray & Dickman, 1994).

Even though the collection of pellet samples for the morphological analysis was made for a longer period, as we sampled during two seasons for approximately three weeks each, against only one entire day of sampling in September 2017, we were able to retrieve more information (e.g. 16 versus 29 arthropod families; 56 considering all three groups) and a more accurate taxonomic description with the metabarcoding analysis of the diet of *T. (boettgeri) bischoffi.* If only the data based on morphological analysis of the pellets was considered, we would conclude that this species feeds exclusively on arthropods; however, with metabarcoding, we could evidence the importance of plants for the diet of this threatened gecko and the presence of seven vertebrate OTUs as prey items. Plants generally are not identified using classic methods, as are mainly composed of soft parts that are more easily entirely digested, and therefore are difficult to observe in the pellets. This is also true for some soft-bodied arthropod species, as for example Diptera. Considering morphology this group was only identifiable to the order whereas with metabarcoding we could reach higher taxonomic levels. In general, classical diet methods tend to underestimate the frequency of occurrence of prey with parts that are totally digested (Brown *et al.*, 2012), therefore there is a tendency to more easily detect hard-bodied groups such as Coleoptera.

However, using metabarcoding methodologies it's still not possible to obtain quantitative data on the biomass of prey consumed, rendering impossible the detection of diet shifts such as the ones identified with the morphological analysis. It would make sense that the number of reads of a determined DNA sequence would reflect the amount of food ingested, yet this does not happen (Polz & Cavanaugh, 1998; Acinas *et al.*, 2005). This issue is related primarily with biological factors as the preys ingested can differ in the number of DNA copies for unit mass, and as during the digestion DNA may be differentially degraded depending on the type of prey (Pompanon *et al.*, 2012). Also, the number of reads can be influenced by technical factors, during PCR amplification when the target DNA is exponentially amplified, that is why an accurate marker choice is so important. Additionally, bias can also occur during the extraction of DNA (Martin-Laurent *et al.*, 2001), DNA pooling, sequencing since there is a preference for the amplification of smaller sequences (Porazinska *et al.*, 2010), and during the bioinformatic processing (Amend *et al.*, 2010). This represents a disadvantage in relation to classic methods and for this aspect these can provide more accurate information on the relative abundance of specific items in the diet. Despite these methodological limitations metabarcoding studies have proven to, with the appropriate procedures, allow the successful detection of the range of taxa caught with classic methods and even more ecological information (Shaw *et al.*, 2016).

In conclusion, allying classical and DNA based studies we can have a more comprehensive description of species diet spectrum as well as other important data for the conservation of threatened species, as is the case of this insular gecko. NGS studies represent a revolutionary tool for conservation research and management more and more used for a variety of cases (Allendorf & Luikart, 2009). Metabarcoding methods in particular are important as they can deliver holistic results on species composition, diversity, ecological networks, among others, at relatively low costs (Taylor & Gemmell, 2016). Moreover, metabarcoding can provide a high amount of data in a short time without relaying in taxonomic experts, giving a great help to raise the success of institutions responsible to the conservation of biodiversity (Ji *et al.*, 2013), especially in areas that are of difficult access and that require urgent actions as it is the case of many remote islands belonging to biodiversity hotspots (Taylor & Harris, 2012; Thomsen & Willerslev, 2015).

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7. Supplementary Material

Appendix I – details on the molecular methods

1.1. DNA amplification

The primers used for DNA amplification and the PCR reagents and conditions are detailed in Table S1 – S3, respectively.

Table S1. Primer sequences forward and reverse used in this study.

Primer Name	Fragment length (bp)	Primer F	Primer R	Reference
12sv5	73-110	12sv5F TAGAACAGGCTCCTCTAG	12sv5R TTAGATACCCCACTATGC	(Riaz *et al.*, 2011)
IN16STK-mod	~ 110	IN16STK-1F-mod TRAACTCAGATCATGTAA	IN16STK-1R-mod TTAGGGATAACAGCGTWA	This manuscript (based on Kartzinel and Pringle, 2015)
g/h	10-143	g_F GGGCAATCCTGAGCCAA	h_R CCATTGAGTCTCTGCACCTATC	(Taberlet *et al.*, 2007)
e/f	~146	e_F GGTTCAAGTCCCTCTATCCC	g_R ATI'TGAACTGGTGACACGAG	(Taberlet *et al.*, 1991)

Table S2. Reagents and respective volumes (µL) used in PCRs for the different primer sets.

Reagents	**12sv5**	**IN16STK-mod**	**g/h**	**e/f**
QIAGEN Multiplex PCR Master Mix	10.4	10.4	10.4	4
Forward Primer (10µM)	0.4	0.4	0.4	1
Reverse Primer (10µM)	0.4	0.4	0.4	1
Blocking Primer (10µM)	8	-	-	-
BSA (20mg/ml)	-	-	-	0.5
Ultra-pure Water	2.8	10.8	10.8	16.4
DNA sample	3	3	3	3
Total	25	25	25	25

Table S3. PCR condition used in amplification of DNA. T= temperature; t= time, NC= Number of cycles

12sv5			**IN16STK-mod**			**g/h**			**e/f**		
T(°C)	**t**	**NC**	**T(°C)**	**t**	**NC**	**T(°C)**	**t**	**NC**	**T(°C)**	**t**	**NC**
95	15'	-	95	15'	-	95	15'	-	94	10'	-
95	30"		95	30"		95	30"		94	60"	
52	30"	40x	45	30"	40x	52	30"	40x	50	3'	30x
72	30"		72	30"		72	30"		72	60"	
72	10'	-	72	10'	-	72	10'	-	72	8'	-

1.2. Library preparation

Library preparation was carried out following the Illumina MiSeq protocol 16S Metagenomic Sequencing Library Preparation (Illumina, 2013). Before sequencing, PCR products were cleaned using Agencourt AMPure XP beads (Beckman Coulter) to remove free primers and primer dimers, following two cleaning steps with ethanol and a final dilution using 10nM Tris. The purified products were quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific), and subsequently normalized to 10 ng/µL. Samples amplified with different barcodes were pooled together. Afterwards, an indexing PCR was performed for the incorporation of the Illumina-compatible indexing primers to each pool, using the Nextera XT Kit (Illumina), allowing individual identification of each amplified product. PCR reactions and cycling conditions were similar to the ones of the first PCR except that only 8 cycles of denaturing, annealing and extension were done, with annealing at 55 ºC. The indexed PCR products were again cleaned, quantified and pooled at equimolar concentrations (15 nM). The final pool was quantified by qPCR (KAPA Library Quant Kit qPCR Mix, Bio-Rad iCycler), diluted to 4 nM, and run in a MiSeq sequencer (Illumina) using a 2x150 bp MiSeq Reagent Kit for an expected average of 12,000 paired-end reads per sample.

1.3. Bioinformatics

The software package Obitools (https://git.metabarcoding.org/obitools/obitools) was used for general sequence processing. Forward and reverse sequences were aligned (command illuminapairedend) and discarded if the overlapping quality was less than 40. Reads were then assigned to samples and primers and barcodes were removed (command ngsfilter), this allowed a total of four mismatches to the expected primer sequence. Lastly, the reads were collapsed into unique haplotypes and singletons (haplotypes with only one read) and the potentially erroneous haplotypes resultant from PCR errors were removed (command obiclean), resulting in the removal of haplotypes that differ by 1 bp from more abundant haplotypes. This way any 'A' haplotype differing one base-pair from a 'B' haplotype, with an absolute read count lower than 'B', and that was not found without the presence of 'B' in any sample, was removed. The samples with less than 100 reads in total after this step, were considered to have failed and removed. For the remaining ones, haplotypes representing less than 1% of the total number were removed from each sample (Mata *et al.*, 2016). Haplotypes were identified by comparing the final set against the GenBank online database (https://www.ncbi.nlm.nih.gov/), as well as the obtained reference samples. The sequences with less than 90% of similarity between known species were classified only to class level, the ones with similarity between 90-95% were classified to the family level, and sequences presenting more than 95% of similarity between known species were classified to species or genus level. After identifying all the haplotypes, the ones with a high probability of resulting from lab contaminations were discarded.

3. General Conclusions

3.1. Conservation and future research

The main objective of this study was to assess the diet composition of endemic geckos from Macaronesia. Our two study species, *Tarentola gigas* and *Tarentola (boettgeri) bischoffi,* are both endemic of isolated island systems classified as natural reserves, where access is restricted. Mainly due to the lack of information on population sizes, restricted areas of distribution and human pressures, these taxa are both threatened, respectively classified as Endangered (Vasconcelos, 2013) and Vulnerable (Cabral *et al.*, 2005). Although there are already some studies based on direct observations and morphological examination of scats to infer diet profiles and other ecological patterns, little information was available to the accurate conservation of these species. In order to develop systematic management strategies, it was necessary to obtain more data to answer several ecological questions, such which role these endemics play in the ecosystem. Through our metabarcoding approach, we were able to reveal a fresh range of prey items that formerly went unnoticed in these *Tarentola* diets with a reasonable taxonomic resolution.

Even though inhabiting different archipelagos, our geckos presented similar diet spectrums. Both revealed to be generalist species, feeding on plants, invertebrates and even vertebrates. As the systems both belong to the Macaronesia biogeographical region, presenting similar origins and habitats, and belonging to the same genus, it would be expected these species to have similar ecological needs, but with some differences based on their large size discrepancies. Plants remained overlooked for both species through classic approaches, but with metabarcoding it was possible to reveal that this group is very important for these geckos. However, with metabarcoding is not yet possible to quantify the mass of item ingested to have a correct assessment of the significance of plants, so further developments are expected to improve this method. In this way, it will be possible to uncover if these endemics depend on plant species or if mostly ingest them when searching for invertebrates. It also needed to address which role these reptiles play for plant communities, either as pollinators, seed dispersers or both and to which extent. Invertebrates were already reported as important for both species, although in our study metabarcoding we improved the description of the taxa ingested to the family level and sometimes even to the species level. Nonetheless, for an even more detailed description of the consumed OTUs, we need to first improve our reference database including all potential arthropod present in these islands, especially in Desertas. For that, it would be required a longer sampling period, preferentially covering both wet and dry seasons. In addition, the two *Tarentola* species appear to have trophic links with endemic seabird species. This was already stated for *T. gigas* (Schleich, 1980; Donald *et al.*, 2003), as they are often found using seabird shelters during the day for refuge, and feeding on regurgitations and other remains, like eggs and juveniles. However, for *T. (boettgeri) bischoffi* this behaviour was never reported in the literature and no feathers or other vestiges were found in the morphological examination, even though it was registered in the other endemic sympatric lizard *Teira duguesii* (Aguilar, 2016). Therefore, it would be of great interest to understand how this species interacts with these bird colonies and if they are sporadically eating bird remains or actually rely on these nutrients. In addition, it would be very interesting to explore if these reptiles provide is a phytosanitary service by feeding on parasites, and carcases, preserving diseases in bird populations.

Overall, diet studies reliant on prey identification from faecal samples represent a snapshot of the last ingested meal. Considering that diet composition can differ with prey availability which fluctuates with seasonal variations, for a precise description of our species diet it would be recommended to increase the sampling effort across several time periods. However, due to the remoteness of the study areas and the difficult access, with our DNA based approach, it was possible to recover a significant amount of data for the species conservation in a relatively short time period. Moreover, we can support that our metabarcoding approach can provide important data to supplement previous diet assessments, delivering many times higher taxonomic resolution. Therefore, allying direct observations, morphological methods and metabarcoding approaches is the best option to give accurate representations of diet composition.

Furthermore, for the accurate management of these endemics, future research is needed to address other issues. For a successful reintroduction of *T.gigas* on Santa Luzia, it is necessary to assess the genetic and morphological differences of the two subspecies. Additionally, for this species, it would be interesting to compare our diet results with the ones already available from the endemic and syntopic *Tarentola raziana* (Seguro, 2017). For *T. (boettgeri) bischoffi* it is necessary to develop more phylogenetic studies in order to understand the evolutionary path of this species and their relatives in the Canary Islands. In addition, the study of the niche overlap of this endemic with the Madeiran wall lizard *Teira dugesii* on Selvagem Grande needs to be explored. With the eradication of mammals in the island the populations have grown, and due to the greater fierceness and body size of this lizard, there can be predation episodes on the geckos.

3.2. Final thoughts regarding metabarcoding

Even though metabarcoding is a great approach, not only for diet studies but also to recall valuable information for species conservation, there are still aspects of sampling to bioinformatics that need to be worked on. Above the challenges in selecting of the most precise primer sets to uncover the maximum of diet items and the bias caused by errors along the laboratory procedures (e.g. amplification and sequencing errors) (Symondson, 2002; Pompanon *et al.*, 2012), metabarcoding dietary studies are somehow affected by the deficiency of reference sequences. More accurate and with higher resolution taxa identification would be possible if there were complete DNA reference collections. In this study, we started the construction of reference databases for both island systems that were very helpful in the correct identification of some diet items, however, there is still most work to do in order to have a reliable and complete representation of the flora and fauna of these poorly studied areas.

Another current limitation is the possibility of false inferences due to contaminations, therefore a careful interpretation of doubtful taxa should be carried out (Yu *et al.*, 2012). In order to reduce these false positives, first of all, it is indispensable to be careful in the collection of samples in the field (McInnes *et al.*, 2017), and secondly follow rigorous laboratory protocols to prevent contaminations (Champlot *et al.*, 2010). Also, in order to provide measures of contamination levels, it is essential to keep controls and blanks in all procedure steps (De Barba *et al.*, 2014). Moreover, multiple amplifications of the same pellet are recommended and the selection of appropriate thresholds when eliminating sequences of low frequencies is very important. Both to avoid the risk of discarding rare items and to avoid the keeping of false positives (Pompanon *et al.*, 2012).

The use of blocking primers in metabarcoding studies is very advantageous as it enhances the change of detecting prey by blocking the predator DNA, however, the use of high annealing temperatures for these primers can also prevent the amplification of some prey DNA (Vestheim & Jarman, 2008). As well, the use of these primers inhibits the detection of cases of cannibalism or ingestion of other closely related species, as was the case in our manuscript I. Even though there were suspicions that *T. gigas* could ingest other reptiles due to is high dimensions, with metabarcoding we could not verify that. While blocking primers are a widely used method to eliminate predator DNA, there are other options. One alternative can be the use of beads or gel excision, for example, to remove predator DNA that is expected to have high molecular weight. Prey DNA in principle would not be removed, as it is expected to be more fragmented due to the degradation during the digestion process (Krehenwinkel *et al.*, 2017).

In conclusion, as the technological procedures evolve in accelerated rates, these and other metabarcoding issues are being solved. It is expected that these tools for conservation will continue to improve, reducing the costs and providing high-quality data to support management plans of excellence.

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5. Supplementary Material

Appendix I – other works in progress

Intricate trophic links between threatened vertebrates confined to a small island in the Atlantic Ocean

Ricardo J. Lopes, Catarina J. Pinho, Bárbara Santos, Mariana Seguro, Vanessa A. Mata, Bastian Egeter, Raquel Vasconcelos

Abstract

Trophic networks in small isolated islands are in a fragile balance, and its disturbance can easily contribute towards the extinction vortex of species. Here we show, in a small Atlantic island (Raso) in the Cabo Verde Archipelago, using DNA metabarcoding, the extent of trophic dependence of the Endangered giant wall gecko *Tarentola gigas* on endemic populations of vertebrates, including one of the rarest bird species of the world, the Critically Endangered Raso lark *Alauda razae*. We show that the Raso lark, Iago sparrow *Passer iagoensis*, Cabo Verde shearwater *Calonectris edwardsii* and the Bulwer's petrel *Bulweria bulwerii* are the most frequent vertebrate signatures found in giant wall gecko faeces. This work provides the first integrative assessment of their trophic links, an important issue to be considered for the long-term conservation of these small and isolated island ecosystems.

Appendix II – oral communications in congresses

XV Portuguese-Spanish Herpetology Congress/ XIX Spanish Congress of Herpetology Biology and Conservation of Herps In the Anthropocene. Salamanca, Spain.

What is the Giant Wall Gecko having for dinner? Conservation genetics for guiding reserve management in Cabo Verde.

Catarina J. Pinho, Bárbara Santos, Vanessa Mata, Mariana Seguro, Ricardo Jorge Lopes & Raquel Vasconcelos

Abstract

The Cabo Verde Archipelago belongs to the biogeographical region of Macaronesia and holds its highest number of endemic reptiles. An emblematic Cabo Verdean reptile is the giant wall gecko *Tarentola gigas*, which is presently one of the largest geckonids in the world and restricted to the uninhabited Branco and Raso islets. It is classified as Endangered, mainly due to its reduced distribution, and because it is locally Extinct on Santa Luzia Island, yet little information is known about its diet and behaviour. Regarding diet, due to the scarcity of insects and other small prey on the islets, *Tarentola gigas gigas* population from Raso is thought to have strong trophic links with seabirds, and also thought to probably be the major natural predator of eggs of the Critically Endangered Raso lark *Alauda razae*. The other subspecies, *Tarentola gigas brancoensis* from Branco, presumably relies primarily on colonies of the Near Threatened endemic Cabo Verde shearwater *Calonectris edwardsii*. With this study, we intended to provide useful information to guide the authorities in the reintroduction of this threatened gecko on Santa Luzia, which presently is the largest reserve of the country, by revealing the best source population. For that, we have identified the main bird, plant and arthropod species preyed by both subspecies of *T. gigas* using Next Generation Sequencing methods (metabarcoding of faecal pellets), and compared them with the species known to occur on Santa Luzia. Results have revealed that plants have a significant role as preys and identified vertebrate and invertebrate species with much higher taxonomic resolution than traditional methods. The information revealed by these ecological networks is important for the development of conservation plans on these protected areas.