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(BIOLOGIA VEGETAL)

FENOLOGIA, ECOLOGIA REPRODUTIVA E RAZÃO SEXUAL DE *Zamia boliviana* (Brongn.) A. DC. (CYCADALES, ZAMIACEAE): UMA CICADA TROPICAL RARA E AMEAÇADA

ROSANE SEGALLA SOARES

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de doutora em Ciências Biológicas, ênfase em Biologia Vegetal

JUNHO - 2020

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Rio Claro, 03 de junho de 2020

DEDICATÓRIA

To the most learned and distinguished Cycad Scientists;
To the youngest Cycad Biologists;
To the Lovers and Cycad Guardians;
I dedicate.

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Não tenho a anatomia de uma *Eumaeus* pra receber
em mim os perfumes das flores e do azul.
Mas eu recebo.
É uma bênção.

Às vezes se tenho uma tristeza,
As *Cyanocorax* me namoram mais de perto.
Fico enamorada.
É uma bênção.

Logo dou as cícadas ornamentos de ouro
para que se tornem peregrinas do chão.
Elas se tornam.
É uma bênção.

Até alguém já chegou de me ver passar
a mão nos cabelos de Deus!
Eu só queria agradecer.

(Modificado de Barros, Manoel de. Poesia completa, São Paulo: Leya, 2013. p. 465).

EPÍGRAFE

"A amorosidade de que falo, o sonho pelo qual brigo e para cuja realização me preparo permanentemente, exigem em mim, na minha experiência social, outra qualidade: a coragem de lutar ao lado da coragem de amar!"

(Paulo Freire)

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RESUMO GERAL – Cicadáceas são plantas que ocupam um lugar especial na botânica. A valoração biológica, social e cultural deriva do antigo registro fóssil, que remontam mais de 250 milhões de anos e dos atributos cênicos e harmônicos que elas representam. As cicadáceas são predominantemente dioicas, com cruzamentos e sincronismos fenológicos obrigatórios. A ausência desses mecanismos torna indivíduos efetivamente estéreis. No curso de sua evolução, tais plantas desenvolveram interações com agentes bióticos, algumas indissociáveis como os mutualismos envolvidos na polinização, os serviços de dispersão de sementes e os antagonismos, como a herbivoria. Nesta tese elegemos *Zamia boliviana*, uma cicadácea do Cerrado, para descrever a fenologia reprodutiva, a razão sexual e as interações mutualísticas e antagônicas de suas populações no ambiente natural. Avaliamos o desempenho reprodutivo, mostrado pelo padrão da vida útil de estróbilos e da análise do padrão fenológico e da razão sexual, bem como, a natureza das interações cicadácea-insetos. Discorremos sobre como podemos associar as descobertas ao entendimento e conservação deste sistema biológico, à luz do sucesso ancestral da planta e do regime dos ecossistemas contemporâneos em que se desenvolve. Argumentamos que as condições bioclimáticas do Cerrado e as interações bióticas envolvidas com a cicadácea, têm sido primordiais para sua sobrevivência. A abrangência de atributos funcionais desses habitats permite que essa espécie persista como "gimnospermas em meio à indubitável diversidade de angiospermas" tropicais (Forzza et al. 2010; BFG, 2015), em ecossistemas impulsionados por processos determinísticos, efeitos fundadores e estocásticos. Em uma revisão de literatura da família Zamiaceae (Capítulo I), descobrimos que o número de publicações dobrou no século XXI, concentrando-se principalmente em genética, taxonomia e sistemática, morfologia, interações ecológicas e um interesse crescente em ecologia e conservação populacional. Investigações sobre esses tópicos e estratégias de conservação, especialmente para espécies de *Zamia* na América do Sul, foram apontadas como necessárias. No estudo fenológico (Capítulo II), identificamos padrão reprodutivo sazonalmente sincrônico, com sobreposição predominante das fases de emergência, derramamento de pólen e receptividade dos estróbilos, igualmente compatíveis entre os sexos, populações e anos subsequentes. A vida útil dos estróbilos é semelhante a distinção morfológica e temporal típica de *Zamia* spp., com marcada diferença no desenvolvimento de estróbilos ovulíferos, em relação aos poliníferos. O estudo de razão sexual (Capítulo III), mostrou viés predominantemente masculino para a maioria das populações examinadas. Esse resultado foi atribuído, principalmente, aos custos diferenciais dos sexos. Interações antagônicas especializadas e obrigatórias são típicas das cicadáceas em todo o mundo, como as de *Eumaeus minyas* Hübn. (Lepidoptera: Lycaenidae) (Capítulo IV) e também foram registradas para *Z. boliviana*.

Observações periódicas em populações da cicadácea e a condução de experimentos no ambiente natural mostraram insetos visitantes (visitantes sazonais e oportunisticamente não confiáveis), seu comportamento e eficiência como vetores de polinização (Capítulo V). Os resultados evidenciaram que o vento não é necessário para a polinização dessa cicadácea. Por outro lado, revelamos a existência de mutualismo obrigatório e especializado envolvendo uma espécie de besouro e essa cicadácea, um mecanismo também conhecido para outras *Zamia* spp.. A cicadácea hospedeira é polinizada por uma nova espécie de *Pharaxonotha* (Coleoptera: Erotylidae), descrita no decorrer desta tese. *Pharaxonotha cerradensis* Skelley and Segalla mantém seu ciclo de vida em associação com as estruturas reprodutivas de *Z. boliviana* e a sazonalidade bioclimática do Cerrado. Provavelmente, a combinação de fatores físicos, químicos e biológicos (e. eg.: bioclimáticos, morfológicos, adaptativos, defensivos e interações) permitiu que essa e outras *Zamia* spp. tenham sobrevivido a outras plantas sub-históricas. Nossa pesquisa mostra e corrobora estudos anteriores sobre a biologia e ecologia de *Zamia* spp. e aponta para a necessidade de pesquisas com espécies dioicas e suas interações na América do Sul. Esse conhecimento é fundamental para entender e conservar sistemas biológicos co-dependentes de espécies endêmicas, raras e de vida longa, como as linhagens relíquias e ecologicamente restritas de *Zamia* spp..

Palavras-chave: Conservação de cicadáceas, Ciclo de vida de besouros, Interações ecológicas, Herbívoros obrigatórios, Plantas hospedeiras, Tempo reprodutivo, Sistema reprodutivo, Viés sexual, Custo de reprodução, Vida útil de estróbilos

GENERAL ABSTRACT – Cycads are plants that occupy a special place in botany. The biological, social and cultural relevance derive from the ancient fossil record, which dates back more than 250 million years and the scenic and harmonic attributes that represent. Cycads are predominantly dioecious, with obligate outcrossing and phenological synchronism. In the absence of such mechanisms, individuals become effectively sterile. During their evolution, cycads have developed interactions with biotic agents, including some inextricable relationships such as mutualisms involved in pollination, seed dispersal services and antagonisms, such as herbivory. In this dissertation, we chose *Zamia boliviana*, a cycad from the Cerrado, to describe the reproductive phenology, sex ratio and mutualistic and antagonistic interactions of *Zamia* populations in the natural environment. We evaluated *Zamia* reproductive performance, including the lifespan pattern of strobili, the phenological pattern and sex ratio, as well as the nature of cycad-insect interactions. We discussed how we could associate our findings with the current understanding and conservation of this biological system, taking into consideration the past success of the plant and the contemporary ecosystem regime in which it develops. We argue that the bioclimatic conditions of the Cerrado ecosystem and the biotic interactions involved in cycad development have been essential for its survival. The range of functional attributes of these habitats allows this species to persist as "gymnosperms amid the undoubted diversity of tropical angiosperms" (Forzza et al. 2010; BFG, 2015) in ecosystems driven by deterministic process and founding and stochastic effects. In a literature review about the Zamiaceae family (Chapter one), we found that the number of publications doubled in the 21st century, and they were mostly focused on genetics, taxonomy and systematics, morphology, ecological interactions, with an increasing interest in population ecology and conservation. Investigation about these topics and conservation strategies, especially for South America species of *Zamia*, were pointed out as necessary. In the phenological study (Chapter two), we identified a seasonally synchronous reproductive pattern, with predominance of emergence stage, pollen release and strobili receptivity, which were equally compatible between sexes, populations and subsequent years. The lifespan of strobili resembles the morphological and temporal distinction typical of *Zamia* spp., with a marked difference in the development of ovuliferous strobili in comparison to polleniferous strobili. The sex ratio study (Chapter three) showed a predominantly male bias for most of the populations examined. This result was mainly attributed to differences in reproductive costs. Specialized and obligate antagonistic interactions are typical of cycads around the world, such as those of *Eumaeus minyas* Hübner (Lepidoptera: Lycaenidae) (Chapter four) and those registered for *Z. boliviana*. Periodic observations of cycad populations and the conduct of experiments in the natural environment revealed insect visitors (seasonal visitors and opportunistically unreliable visitors), their behavior and the efficiency as vectors of pollination (Chapter five). The results demonstrated that wind is not necessary for pollination of this cycad. On the other hand, we revealed the existence of obligate and specialized mutualism between a species of beetle and this cycad, which is a mechanism also known in other *Zamia* spp. The host cycad is pollinated by a new species of *Pharaxonotha* (Coleoptera: Erotylidae) as described in this dissertation. *Pharaxonotha cerradensis* Skelley and Segalla maintains its life cycle in association with the reproductive structures of *Z. boliviana* and the bioclimatic seasonality of the Cerrado. It is likely that the combination of physical, chemical and biological factors (e.g., bioclimatic, morphological, adaptive, defensive and interactions) allowed the survival of this and other *Zamia* spp. to other understory plants. Our research corroborates previous studies on the biology and ecology of *Zamia* spp. and points to the need for research with dioecious species and their interactions in South America. This knowledge is essential to understand and conserve biological systems co-dependent on endemic, rare and long-lived species, such as the relic and ecologically restricted populations of *Zamia* spp.

Keywords: Cycad conservation, Beetle life cycle, Ecological interactions, Host plants, Obligate-herbivores, Lifespan strobili, Reproductive system, Reproductive timing, Sex bias, Reproductive cost

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CHAPTER 1

Países que ocupen lugares destacados en biodiversidad, incluidas las cícadas, da una pauta para conocer este recurso mejor y así lograr su protección efectiva a largo plazo. Ésta es una responsabilidad que recae sobre todos, dado que cualquier especie que se extingue es una opción a menos para la humanidad.
(Adaptado de Andrew P. Vovides (2000). México: segundo lugar mundial en diversidad de cícadas. CONABIO. Biodiversitas, 31, 6-10).
Photo credits: Rosane Segalla, personal collection.

A review of current knowledge of Zamiaceae, with emphasis on *Zamia* from South America

Rosane Segalla, Francismeire Jane Telles, Fábio Pinheiro, and Patrícia Morellato

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A Review of Current Knowledge of Zamiaceae, With Emphasis on *Zamia* From South America

Rosane Segalla[1,2], Francismeire Jane Telles[3], Fábio Pinheiro[4], and Patrícia Morellato[2]

Abstract

Zamiaceae, a family of the ancient order Cycadales, is distributed throughout the tropical and subtropical regions of both the Old and New Worlds. Here, we present a systematic review of Zamiaceae with emphasis on *Zamia* species from South America. We aim to (a) establish the current knowledge, (b) identify research gaps, and (c) indicate directions for future studies, discussing ecology and conservation of South America species. The search recovered 508 papers, further classified into 11 research topics: taxonomy and systematics, morphology, biochemistry, genetics, phylogeography, population ecology, reproductive biology, ecological interactions, plant propagation, conservation, and reviews. The number of publications doubled in the 21st century, mostly focusing on genetics ($n = 60$), taxonomy and systematics ($n = 52$), morphology ($n = 36$), ecological interactions ($n = 30$), and an increasing interest in population ecology ($n = 29$) and conservation ($n = 32$). Studies are concentrated in North and Central America (54% of all studies) with just 6% (29) addressing South America species of *Zamia*. Overall, studies point out the key role of pollinators in promoting gene flow through pollen dispersal among populations of Zamiaceae. Therefore, investigate natural history, ecology, reproductive biology, genetic, and phylogeography, especially for South America species, are needed. Moreover, the implementation of in situ and ex situ collections and germplasm banks linked to botanical gardens are essential for the conservation and reestablishment of local populations of critically endangered *Zamia* species in South America. Concomitantly, we suggest studies modeling the distribution of *Zamia* species in future climate change scenarios.

Keywords

reproductive system, ecological interactions, cycad conservation, seed dispersal, phylogeography

Introduction

The family Zamiaceae, together with Cycadaceae and Stangeriaceae, form a monophyletic group belonging to the order Cycadales, an ancient lineage of vascular plants (Donaldson, Dehgan, Vovides, & Tang, 2003; Grobbelaar, 2002; Norstog & Nicholls, 1997; Walters, Osborne, & Decker, 2004). Even though Cycadales occupies a key phylogenetic position among current terrestrial plants, exhibiting characteristics that transition between seedless vascular plants and more derived seed plants, ecological and reproductive aspects of Zamiaceae species are still underexplored (Krieg, Watkins, Chambers, & Husby, 2017), especially in the genus *Zamia* from South America. Understanding of *Zamia* evolution, including the evolution of reproductive strategies and mutualistic interactions (Tang, Xu, et al., 2018; Vovides, 2000), are fundamental for their conservation.

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Zamiaceae is composed of seven genera and 235 species, according to the cladistic analysis of Stevenson (1990) and formal classification of Stevenson (1992): *Ceratozamia* Brongniart (31), *Dioon* Lindley (16), *Encephalartos* Lehmann (65), *Lepidozamia* Regel (2), *Macrozamia* Miquel (41), *Microcycas* (Miq.) A. DC. (1), and *Zamia* L. (80) (Calonje, Stevenson, & Osborne, 2019), which are distributed throughout Africa, the Americas, Australia, and the Greater Antilles (Donaldson et al., 2003; Stevenson, 2004a; Taylor, Haynes, Stevenson, Holzman, & Mendieta, 2012; Walters et al., 2004). In the Americas, Zamiaceae species occur from sea level to an altitude of 2,500 m, in well-drained calcareous soils and different types of habitat, such as tropical forests, savannas, dunes, swamps, and deserts (Lopez-Gallego, 2015; Stevenson, 2004a; Whitelock, 2002). The greatest species diversity is found in the genera *Ceratozamia*, *Dioon*, and *Zamia*, all endemic to the New World (Lopez-Gallego, 2015; Stevenson et al., 2003; Stevenson, 2004a; Taylor et al., 2012; Walters et al., 2004). From those, *Zamia* is considered the most diverse genus of the extant cycads in terms of ecology and morphology (Calonje, Stevenson, et al., 2019; Norstog & Nicholls, 1997). Many *Zamia* populations suffer from habitat loss and often occur in disjunct, small populations (Calonje, Meerow, et al., 2019; Mankga & Yessoufou, 2017; Stevenson, 1993; Walters et al., 2004).

Information regarding *Zamia* populations (structure, dynamics, interactions, etc.) is scarce and one of the explanations is the difficult access to populations, given their remote distribution, hindering research and species conservation actions, even though many are endangered and occur outside protected areas (Calonje, Stevenson, et al., 2019; Donaldson, 2003; Lopez-Gallego, 2015; Mankga & Yessoufou, 2017; Stevenson et al., 2003). Most of the areas where *Zamia* species occur in South America are also considered biodiversity hot spots (Stevenson et al., 2003). In this revision, we aimed to gather information regarding the Zamiaceae family based on a systematic survey of studies from different databases. We focused mainly on the ecology and reproductive biology of *Zamia*, especially the species from South America. We aim to (a) establish the current knowledge, (b) identify research gaps, and (c) indicate directions for future studies, emphasizing the ecology and conservation of the understudied South America species of *Zamia*, within the framework of the literature review of Zamiaceae.

Review Method

We searched through the databases of Web of Science and Google Scholar using the term *Zamia** to compile studies on Zamiaceae and *Zamia* up to June 2018. We also searched The Cycad Society, The World List of Cycads, and the International Union for Conservation of Nature using the same term. Indexed publications (DOI, ISSN, and ISBN) as well as those relevant classical papers and monographs not indexed (listed in Files in Data Supplement—FDS) were included in this review. Unpublished data were only included if from the authors of this review. After compilation, we conducted a systematic classification of the studies, summarizing the most important themes pointed out in each paper reviewed, resulting in 11 research topics: taxonomy and systematics, morphology, biochemical (biochemical composition), genetics, phylogeography, population ecology, reproductive biology, ecological interactions (mutualism and antagonism), plant propagation, conservation, and reviews. After classification, for each study, we extracted the following information: year of publication, geographic region (main region or local distribution area), species under study, and if applicable, the interaction type (mutualism/antagonism/other), and interacting agent (name of the interacting species). All studies surveyed and additional information can be found in FDS.

Considering the small number of publications in South America (FDS) and in order to show the current distribution of *Zamia* in this region throughout different vegetation types, according to the classification of Olson et al. (2001), we created a distribution map of *Zamia* species using the data available at the Global Biodiversity Information Facility (2019), SpeciesLink (2018), and publications presenting geographic coordinates or indications of localities of the species (Segalla & Calonje, 2019). Olson's classification was extracted from http://www.worldwildlife.org/publications/ terrestrial-ecoregions-of-the-world) and the manipulation of geospatial data was done using QGIS (http//qgis.osgeo.org). To identify the knowledge gaps and guide future efforts, we also compiled information of *Zamia* species of South America, including their conservation status according to the International Union for Conservation of Nature (IUCN; Calonje, Stevenson, et al., 2019 in World List of Cycads—http://www.cycadlist.org, 2013–2019, and Red List of Threatened Species—IUCN 2010–2019 in https://www.iucnredlist.org), and possible causes for population decline.

The Knowledge of Zamiaceae Over Time, Research Topic, Species, and Continents

We reviewed 508 studies that addressed Zamiaceae according to our review criteria (FDS). The first study dates from 1897 (Webber, 1897), describing the pollen tube dynamics of *Zamia integrifolia* L.f. (Webber, 1897). This study was followed by sparse publications until the 1980s, totaling 34 papers, mostly related to the morphology ($n = 12$), propagation ($n = 5$) and biochemistry

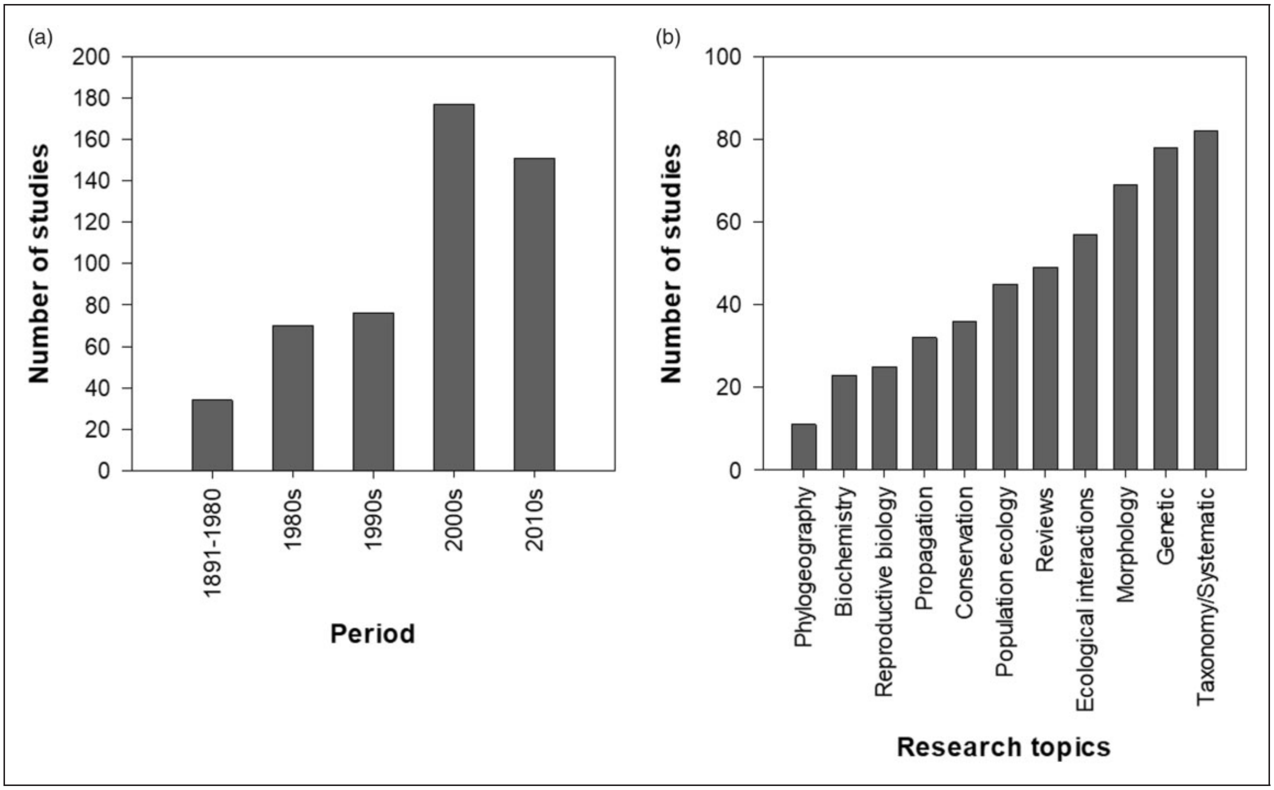

Figure 1. Summary of Zamiaceae review. (a) Number of publications reviewed per decade, between 1871 and June 2018. (b) Number of publications surveyed according to research topics defined in the review: taxonomy and systematics, morphology, biochemical composition, genetics, phylogeography, population ecology, reproductive biology, ecological interactions, plant propagation, conservation, and reviews.

(*n* = 4) of Zamiaceae. We grouped the studies in five periods, considering the first as 1891 to 1980 (Figure 1(a)). From the 1980s, research on Zamiaceae showed a significant increase (*n* = 70) mainly focusing on taxonomy and systematics (*n* = 15), ecological interactions (*n* = 12), and a similar number of studies in morphology (*n* = 13). From the period 1991 to 2000 (*n* = 76), researchers kept their interest in taxonomy and systematics (*n* = 12) and ecological interactions (*n* = 12), but now also branched into genetics (*n* = 11), with biochemistry, morphology, population ecology, and propagation with similar numbers of publications (six to nine studies). The number of publications doubled after the 21st century (*n* = 328), with research focusing on genetics (*n* = 60), taxonomy and systematics (*n* = 52), reviews (*n* = 41), morphology (*n* = 36), ecological interactions (*n* = 30), and a surprisingly increasing interest in population ecology (*n* = 29) and conservation (*n* = 32) (Figure 1(a) and (b)).

Our review revealed that over the decades, the following research topics predominated, with more than 30 publications on each: ecological interactions (*n* = 57), reviews (*n* = 49), population ecology (*n* = 45), conservation (*n* = 36), and propagation (*n* = 32) (Figure 1(b), FDS). Taxonomy and systematics, genetics, and morphology research topics had over 60 publications each, indicating that most studies on Zamiaceae have a more descriptive/comparative aspect. Less studied research topics were reproductive biology (*n* = 25), biochemistry (*n* = 23), and phylogeography (*n* = 11). Currently, a multidisciplinary focus combining morphological, anatomical, and molecular data have been used to approach questions related to the speciation processes, also considering climatic conditions and species biogeography, as described for the genera *Ceratozamia* (Vovides, Stevenson, Pérez-Farrera, López, & Avendaño, 2016) and *Dioon* (Vovides et al., 2018).

The large number of reviews and studies focusing on taxonomy and systematics, morphology, and genetics can be linked to the existence of research centers that contain large herbaria with important cycad collections, such as the South African National Botanical Institute (South Africa), the Montgomery Botanical Center in Miami (USA), and Nong Nooch Tropical Garden (Thailand) (Walters, 2003). However, additional taxonomic work is required to resolve taxonomic issues of species from Brazil, Venezuela, and Colombia (Calonje, Meerow, et al., 2019). The gradual increase in studies focusing on population ecology and conservation is

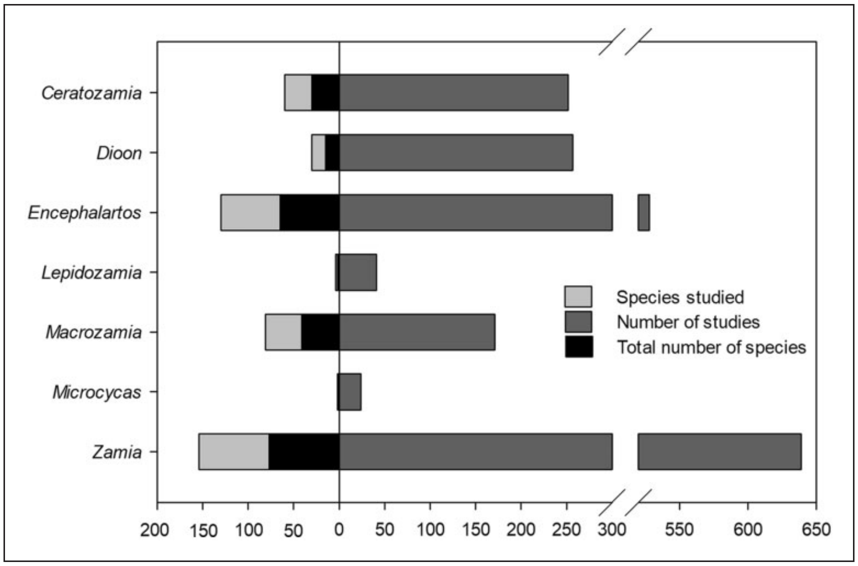

Figure 2. Number of species per genus (black bar), number of species studied (light gray), and of studies per species (dark gray) in the Zamiaceae family.

most likely driven by the growing destruction of suitable habitat for cycad populations around the world, a noticeable factor since the beginning of the 21st century. Finally, the reduced number of studies addressing reproductive biology, biochemistry, and phylogeography could be explained by the fact that these research topics are more expensive both in time and resources, requiring extensive fieldwork or expensive technological analyses not always accessible, especially until recently in the New World.

Considering the diversity of species, the most studied genera of Zamiaceae are *Zamia*, *Encephalartos*, *Ceratozamia*, *Dioon*, and *Macrozamia* (Figure 2). Most of the studies focus on morphology and genetics (FDS). Despite the number of studies, information is still scarce, especially for *Zamia* and *Encephalartos* (FDS). In addition, *Microcycas* and *Lepidozamia* have few species and, thus, studies surveyed (Figure 2, FDS). Most of the Zamiaceae studies are concentrated in the North American continent ($n = 273$), corresponding to 54% of all studies found in our survey, followed by Africa ($n = 73$; 14%), Australia ($n = 54$; 11%), South America ($n = 31$; 6%), Europe ($n = 23$; 5%), and Asia ($n = 14$; 3%). The remaining 7% ($n = 40$) correspond to reviews. Studies identified as being from North America and Europe are classified as such due to the affiliation of the main authors. It is interesting to note that, although the species do not naturally occur in these areas, a considerable part of the studies have been done by researchers from these regions.

Regarding the species from the New World, the vast majority of studies have been conducted in North and Central America (FDS). Mexico, the country with most studies on Zamiaceae, holds the greatest diversity of cycads in the Neotropics with a high percentage of endemic species (Lopez-Gallego, 2015; Nicolalde-Morejón et al., 2014; Vovides et al., 2003). Of the 62 cycad species found in Mexico, 58 are endemic (Calonje, Stevenson, et al., 2019), of which *Zamia furfuracea* L.f., *Dioon edule* Lindl., and *Ceratozamia mexicana* Brongn. are the most studied ones (Nicolalde-Morejón et al., 2014). Mexico is also an exception regarding action plans for conservation. The country has developed local conservation actions based on national collections, botanical gardens, investing in sustainable use of cycad species by means of educational programs (Donaldson, 2003; Lázaro-Zermeño, González-Espinosa, Mendoza, & Martínez-Ramos, 2011; Pérez-Farrera, Quintana-Ascencio, Salvatierra, & Vovides, 2000; Pérez-Farrera & Vovides, 2006; Vovides, Iglesias, Luna, & Balcázar, 2013; Vite, Pulido & Vázquez, 2013). Following this example, Australia and South Africa have also increased the number of cycad related studies (FDS). Such studies and efforts are needed in South America. However, due to the numerous political borders across the geographical distribution of cycads in the Americas, research efforts have evolved slowly (Terry et al., 2012).

Within Zamiaceae, *Zamia* is the genus with the greatest number of species (80), of which 30 are found throughout South America (Figure 3). Despite the fact that many species have been well studied, others still require basic research (Table 1). The rate of habitat loss has increased faster than species distribution data is updated, giving the wrong impression that populations are stable when in fact numbers are declining fast and they are endangered. In addition, the disjunct nature of populations makes it difficult to assess their dynamics or

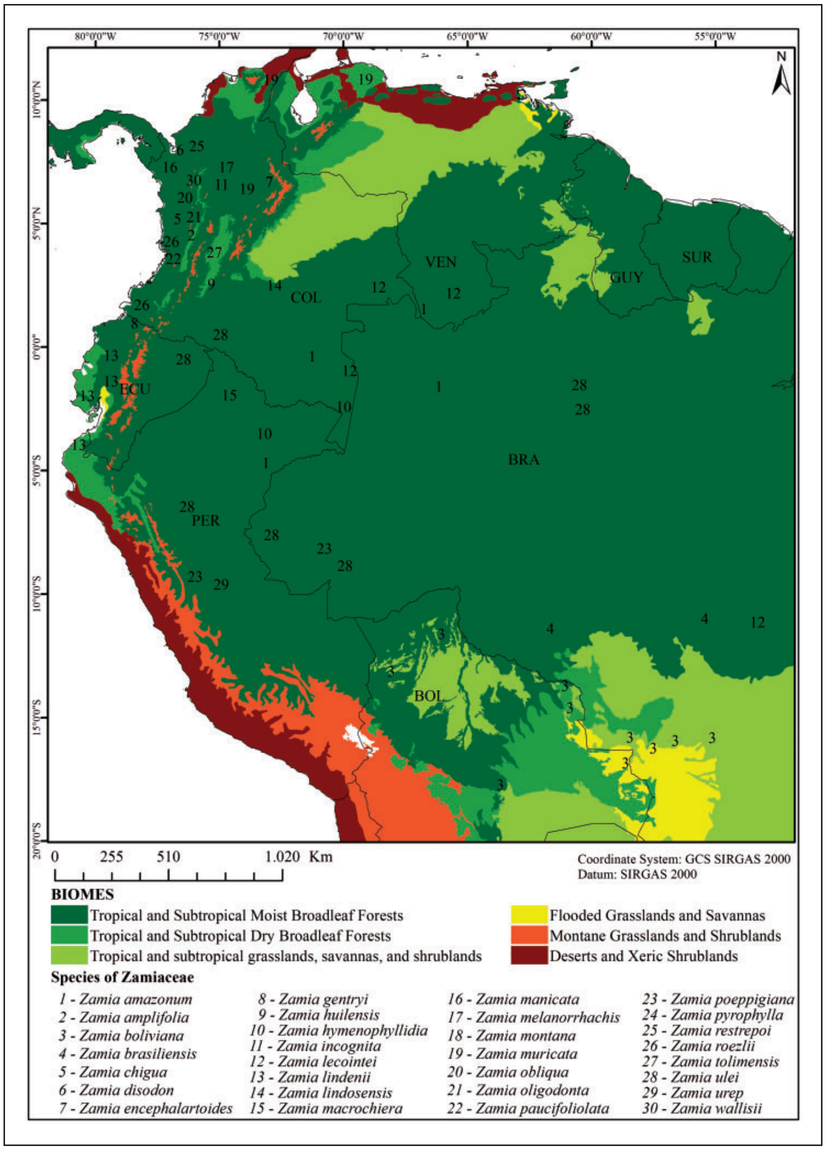

Figure 3. Geographical distribution of the 30 Zamiaceae species of South America considering the different vegetation types where they occur.

stability under different scenarios without extensive fieldwork. This is a critical limitation in South America (Donaldson et al., 2003; Stevenson, 1993). The 30 species of *Zamia* occurring in South America are distributed between Brazil, Colombia, Ecuador, Peru, and Venezuela (Calonje, Stevenson, et al., 2019). Colombia has 21 species of *Zamia* with populations in all biogeographical regions (Lopez-Gallego, 2015). About 62% of these species them considered endemic to South America and are distributed in the floristic elements of Chocó, montane, Río Magdalena Valley, and Amazonian Basin (Lopez-Gallego, 2015; Stevenson, 2004b). Brazil and Bolivia have species inhabiting the ecosystems of the Amazon Basin, Cerrado (Savanna), and the transition areas between the two (Segalla & Calonje, 2019). For species shaing similar regions but crossing geographical borders, research efforts and joint conservation plans should be established between countries.

Reproductive Biology and Ecological Interactions on Zamiaceae

Most of the studies focusing on ecological interactions involve *Ceratozamia*, *Dioon*, and *Zamia*. Thus, we focused on these genera. Basic aspects of the morphology of reproductive structures, common to all cycads, were previously revised somewhere else (Stevenson, 1993; Stevenson, 2004a; Terry et al., 2012).

Table 1. General Overview of Possible Studies With the Genus *Zamia* From South America: Conservation Status of Species, Potential Causes of Population Decline, and Topics for Future Studies.

Zamiaceae Taxa	Conservation status	Causes of population decline	Suggested topics for future studies: Natural history and phenology 1	Population dynamics 2	Reproductive biology 3	Ecological interaction 4	Habitat characteristics 5	Populations reestablishment translocations 6	Ex situ collections/ Germplasm bank 7	Phylogeography 8	Ethnobotany/ Ecotourism 9	Intercountry conservation actions 10	Source
Zamia amazonum D.W. Stev.	Near threatened/ Vulnerable	Loss of habitat	X	X	X	X	X	X	X	X	X	X	1, 9
Zamia amplifolia W. Bull ex Mast.	Critically endangered	Small area of occupation	X	X				X	X		X		1, 9
Zamia boliviana (Brongn.) A.DC.	Near threatened/ Vulnerable	Fragmented population		X			X		X	X	X	X	1, 2, 9
Zamia brasiliensis Calonje & Segalla	Endangered	Small area of occupation	X	X	X	X		X	X	X	X	X	13
Zamia chigua Seem.	Near threatened/ Vulnerable	Loss of habitat/ Other causes		X									1, 7, 9
Zamia disodon D.W. Stev. & Sabato	critically Endangered	Small area of occupation					X	X		X			1, 9
Zamia encephalartoides D.W. Stev.	Vulnerable/ Endangered	Small area of occupation	X	X				X	X		X		1, 9
Zamia gentryi Dodson	Critically endangered	Small area of occupation/ Loss of habitat	X	X			X	X		X			1, 9
Zamia huilensis Calonje, Esquivel, & Stev	Critically endangered	Small area of occupation/ Loss of habitat/ Illegal trade		X		X			X		X		1, 6
Zamia hymenophyllidia D.W. Stev.	Critically endangered	Small area of occupation/ Fragmented population	X	X	X	X		X	X				1, 9
Zamia incognita A. Lindstr. & Idárraga	Vulnerable	Loss of habitat/ Petroleum and Ore exploration		X						X			1, 7, 8, 9
Zamia lecointei Ducke	Near threatened/ Vulnerable	Loss of habitat	X	X						X			1, 9
Zamia lindenii Regel ex André	Deficient data	Loss of habitat	X	X	X	X							3, 9
Zamia macrochiera D.W. Stev.	Critically endangered	Loss of habitat	X	X	X	X		X			X		1, 9
Zamia lindosensis D.W. Stev., D. Cárdenas & N. Castaño	Endangered	Loss of habitat/ Agricultural expansion		X	X	X		X	X	X			1, 9,10
Zamia manicata Linden ex Regel	Near threatened/ Vulnerable	Current land use and occupation	X	X		X							1, 9
Zamia melanorrhachis D.W. Stev.	Endangered	Loss of habitat/Small area of occupation	X	X		X			X				1, 9

(continued)

Table 1. Continued.

Zamiaceae Taxa	Conservation status	Causes of population decline	Suggested topics for future studies Natural history and phenology 1	Population dynamics 2	Reproductive biology 3	Ecological interaction 4	Habitat characteristics 5	Populations reestablishment translocations 6	Ex situ collections/ Germplasm bank 7	Phylogeography 8	Ethnobotany/ Ecotourism 9	Intercountry conservation actions 10	Source
Zamia montana A. Braun	Critically endangered	Small area of occupation/ Loss of habitat	X	X		X		X	X				1, 9
Zamia muricata Willd.	Near threatened/ Vulnerable	Fragmented population/ Current land use and occupation	X	X	X		X			X			1, 7, 9
Zamia obliqua A. Braun	Near threatened/ Vulnerable	Loss of habitat	X	X	X								1, 7, 9
Zamia oligodonta E. Calderón & D.W. Stev.	Endangered	Loss of habitat/ Illegal trade		X	X	X		X	X				1, 12
Zamia paucifoliolata Calonje	Endangered	Loss of habitat/Small area of occupation	X	X	X	X		X	X				1, 9,11
Zamia poeppigiana Mart. & Eichler	Near threatened	Loss of habitat	X	X	X	X							1, 9
Zamia pyrophylla Calonje, D.W. Stev. & A. Lindstr.	Critically endangered	Loss of habitat/Current land use and occupation	X	X	X			X	X				4, 9
Zamia restrepoi (D.W. Stev.) A. Lindstr.	Critically endangered	Loss of habitat/Small area of occupation		X				X	X				1, 7 , 9
Zamia roezlii Linden	Vulnerable	Current land use and occupation		X	X			X	X		X		1, 7
Zamia tolimensis Calonje, H.E. Esquivel & D. W. Stev.	Critically endangered	Fragmented population/ Current land use and occupation	X	X	X			X	X				5, 6, 7, 9
Zamia ulei Dammer	Near threatened	Ore exploration/Loss of habitat	X	X	X	X							1, 7, 9
Zamia urep B. Walln.	Critically endangered	Deficient data	X	X	X	X		X			X	X	1, 9
Zamia wallisii A. Braun	Critically endangered	Loss of habitat/Small area of occupation/ Illegal trade	X	X	X			X					1, 7, 9

Source: (1) International Union for Conservation of Nature (IUCN) 2013–2019, (2) Skelley & Segalla (2019), (3) Lindström (2010), (4) Calonje et al. (2010), (5) Calonje, Kay, and Griffith (2011), (6) Calonje, Esquivel, Morales, Mora-Lizcano, and Stevenson (2012), (7) Lopez-Gallego (2015), (8) Valencia-Montoya, Tuberquia, Guzmán, and Cardona-Duque (2017), (9) Calonje et al. (2019), (10) Stevenson et al. (2018), (11) Calonje et al. (2018), (12) Calderón-Sáenz and Stevenson (2003), and (13) Segalla and Calonje (2019).

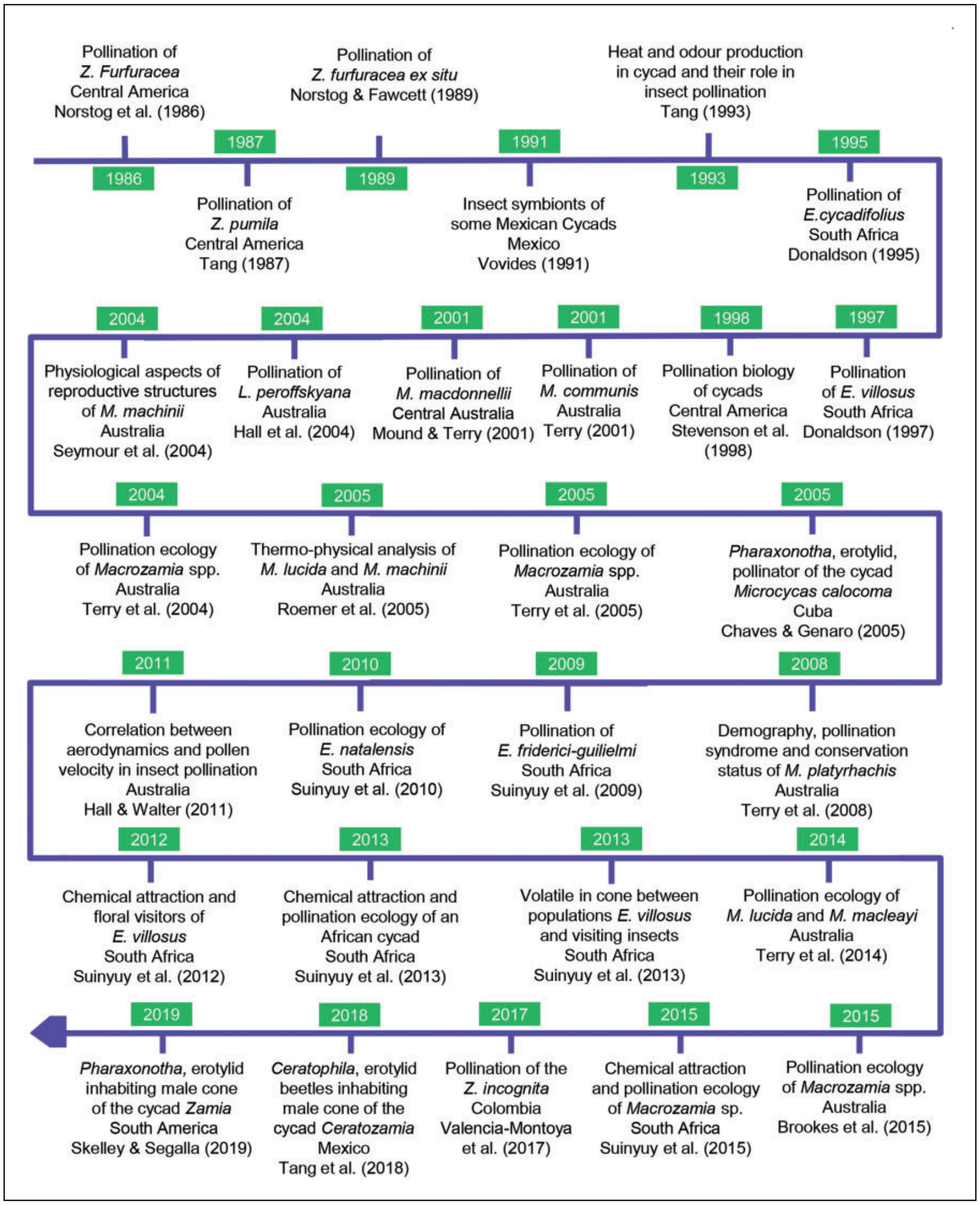

Figure 4. Timeline of main studies considering the reproductive biology of Zamiaceae.

Mutualistic Interactions: Plant-Pollinators

The pollination of different species of Zamiaceae seems to be mediated by host-specific insects, typically Coleoptera and Thysanoptera (Franz & Skelley, 2008; Tang, Skelley, & Pérez-Farrera, 2018; Terry et al., 2004; Terry, Forster, Moore, Roemer, & Machin, 2008; Valencia-Montoya et al., 2017). We prepared a timeline of the main studies addressing the pollination biology of Zamiaceae species (Figure 4). Historically, the pollination of Zamiaceae was attributed exclusively to anemophily. As with other gymnosperms, wind was considered the only facilitating agent of pollination (Terry, Roe, Tang, & Marler, 2009). This idea was refuted only in the 1980s with the classic experimental studies of Norstog, Stevenson, and Niklas (1986); Tang (1987); Norstog and Fawcett (1989); Vovides (1991); and Tang (1993) confirming that beetles of the genera *Pharaxonotha* (Curculionoidea: Erotylidae) and *Rhopalotria* (Curculionoidea: Belidae) are the pollinators of different species of *Zamia*. Such studies stimulated further experimental research, corroborating insect pollination in *Zamia* species. In fact, research on pollination published after these first studies frequently report beetles as pollinators of Zamiaceae, as well as of basal angiosperms (Ollerton, 2017), suggesting an evolutionary process between cycads and beetles acting as pollinators (Walters et al., 2004).

Pharaxonotha has been commonly described as a mutualistic agent of *Zamia* and has also been found in strobili of species from other genera, such as *Ceratozamia*, *Dioon*, and *Microcycas* (Chaves & Genaro, 2005; Franz & Skelley, 2008). Recently, a new species of *Pharaxonotha* Reitter (Coleoptera: Erotylidae) was found inhabiting the male strobilus of *Zamia boliviana* (Brongn.) A.DC. from central South America (Skelley & Segalla, 2019), and a new species of the genus *Ceratophila* Tang, Skelley, and Pérez-Farrera (Erotylidae: Pharaxonothinae) was described inhabiting male strobili of *Ceratozamia* in Mexico (Tang, Xu, et al., 2018). Surveys of Coleoptera inhabiting the strobilus of other cycad genera in the New World, including *Dioon*, *Microcycas*, and *Zamia*, indicate that *Ceratophila* is restricted to *Ceratozamia*, the only known host of these beetles (Tang, Xu, et al., 2018). The existence and nature of insect interactions with cycads species of South America, especially as it relates to ecology and reproductive biology, still need ample effort of investigation.

Molecular and morphological phylogenetic analyses of beetles present in cycads from the New World suggest that pollinator type may impact the population genetic structure of their host species (Tang, Skelley, et al., 2018). The new findings indicate that this is a fruitful avenue of research (Tang, Skelley, et al., 2018), applicable mainly to the conservation of South American cycads. In tropical regions, the size and lifespan of the strobilus is short, limiting the attractiveness of visitors that have long reproductive periods and acts as a barrier to the colonization of certain species (Terry et al., 2012). Strategically, this characteristic promotes greater activity in the beetles to move from one plant to another, favoring pollen dispersion over longer distances (Terry et al., 2012). This hypothesis remains to be tested for South American cycads.

Mutualistic Interactions: Seed Dispersers and Consumers

Seed dispersal by animals is a facultative mutual relationship relevant to the gene flow and maintenance of plant species. Birds, rodents, and probably many other animals disperse cycad seeds by ingesting the sarcotesta and dropping the stony layer and its contents away from the mother plant (Hill & Osborne, 2001; Taylor & Holzman, 2012), but these events need to be better studied in Zamiaceae species (Lopez-Gallego, 2015). Seed dispersal maintains the local genetic structure of species even more than pollen dispersal (Dow & Ashley, 1996; Dyer, 2007; Ortego, Bonal, & Muñoz, 2010). Although pollen dispersal may promote high diversity at a global scale, seed dispersal acts locally, determining the structure of populations (Cabrera-Toledo, González-Astorga, & Flores-Vázquez, 2012). Table 2 summarizes the studies addressing seed dispersal agents in Zamiaceae species and shows that in general, seed dispersal mechanisms need to be investigated for most species.

The relationship between dispersal and recruitment is still poorly understood for most Zamiaceae species. Gregory and Chemnick (2004) observed that in *Dioon* species most seeds germinating near the mother plant do not survive. According to these authors, seedling survival is determined by seed storage period and depth of burial, as seeds that are buried deeper in the soil are more likely to avoid predation and germinate successfully. Recruitment of seeds next to the mother plant also suggests that seed dispersal by animals is not as effective (Pérez-Farrera et al., 2000). For example, *Ceratozamia matudae* Lundell interacts very little with predators and dispersers due to the large size of its seeds (2–3 cm in diameter) and due to its high concentration of neurotoxins (Pérez-Farrera et al., 2000). The *C. matudae* plants are usually found in areas with steep topography (Pérez-Farrera et al., 2000) and gravity might be responsible for the limited local seed dispersal (Jones, 1993). Potential differences in pollen or seed dispersal distances between native- and degraded-forest habitats as in *Zamia fairchildiana* L.D. Gómez populations are difficult to evaluate, given the limited knowledge of its pollination and dispersal biology (Lopez-Gallego & O'Neil, 2010). Other relationships with species of *Zamia*, such as opportunistic associations with ants as removal agent of fresh sarcotesta (Lázaro-Zermeño et al., 2011), are also important as maintainers of ecological services, but are poorly understood. The dispersal of seeds and other aspects of the natural history of *Zamia* species, as well as others around the world, require further research (Lopez-Gallego, 2015).

Antagonist Interactions: Herbivores and Seed Predators

Zamia species, like many other plant species, produce a variety of secondary toxic substances (allelochemicals) to defend themselves against antagonists, mainly herbivores. Dimeric flavones, the nitrogen-containing methylazoglucosides cycasin, macrozamin, and several neocycasins are among the most important allelochemicals, and palatable to only a few animal species (Brenner, Stevenson, & Twigg, 2003; Prado, 2011; Schneider, Wink, Sporer, & Lounibos, 2002). Table 3 summarizes the studies addressing antagonist interactions, such as herbivory and predation, in Zamiaceae from the New World. In general, studies indicate that there is a high dependence by animals on the host plant (Table 3). Nonetheless, the mechanisms involved in the

Table 2. Compilation of Studies on Seed Dispersal and Predation on Different Species of the Zamiaceae Family.

Taxa de Zamiaceae	Seed dispersers and predators	Geographical region/Country	Source
Ceratozamia matudae Lundell	*Peromyscus mexicanus* (Saussure, 1860); *Pecari tajacu* Linnaeus, 1758	Mexico	Pérez-Farrera et al. (2000)
Ceratozamia mirandae Vovides, Pérez-Farr. & Iglesias	Peccaries (Tayassuidae)	Mexico	Pérez-Farrera et al. (2006)
Dioon edule Lindl.	*P. mexicanus*	Mexico	Vovides et al. (2003)
Dioon merolae De Luca, Sabato & Vázq.Torres	*P. mexicanus*	Mexico	Lázaro-Zermeño, González-Espinosa, Mendoza, and Martínez-Ramos (2011)
Dioon spinulosum Dyer ex Eichler	Birds, coatis, and rodents	Mexico	Chemnick (2013)
Encephalartos barteri subsp. *barteri*	Big mammals and large flying birds	Benin/Africa	Ekué et al. (2008)
Macrozamia lucida L.A.S. Johnson	*Trichosurus Vulpecula*, *Rattus fuscipes*	Queensland, Australia	Snow and Walter (2007)
Macrozamia miquelii (F. Muell.) A.DC.	*Trichosurus vulpecula*	Queensland, Australia	Hall and Walter (2013)
Macrozamia riedlei (Gaudich.) C.A. Gardner	Birds, parrots, and marsupials	Western Australia	Burbidge and Whelan (1982)
Zamia amblyphyllidia D.W. Stev.	Small and medium mammals	Puerto Rico	Negron-Ortiz and Breckon (1989)
Zamia fairchildiana L.D. Gómez	*Saltator* spp. (Cardinalidae), *Ramphocelus passerinii* Bonaparte, 1831	Costa Rica	Gómez (1993)
Zamia lindenii Regel ex André	*Dasyprocta punctata* Gray, 1842	Equador	Lindström (2010)
Zamia pumila L.	Mockingbird (Mimidae)	Florida/USA	Eckenwalder (1980)

Note. Interaction species, geographical region of study and references are provided.

interaction between the antagonist agents and the chemical substances are not fully understood (Prado, 2014).

Antagonist interactions with Lepidoptera and Coleoptera have been observed for many Zamiaceae species. For example, butterflies of the genus *Eumaeus* (Lepidoptera: Lycaenidae) are obligate cyclic herbivores that consume both vegetative and reproductive parts of many Neotropical Zamiaceae (Figure 5(a)–(c)), while beetles (Coleoptera: Chrysomelidae and Aulacoscelinae) act as predators (Cascante-Marín & Araya, 2012; Castillo-Guevara & Rico-Gray, 2002; Contreras-Medina et al., 2003; Koi & Daniels, 2015; Pérez-Farrera & Vovides, 2004; Prado et al., 2011; Ruiz-García et al., 2015; Taylor et al., 2008). Those studies are limited, however, and only provide brief descriptions of the observed interactions.

The different adaptations needed to overcome the toxicity of cycads are not restricted only to herbivory or predation but also to the possible gains by the insect using the plant's secondary metabolites (Prado, 2011). The aposematic traits of *Eumaeus* larvae which feed on *Zamia* species, suggest a long evolutionary association where insects are tolerant to the plant's defenses while exploring the resources free from competition (Castillo-Guevara & Rico-Gray, 2002; Schoonhoven, van Loon, & Dicke, 2005). Prado, Rubio-Mendez, Yañez-Espinosa, and Bede (2016) recommend studies on the life cycles of both plants and herbivores to evaluate preference, performance, and levels of damage throughout different ontogenetic stages between male and female individuals.

Biogeographic Studies as a Conservation Strategy for Zamia Species

Variability is a basic requirement for plant survival and adaptive evolution. Populations that are genetically related have higher degrees of endogamy, which brings negative consequences for future generations (Linhart, 2014). Gene transfer between populations is even more important given the decline of pollinator populations, increase in habitat loss and fragmentation, and shifts in species distribution due to climate change (Gutiérrez-Ortega, Yamamoto, et al., 2018b; Liu, Compton, Peng, Zhang, & Chen, 2015). Indeed, several studies have detected low genetic diversity and high levels of inbreeding in Zamiaceae, particularly in the Australian species of *Macrozamia* (Sharma, Jones, Forster, & Young, 1998; Sharma et al., 1999, 2004). Studies of genetic variation and structure in cycad populations have given variable results, mainly in Asian *Cycas* species (reviewed by Liu et al., 2015), which

Table 3. Compilation of Studies (1870–2019) Regarding Antagonistic Interactions Involving Zamiaceae Species From the New World.

	Zamiaceae				
Agent	Genus	Taxa	Behavior of the agent	Geographical region	Source
Aulacoscelis appendiculata (Cox & Windsor, 1999), (Coleoptera: Chrysomelidae)	*Zamia*	*Zamia elegantissima* Schutzman, Vovides & R.S. Adams	Predation	Panama	Prado, Ledezma, Cubilla-Rios, Bede, and Windsor (2011)
Aulacaspis yasumatsui (Hemiptera: Sternorrhyncha: Diaspididae)	*Dioon*	*Dioon califanoi* De Luca & Sabato	Parasitism	Mexico	Howard et al. (1999)
		Dioon edule Lindl.	Parasitism	Mexico	Howard et al. (1999)
		Dioon merolae De Luca, Sabato & Vázq.Torres	Parasitism	Mexico	Howard et al. (1999)
		Dioon spinulosum Dyer ex Eichler	Parasitism	Mexico	Howard et al. (1999)
		Dioon tomasellii De Luca, Sabato & Vázq.Torres	Parasitism	Mexico	Howard et al. (1999)
		Dioon rzedowskii De Luca De Luca, A. Moretti, A. Moretti, Sabato, Sabato & Vázq.Torres & Vázq.T	Parasitism	Mexico	Howard et al. (1999)
	Microcycas	*Microcycas calocoma* (Miq.) A.DC.	Parasitism	Cuba	Howard et al. (1999)
Aulacoscelis vogti (Monrós, 1959), (Coleoptera: Orsodacnidae)	*Dioon*	*Dioon edule* Lindl.	Predation	Mexico	Prado et al. (2011)
Eumaeus atala (Poey, 1832), (Lepidoptera: Lycaenidae)	*Zamia*	*Zamia integrifolia* L.f.	Herbivory	Florida	Schneider et al. (2002)
Eumaeus childrenae (G. Gray, 1832), (Lepidoptera: Lycaenidae)	*Zamia*	*Zamia fischeri* Miq.	Herbivory	Mexico	Contreras-Medina, Ruiz-Jiménez, and Vega (2003)
		Zamia cremnophila Vovides, Schutzman, & Dehgan	Herbivory	Mexico	Jiménez-Pérez et al. (2017)
	Ceratozamia	*Ceratozamia matudae* Lundell	Herbivory	Mexico	Pérez-Farrera and Vovides (2004)
E. childrenae	*Dioon*	*Dioon merolae* De Luca, Sabato & Vázq.Torres	Herbivory	Mexico	Lázaro-Zermeño et al. (2011)
Eumaeus godartii (Boisduval, 1870), Lepidoptera: Lycaenidae)	*Zamia*	*Zamia fairchildiana* L. D. Gómez	Herbivory	Costa Rica	Lopez-Gallego (2007)
		Zamia skinneri Warsz. ex A. Dietr.	Herbivory	Panama	Taylor, Haynes, and Holzman (2008)
		Zamia acuminata Oerst. ex Dyer	Herbivory	Costa Rica	Cascante-Marín and Araya (2012)
		Zamia stevensonii A.S. Taylor & Holzman	Herbivory	Panama	Taylor and Holzman (2012); Prado et al. (2014)
		Zamia incognita A. Lindstr. & Idárraga	Herbivory	Colombia	Lopez-Gallego (2015); Valencia-Montoya et al. (2017)
Eumaeus minyas (Hübner, 1809), (Lepidoptera: Lycaenidae)	*Zamia*	*Zamia neurophyllidia* D.W. Stev.	Herbivory	Costa Rica	Clark et al. (1992)
		Zamia loddigesii Miq.	Herbivory	Mexico	Castillo-Guevara and Rico-Gray (2002)
		Zamia encephalartoides D.W. Stev.	Herbivory	Colombia	González (2004)
		Zamia boliviana (Brongn.) A.DC.	Herbivory	Brazil	Segalla and Morellato (2019)

(continued)

Table 3. Continued.

Agent	Zamiaceae		Behavior of the agent	Geographical region	Source
	Genus	Taxa			
Eumaeus toxea (Godart, 1824) (Lepidoptera: Lycaenidae)	*Zamia*	*Zamia poeppigiana* Mart. & Eichler	Herbivory	Mexico	Ruiz-García, Méndez-Pérez, Velasco-García, Sánchez-de la Vega, and Rivera-Nava (2015)
Eumaeus sp.	*Zamia*	*Zamia lindenii* Regel ex André	Herbivory	Equador	Lindström (2010)
Eumaeus sp.	*Zamia*	*Zamia pyrophylla* Calonje, D. W. Stev. & A. Lindstr.	Herbivory	Colombia	Calonje et al. (2010)
Eumaeus sp.	*Zamia*	*Zamia huilensis* Calonje, H.E. Esquivel & D.W. Stev.	Herbivory	Colombia	Calonje et al. (2012)
Eumaeus sp.		*Zamia nana* A. Lindstr., Calonje, D.W. Stev. & A. S. Taylor	Herbivory	Panama	Lindström et al. (2013)
Eumaeus sp.	*Ceratozamia*	*Ceratozamia subroseophylla* Mart.-Domínguez & Nic.-Mor.	Herbivory	Mexico	Martínez-Domínguez et al. (2016)
Janbechynea elongata (Coleoptera: Polyphaga: Orsodacnidae)	*Ceratozamia*	*Ceratozamia huastecorum* Avendaño, Vovides & Cast.-Campos	Herbivory	Mexico	Reyes-Ortiz et al. (2016)
Janbechynea paradoxa (Monrós, 1953), (Coleoptera: Chrysomelidae)	*Zamia*	*Zamia boliviana* (Brongn.) A.DC.	Predation	Bolivia	Prado et al. (2011)
Seirarctia echo (J. E. Smith, 1797), (Lepidoptera: Erebidae)		*Zamia pumila* L.	Herbivory	EUA	Negrón-Ortiz and Gorchov (2000)

Note. The interacting agents (plant–animal species), behavior, geographical region of the study, and references are shown.

may be related to its occurrence on islands (Keppel, Lee, & Hodgskiss, 2002) and in naturally fragmented landscapes as this may facilitate pollen and seed movement in some species but not in others (Xiao, Ge, Gong, Hao, & Zheng, 2004; Xiao et al., 2005).

In general, cycads evolved under limiting local conditions and many populations are critically small, as described by Silva, Donaldson, Reeves, and Hedderson (2012) for *Encephalartos latifrons* (Lehmann). The high levels of genetic diversity within *E. latifrons*, despite the weak genetic structure, suggest that remaining subpopulations are remnants of the original panmictic population with high levels of gene flow (Silva et al., 2012). Such patterns may be extended to other cycad species that had originally small species distribution (Silva et al., 2012). Indeed, genetic studies may clarify many demographic questions in Zamiaceae by explaining the relative role of pollen and seed dispersal in keeping the genetic integrity and cohesion of *Zamia* populations, and how such species cope with demographic oscillations such as founder effects, bottlenecks, and genetic drift. Investigating these subjects inform decision makers about which populations are likely to be most important for conservation initiatives, and which management procedures are most appropriate for keeping its evolutionary potential (Frankham, 2010; González-Astorga, Vovides, Cruz-Angon, Octavio-Aguilar, & Iglesias, 2005; González-Astorga, Vovides, Ferrer, & Iglesias, 2003; Cabrera-Toledo et al., 2010). Genetic techniques are important conservation tools when associated with other biological, ecological, and biogeographical information (Miyaki & Alves, 2006). Recently, the number of phylogenetic analyses with Zamiaceae has increased (Gutiérrez-Ortega, Kajita, & Molina-Freaner, 2014; Gutiérrez-Ortega, Jimenez-Cedillo, et al., 2018a; Gutiérrez-Ortega, Yamamoto, et al., 2018b), offering an unprecedented amount of molecular data. Such phylogenetic inferences will deepen our understanding about the evolution of niche conservatism, morphological traits associated with specific reproductive strategies, and speciation rates, as observed in other plant groups (Cardoso-Gustavson et al., 2018; Vasconcelos et al., 2019). Since cycads represent an old lineage of seed plants, new evidence on diversification mechanisms obtained by using multiple approaches would provide a solid framework for the comprehension of plant evolution and speciation pathways and also contribute to the conservation of this

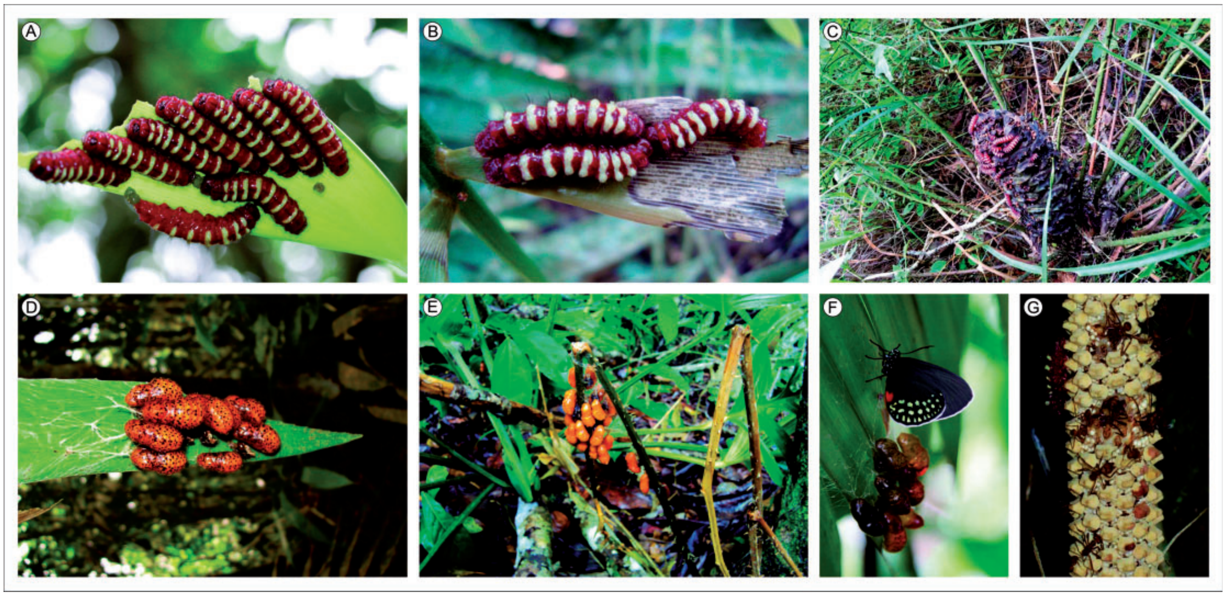

Figure 5. Ecological interactions with Zamiaceae: (a) Larvae of *Eumaeus* sp. in *Z. huilensis*; (b) Larvae of *Eumaeus* sp. in *Z. incognita*; (c) Larvae of *Eumaeus* sp. in *C. mirandae*; (d) Pupae of *Eumaeus* sp. in *Z. incognita*; (e) Pupae of *Eumaeus* sp. in *Z. manicata*; (f) *Eumaeus* sp. adult in *Z. encephalartoides*; and (g) *Atta* sp. (Formicidae), larvae of *Eumaeus* sp. and *Pharaxonotha* sp. in pollen strobili of *Z. incognita*. Photo credits: (a) and (f)—Machael Calonje; (b), (d), and (g)—Arturo Aristizabal; (c)—Chip Jones; and (e)—Cristina Lopez-Gallego. Source: Calonje, Stevenson, et al. (2019). License: CC BY-NC-SA 4.0.

charismatic but threatened plant group (Gutiérrez-Ortega, Yamamoto, et al., 2018b).

Challenges and Perspectives, With Emphasis on *Zamia* From South America

Not only *Zamia* species tend to be rare in their habitats (low abundance and restricted geographic distribution) compared with other species of tropical plants (Lopez-Gallego, 2015), but they are also mainly endemic, with populations growing mostly in remote areas with restricted access (Calonje, Meerow, et al., 2019; Stevenson, 1993). Although most of these distribution areas are considered biodiversity hot spots, many populations of *Zamia* occur outside protected areas (Donaldson, 2003; Mankga & Yessoufou, 2017; Skelley & Segalla, 2019). Shifts in land use, overexploitation of plant populations as ornamentals, reduction or loss of pollinators, and other interactors due to insecticides and herbicides are some of the threats faced by populations (González, 2004; Lopez-Gallego, 2015; Taylor et al., 2012). To preserve the multiple aspects of biodiversity hot spots, a biogeographic approach, associated with the current state of species conservation, population dynamics, and ecology (Lopez-Gallego, 2015; Mankga & Yessoufou, 2017) is necessary. However, implementation of such studies still represents a challenge for South American cycads (Schutzman, 2004).

Cross-pollination, mandatory in dioecious species (Canuto, Alves-Ferreira, & Côrtes, 2014), benefits plant populations in many ways, mainly by increasing genetic diversity (Nybom, Weising, & Rotter, 2014). However, *Zamia* populations may be negatively affected considering their dioecious reproductive system, suffering both biotic (presence of pollinators and dispersers) and abiotic pressures (related to habitat loss and fragmentation), which may prevent gene flow between populations (Barrett, 2010; Donaldson, 2003; Laidlaw & Forster, 2012; Liu et al., 2015). Generally, *Zamia* species that are critically threatened (Table 1), with less than 250 adult individuals, are found in small isolated fragments (Stevenson, Vovides, & Chemnick, 2003). This is particularly problematic because (a) all species are dioecious, (b) isolated plants rarely reproduce, and (c) pollination depend on specialized vectors, with reproductive success determining plant populations persistence, increasing the chances of extinction when in reproductive disadvantage (Donaldson et al., 2003; Mora, Yáñez-Espinosa, Flores, & Nava-Zárate, 2013; Stevenson et al., 2003). Studies suggest that environmental differences as a result of anthropogenic disturbance in forest habitats of *Z. fairchildiana* can significantly affect the life history of subpopulations, particularly their growth rate and allocation to fecundity, and the availability of mates for a female in a given reproductive season (Lopez-Gallego & O'Neil, 2010). Calonje et al. (2011) emphasize the importance of understanding the reproductive biology of cycads and propagation techniques, storage and viability of pollen, manual pollination, seed storage, and germination, in order to increase the availability of these rare plants and reduce the demand for wild-collected plants in areas where they are economically and culturally relevant.

Despite the current political and environmental scenarios of many countries where *Zamia* species occur in South America, conservation actions and strategies must be integrated into a larger action plan across borders, involving the countries and subregions to facilitate better outcomes for conservation, not only of their cycads, but also the Neotropical flora and interactions associated with them. Except for Colombia, which has recently developed a plan of action for the conservation of cycads, the remaining species of *Zamia* in South American countries lack conservation plans in their territories. Countries such as Colombia and Brazil suffer from constant agrarian and economic conflicts, causing irreversible modifications of habitats of many species of cycads (Lopez-Gallego, 2015; Segalla & Calonje, 2019; Skelley & Segalla, 2019). Initiatives such as the National Program of Cycads of Mexico are recommended in South America. This project proposes priorities such as ex situ and in situ conservation, sustainable management, ethnobotanical education, and law enforcement for conservation of the Zamiaceae species. A special subcommittee has been established and the cycads have been listed, among other threatened flora and fauna, as a national conservation priority (Lillo et al., 2000). These policies and actions are very important for small endemic populations and can help in the establishment of sanctuaries or protected areas with a high number of adult individuals and less disturbed habitats (González-Astorga et al., 2005). Cycad Specialists in the New World, especially in South America, have the challenge of implementing conservation actions to protect the species of cycads in a scenario marked by a large geographical extension of their territories (Calonje, Meerow, et al., 2019), sociocultural and economical conflicts, difficulties in accessing remote populations, associated with a lack of government incentives and funding for research. More efforts should be made to preserve South American populations and equally their interactions with other organisms (Lopez-Gallego, 2015; Walters et al., 2004; Mankga & Yessoufou, 2017).

Concluding Remarks

This review has shown that the number of studies involving species in the family Zamiaceae has increased over the decades, especially within the genera *Encephalartos* (Africa), *Lepidozamia* and *Macrozamia* (Australia), *Dioon* (Honduras and Mexico), and *Zamia* (Isthmus region, Central and South America). However, despite the importance of these species to biodiversity and the maintenance of ecological interactions (Franz & Skelley, 2008; Segalla & Calonje, 2019; Skelley & Segalla, 2019; Tang, Xu, et al., 2018; Valencia-Montoya et al., 2017), their intrinsic value to science and society and the continuing decline of especially Lepidoptera, Hymenoptera, and Coleoptera species (Sánchez-Bayo & Wyckhuys, 2019), we lack the basic knowledge for many cycad species, especially when considering the genus *Zamia* in South America. As an attempt to direct future research with *Zamia* species on the South American continent, we list the topics that we consider most important, in order to contribute not only to the acquisition of basic information, but also to applied fields such as restoration and conservation of cycads in this region.

1. Species distribution—records from floristic studies, mapping the current distribution of species, and modeling species occurrence taking into consideration environmental and anthropic factors, current climate data and future scenarios. These models can be useful to understand how environmental conditions influence the occurrence and abundance of *Zamia* species and may predict and indicate environmental suitability for conservation actions. Support and recommendations for studies of this nature are found at: Rodríguez, Brotons, Bustamante, and Seoane (2007); Pearson (2007); Feeley and Silman (2010); Guisan et al. (2013); Velazco, Galvão, Villalobos, and Marco Júnior (2017); and Gomes et al. (2018). Other studies applied for cycads conservation plans are found at: Lillo et al. (2000); Vovides, Pérez-Farrera, and Iglesias (2010); Lopez-Gallego, Calonje, and Idárraga-Piedrahíta (2011); Lopez-Gallego (2015); and Mistry, Schmidt, Eloy, and Bilbao (2018) Vite, Pulido & Vázquez (2013).
2. Population monitoring—to better understand population dynamics, including the potential short- and long-term effects of habitat modifications on the life history and spatial distribution, genetic variation, sex ratio, phenology, and the relationship with abiotic factors. The studies with *Macrozamia riedlei* (Gaudich.) C.A. Gardner (Ornduff, 1985, 1991); *Zamia skinneri* Warsz. ex A. Dietr. (Clark & Clark, 1987); *Encephalartos transvenosus* Stapf & Burtt Davy (Grobbelaar, Meyer, & Burchmore, 1989); *Ceratozamia matudai* (Pérez-Farrera & Vovides, 2004); *C. mirandae* Vovides, Pérez-Farr. & Iglesias (Pérez-Farrera et al., 2006); *Macrozamia macdonnellii* (F.Muell. ex miq.) A.DC, Preece, Duguid, and Albrecht (2007); *Z. fairchildiana* (LopezGallego & O'Neil, 2010, 2014); *Z. fairchildiana* (Lopez-Gallego, 2013); *Dioon edule* (Mora et al., 2013); *Z. furfuracea* (Octavio-Aguilar, Iglesias-Andreu,

Cáceres-González, & Galván-Hernández, 2017); and with *Z. portoricensis* Urb. (Lazcano-Lara & Ackerman, 2018) are good models to follow for future studies related to these topics and offer useful insights for conservation strategies.

3. Reproductive ecology—detailed studies of pollination mechanisms, maturation of reproductive structures, seed germination ecology, seedling recruitment, and the relationship between strobilus temperature and attractiveness to pollinators. Studies involving cone odor, thermogenesis, and cycad pollinators as for *Encephalartos* (Suinyuy, Donaldson, & Johnson, 2013a, 2013b, 2015) and *Macrozamia* of the Old World (Terry et al., 2004; Terry, Roemer, Walter, & Booth, 2014; Terry, Roemer, Booth, Moore, & Walter, 2016) and *Zamia* of the New World (Valencia-Montoya et al., 2017) are recommended for cycads of South America. Further studies on chemical signal perception and cognition of the beetle pollinators, together with efforts to resolve the detailed phylogeny of cycads and their associated pollinators, will improve our understanding of cycad-insect mutualism (Suinyuy et al., 2015).
4. Investigation of ecological interactions of different species, including the levels of specialization of each interacting organism, as well as the impact on the fitness of individuals, with consequences for future generations. Conservation plans need to ensure the continued existence of key interactions with root symbionts, pollinators, and dispersal agents, but it is equally important to conserve cycads because they have a key function in the life histories of other organisms (Walters et al., 2004). Once these processes are uncovered, resulting data will probably have a significant impact on *taxa* conservation.
5. The use of genetic markers to estimate levels of gene exchange based on pollen and seed dispersal in order to access the role of pollinators and frugivores in keeping genetic diversity; detecting demographic processes such as founder events and bottlenecks, and estimate the importance of evolutionary forces such as drift, gene flow, and selection in shaping the current patterns of genetic structure and diversity observed in natural populations, as recommend by Gutiérrez-Ortega, Jimenez-Cedillo, et al. (2018a) and Gutiérrez-Ortega, Yamamoto, et al., (2018b).
6. The establishment of ex situ collections and monitoring of species reproduction and germination/propagation after disturbances, both natural and human induced, as indicated by Vovides et al. (2010); Calonje et al. (2011); Vovides et al. (2013); Griffith et al. (2015); and Griffith et al. (2017) for *Zamia* species. The implementation of in situ and ex situ collections and germplasm banks linked to botanical gardens are useful to promote the value of tropical cycads and may stimulate scientific and educational research aimed at the conservation of the group.

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Supplemental material

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CHAPTER 2

A gente só encanta quando se encanta. Se eu não estiver encantado com o meu objeto de conhecimento, eu não posso encantar o outro. No sentido não de fetiche, mas de sedução gnosiológico.
(Mário Sergio Cortella)

Photo credits: Rosane Segalla, personal collection.

Phenology of a *Zamia* from a seasonal biological Hotspot in South America

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Background and Aims: The dioecy condition, common to all cycads, requires an obligatory outcrossing in which the absence of potential mates of the opposite sex should render an individual sterile. This process makes reproductive synchronization essential for the reproductive success of the species in this group. Phenological studies of dioecious species from diverse phylogenetic lineages have been encouraged for future analysis. Our aim was to describe the phenophases and lifespan of strobili of *Zamia boliviana* (Brongn.) A. DC. (Cycadales, Zamiaceae) and their reproductive phenology in Central South America, Brazil. We aimed to investigate the potential variation between microsporangiate and megasporangiate individuals and their populations.

Methods: The description of the lifespan of polleniferous and ovuliferous strobili was based on observations, records and systematic measurements of these organs' development. The phenological study covered three reproductive cohorts and two populations of cycad.

Key results: The lifespan of polleniferous strobili comprises four phases and ~ 50 days for the cycle's completion, while ovuliferous strobili go through seven phases and take ~ 330 days until seed dehiscence. Both sexes produced strobili from June to October. We identified a seasonally synchronous pattern in the reproductive phenology this cycad, with major overlap in the phases of emergence, pollen release and receptivity of strobili, equally compatible between sexes, populations and subsequent years.

Conclusions: The reproductive events of *Z. boliviana* follow the seasonality of the Cerrado. Synchronization between the period of production of strobili and activity peaks was found for both sexes, and seed dehiscence in dry season, but with temporal distinction in the development of ovuliferous strobili from the phase of receptivity onward, in relation to that of polleniferous strobili.

Keywords: Circular statistics, Cycadales, Dioecy, Phenograms, Reproductive timing, *Zamia* lifespan

INTRODUCTION

Phenology is an integrative environmental science that has achieved a prominent position in current global-change research (Morellato et al., 2016). Admittedly, it is a science capable to monitor, understand and predict the timing of recurrent biological events related to climate and its effects on different species of plants and animals (Morellato et al., 2016), such as in climate change (IPCC 2019) and cumulative forest-loss scenarios (Qin et al., 2019). Indeed, the climate change is considered to have a significant effect on the phenological cycles and synchrony of plants, which could ultimately affect the diversity and abundance of plant species (Gordo and Sanz 2010; Ovaskainen et al., 2013).

Studies examining the phenology of dioecious species with different pollination modes in different places of the world and from diverse phylogenetic lineages have been encouraged so that general patterns can be analyzed in the future (Munguia-Rosas et al., 2011). These descriptions are relevant for understanding the influence of sexual selection on the evolution of sexual dimorphism or sex allocation and sexual selection theories in plants (Munguia-Rosas et al., 2011; Escobedo-Sarti and Mondragón 2016). Generally, the variation in the reproductive success of plants is affected by ecological conditions (Dorken and Pannell 2008), number of reproductive adults, pollen availability, seed dispersal and proximity of potential mates (Octavio-Aguilar et al., 2017a; Lazcano-Lara and Ackerman 2018), as well as by differential reproductive investment, particularly in long-lived species (Field et al., 2012). Sexually dimorphic plants usually exhibit differences in competitive ability between genders in reproductive investment, with some individuals investing a lot in reproduction (generally females), and others, much less (generally males) (Obeso 2002; Zhang et al., 2014).

Cycads have prolonged sexual maturation periods and asynchronous reproductive activity within a single or multiple reproduction cohorts (Norstog and Nicholls 1997). Indeed, female cycads invest more in reproduction than males and may not have the resources to generate strobili every year (Clark and Clark 1987; Tang 1990; Calonje et al., 2011). As a result, it is expected that male individuals exhibit earlier and longer strobili emergence periods as well as greater number of strobili, compared to female individuals (Grobbelaar 2002). This differential behavior may presumably cause phenological differences between sexes (Martínez-Domínguez et al., 2018). In addition to these differential characteristics, the dioecy condition, common to all cycads, requires an obligatory outcrossing mechanism, in which the absence of potential mates of the opposite sex should render an individual sterile (Jones, 2002; Käfer et al., 2016). This process makes reproductive synchronization essential for the reproductive success of the species in this group (Heilbuth 2000; Clugston et al., 2016; Martínez-Domínguez et al., 2018). Asynchronous phenological patterns can cause unsuccessful reproductions in small and isolated cycad populations, negatively impacting the fitness of these populations and threatening their survival (Schneider et al., 2002; Lopez-Gallego, 2007; Reed et al., 2012; Laidlaw and Forster 2012; Okubamichael et al., 2016; Clugston et al., 2016; Velasco-García et al., 2016; Octavio-Aguilar et al., 2017ab; Segalla et al., 2019).

Despite the importance and application of the knowledge of changes in the life cycle of organisms (e.g., phenology), plant reproduction triggers in highly seasonal ecosystems (Morellato et al., 2013), such as the Cerrado (Brazilian savanna), remain poorly understood. In these regions, the phenological examination included few species (Mendoza et al., 2017), and it is even more scarce for cycads, which remain largely under-researched in their natural habitat. These plants coexist with the scenario of extensive and frequent habitat loss in tropical ecosystems (Qin et al., 2019). Most of these habitats are considered unique shelters for endemic dioecious species, such as those of the genus *Zamia* (Lopez-Gallego 2015; Segalla et al., 2019). The limited knowledge of the biological patterns and ecological processes that sustain their populations (Krieg et al., 2017) make studies on the phenology of cycads strategically and biologically significant and urgent in South American *Zamia* populations (Clugston et al., 2016; Martínez-Domínguez et al., 2018; Segalla et al., 2019). Phenological knowledge can open a world of possibilities asking new questions and represents an expansion of the possibilities of answers to evolutionary questions, it is relevant also, for strategies of conservation and management of native ecosystems (Gorelick and Marler 2012; Morellato et al., 2016). Describing and analyzing the phenology of tropical plants and detecting triggers demands considering the circular nature of recurrent life-cycle events requer the use of appropriate statistical metrics (Morellato et al., 2010; Staggemeier et al., 2019). Among these, circular statistics stands out, being highly recommended for the investigation and better interpretation of phenological patterns (Morellato et al., 2010).

Here, we investigate the phenology *Zamia boliviana* (Brongn.) A. DC. (Cycadales, Zamiaceae), a cycad species native to Cerrado habitats of Bolivia and Brazil. Our objective was to describe and compare the reproductive phenology of *Z. boliviana* in two populations to describe the phenophases of the reproductive system's lifespan and answer the following question: 1) Does the reproductive phenology of *Z. boliviana* (time, duration, synchrony) vary between the microsporangiate (male) and megasporangiate (female) genders within and between populations? We predict that the phenological pattern will be synchronous between the male and female sexes and their populations, as a strategy for the reproductive success of dioecious species, which are intrinsically dependent on synchrony, but with male individuals exhibiting a higher interannual frequency of production of strobili, in addition to earlier and more disjunctive emergence compared to megasporangiated individuals. We also expect that the events will be seasonal, annual to interannual in the microporangiated individuals and interannual to seasonal in the megasporangiated individuals, with the seed maturation period lasting until the next season (cohort) due to the characteristics of the lifespan and the differential costs between sexes, as predicted for dioecious and long-lived species (Delph 1999; Zhang et al., 2014; Labouche and Pannell 2016) and *Zamia* spp. (Jones 2002; Tang 1990; Clugston et al., 2016).

MATERIAL AND METHODS

Study region

The cycad *Z. boliviana* can be found within the intertropical zone in the central portion of South America (Fig.1), characterized by high total solar radiation incidence throughout the year (SEPLAN 2000) and equatorial and tropical hot climates, with little seasonal and annual temperature variation (Köppen 1918; Kottek et al., 2006). Mean annual temperatures range from 22 ºC to 26 °C, low temperatures from 18 ºC to 23 ºC, and high temperatures from 25 ºC to 30 ºC (based on data from a GIS analysis using bioclimatic variables from CHELSA – Karger et al., 2016). The region has two distinct seasons: a dry season (May to September), with average rainfall of 42.3 mm, and a wet season (October to April), with average rainfall of 186 mm (Karger et al., 2016). This cycad is restricted to an area within the border between Bolivia (Beni, Cochabamba, La Paz, and Santa Cruz) and Brazil, in the state of Mato Grosso (MT) (Fig. 1), with an elevation range from 130 m to 450 m above sea level, and different types of Cerrado (Brazilian savanna) (Stevenson 2004; Jørgensen et al., 2014; Skelley and Segalla 2019; Segalla and Calonje 2019).

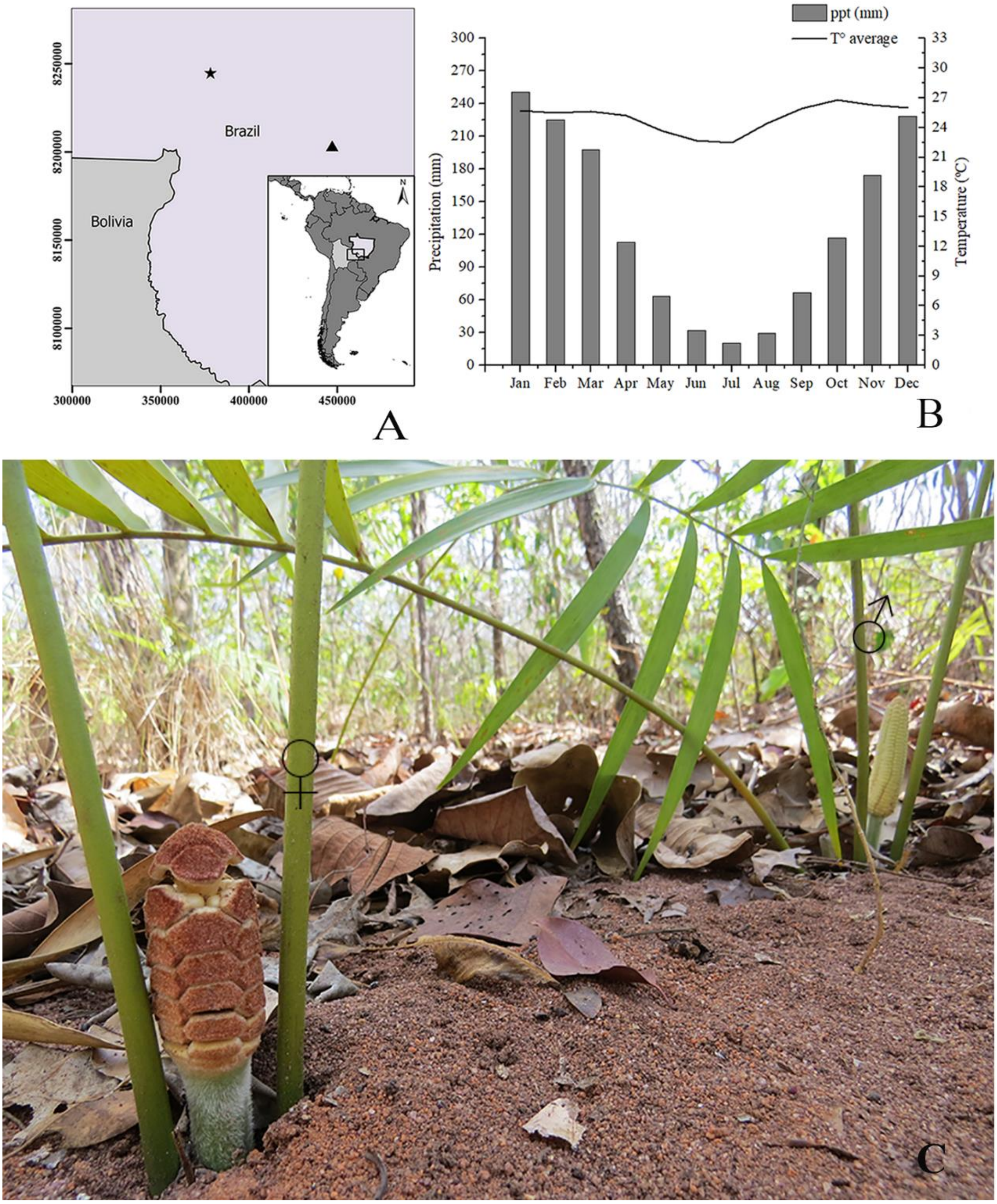

Figure 1. *Zamia boliviana* in its region of occurrence. A. Central Portion of South America, State of Mato Grosso, Brazil. The star indicates the population studied in Glória do Oeste and the triangle indicates the population of the municipality of Cáceres. B. Climatic diagram (mean rainfall and temperature) of the region of occurrence of the studied populations, from 1979 to 2013 (Source: Bioclimatic variables from CHELSA – Karger et al., 2016). C. Female and male plants in habitat. Note: Specific geographical coordinates of the areas of populations are omitted from this chapter to minimize the risk of illegal extraction of this species from the wild. Credits: Rosane Segalla, personal collection.

Zamia boliviana is found in sandy to rocky soil, well-drained Oxisols, Entisols, Inceptisols and Ultisols, and also in Litolic soils associated with rocky outcrops, generally characterized as alkaline, with low native fertility but good structure (SEPLAN 2000; Skelley and Segalla 2019). The habitat of the cycad is relatively flat, or with minor changes in topography (SEPLAN 2000; Segalla, R. – pers. obs.). Slopes predominate in the region of Chapada dos Guimarães, in the state of Mato Grosso, with the formation of deep valleys (SEPLAN 2000). *Zamia boliviana* forms clumps of individuals of both sexes, with different densities and successional stages or different reproductive ages (Segalla, R. – pers. obs.). Most of the population of the cycad's areas of occurrence has been destroyed or suffered drastic reductions in the population, mainly due to the intense fragmentation caused by the expansion of agricultural lands and agribusiness activities in both countries (Skelley and Segalla 2019).

Phenological sampling pattern

To investigate the phenological pattern of *Z. boliviana*, we sampled populations from distinct areas of the Cerrado in the region of the Brazil-Bolivia border in the state of Mato Grosso (MT), (Fig.1), specifically in the municipalities of Glória do Oeste (VB – Vale Bonito = 39.605 ha) and Cáceres (VC – Vale do Chapadão = 15.376 ha,), with approximately 80 km of distance between them. Phenological monitoring started in January 2017, after a pilot project carried out from August to December 2016 to better understand the biological system and define the phenophases. The study covered three reproductive cohorts of the cycad: 2017; 2018 and 2019. Sampling was carried out in fixed plots (VB = 1.300 m^2 and VC = 500 m^2), following the haphazardly criteria, in predominantly uniform patches of individuals, and by actively searching for and marking adult plants within the plots. The inclusion criterion of individuals was made according to plant size (≥ to 0.80 m tall) and the number of leaflets per leaf (≥ 12). The sampling included 197 individuals in VB (0.14 ind. m^2), 60 of them microsporangiated and 45 megasporangiated, and 216 individuals in VC (0.43 ind. m^2), 79 of them microsporangiated and 48 megasporangiated. The observations were performed monthly and included the phenophases of the strobili, which are reproductive organs, from their emergence from the soil to the senescence of the polleniferous strobili and seed dehiscence of the ovuliferous strobili. In each plot, we registered the phenological categories for the Zamiaceae species by adapting the methodology of Hall et al. (2004); Clugston et al. (2016) and Martínez-Domínguez et al. (2018). The complete morphological description of the categories is presented in the results. We applied two methods of analysis the data collected, according to criteria defined by Bencke and Morellato (2002): (a) Percentage of Fournier intensity – method proposed by Fournier (1974), where from the values obtained in the field through of a semi-quantitative interval scale of five categories (0 to 4) is calculated the percentage of intensity of each phenophase, ideal to describe phenophases and graphic representation and for calculations and comments (see Fournier 1974, Bencke and Morellato 2002); (b) Activity index (or percentage of individuals) – used to estimate synchrony, indicating the proportion of sampled individuals who are manifesting particular phenological event.

Strobili: morphology, development and lifespan

To describe the morphological development of the lifespan of the polleniferous (n=6) and ovuliferous strobili (n=6), we made observations and measurements of these reproductive organs from their emergence from the soil to the phase preceding pollen release, and daily from the pollen release phase until complete pollen dehiscence or senescence. The ovuliferous strobili were followed weekly from their emergence from the soil until the end of the phase of receptivity or closing of cracks. Then, monthly until seed dehiscence. We consider senescence followed by dehydration and disintegration, and dehydration followed by apical detachment of the seeds, as the final phases of the reproductive events of polleniferous and ovuliferous strobili, respectively. The photographic records of the strobili were treated and organized on boards according to their respective phenophases and the organ growth dynamics was demonstrated in graphs.

Statistical analysis

To estimate the activity and intensity of phenological seasonality in each of the phenophases for both male and female individuals, we used circular statistics to compare the average angle of occurrence of each population and phenophase in subsequent years. Intensity refers to the measure of how intense the phenophase was in the individuals who went through it, while activity refers to the percentage of individuals who went through a certain phenophase, regardless of its intensity. For each of these components, months were converted into angles, with 0º = January, successively up to 330º = December, in 30º intervals, and the following were estimated: (1) the mean angle, (2) the angular standard deviation, (3) the length of vector r, and (4) we also tested the significance and uniformity of each phenophase in each year using the Rayleigh test (z), as well as the (5) significance of the same data for the circular distribution of von Misses using the Watson test (Zar 1999). These two tests were performed using the *rayleigh.test* and *watson.test* functions, respectively, both from the circular package (Agostinelli and Lund 2013). To test whether each phenophase had a similar intensity and activity index in subsequent years, we compared angles using the Watson-Williams (F) test for activity and the Watson-Wheeler test for intensity, according to the premises of circular statistics (Zar 2010). Additionally, we verified whether the reproductive phenophases of both sexes were seasonally similar and determined the angular dispersion between microsporangiated and megasporangiated individuals. We considered only the Pollen release (PR) and Open/receptive (OR) phenophases, as they are critical for the effectiveness of pollination. For this comparison, we used the *wallraff.test* function, which is also available in the circular package (Agostinelli and Lund 2013).

RESULTS

Zamia boliviana is a small perennial plant with average height of 0,80 cm, subterranean stem or xylopodium (perennial thickened woody axis of the underground system), and up to 1 to 3 leaves in each crown (Fig. 1-C). The reproductive structures of this cycad begin developing underground, but most of the development occurs above ground. Female and male individuals were similarly measured, but there may be differences in the number of strobili produced by each gender; female plants produced only one strobilus per xylopodium, while male plants produced 1-5 strobili per xylopodium or branch (apical meristem) of xylopodium. In the studied populations, we found 197 adult individuals in the VB plot, of which only 105 (53.30 %) produced reproductive structures: 60 (57.14 %) were male and 45 (42.86 %) were female. In the VC plot, we registered 216 adult plants, of which 127 (58.79%) produced reproductive structures; 79 (62.20 %) were male, and 48 (37.80 %) were female.

Strobili's biology: Morphology, lifespan and growth dynamics

According to our classification, polleniferous strobili go through four stages of development over their lifespan, while ovuliferous strobili go through eight stages, with morphological and time variations inherent to each reproductive organ (Table 1; Fig. 2AB) and distinctions from the post-receptivity phase onward. Male and female individuals of this cycad underwent a single strobili emergence period, which lasted four months (June-

October). In the VB and VC populations, the soil emergence of polleniferous and ovuliferous strobili began in July, but while the polleniferous strobili completed their cycle less than 60 days after emergence, the ovuliferous strobili took 300-350 days to complete their cycle, with seed dispersal beginning in April and lasting through August (Table 1; Fig. 3AF).

Table 1. Phenological phases across two populations of *Z. boliviana* in Mato Grosso, Brazil.

Strobili phases	**Description**	**Duration in days**
Ovuliferous strobili		
1. Immature emerging – IE	A1. Organ emerges from the ground surrounded by cataphylls	30-35
2. Immature fully emerged – IFE	B1. Organ immature emerging ground B2. Exposure and growth of peduncle B3. Development and maturation of ovules B4. Pre-receptive phase	10-15
3. Open and receptive – OR	C1. Slight fissures open between the megasporophylls (base/apex) C2. Prominent ovules, translucent white coloring C3. Pollination phase	7-15
4. Closed post-receptive – CPR	D1. Slit closure D2. Late ovule – Pollinated ovules develop	15-20
5. Seed in development – MSD	E1. Increased rigidity of the megasporophylls E2. Gradual change in color (white to yellow to orange) E3. Stop growth E4. Increased rigidity of the sclerotesta E5. Maturation of sarcotesta	270-300
6. Seed dehiscent – MD	F1. Dehydration of central axis and disruption of sterile apex F2. Start release of seeds (can last for up to 30 days)	~ 300-330
7. Senescent/dry – MSD	G1. Disintegration of the central axis, persistent peduncle	Indefinite
Polleniferous strobili		
1. Immature emerging – IE	A1. Organ emerges from the ground surrounded by cataphylls	25-30
2. Immature fully emerged – IFE	B1. Peduncle growth B2. Growth of microsporophylls and microsporangia	20-25
3. Pollen release – PR	C1. Microsporangia in maturation (base to the apex) C2. Central axis, condensed, microsporangia visible, but closed C3. Central axis elongates/distension C4. Microsporophylls separate (base to the apex), pre-release pollen C5. Beginning of the opening of the sporangia	2-4
4. Senescent/dry – SD	D1. Dehydration of central axis and organ curvature D2. Conical structure disintegrating	Indefinite

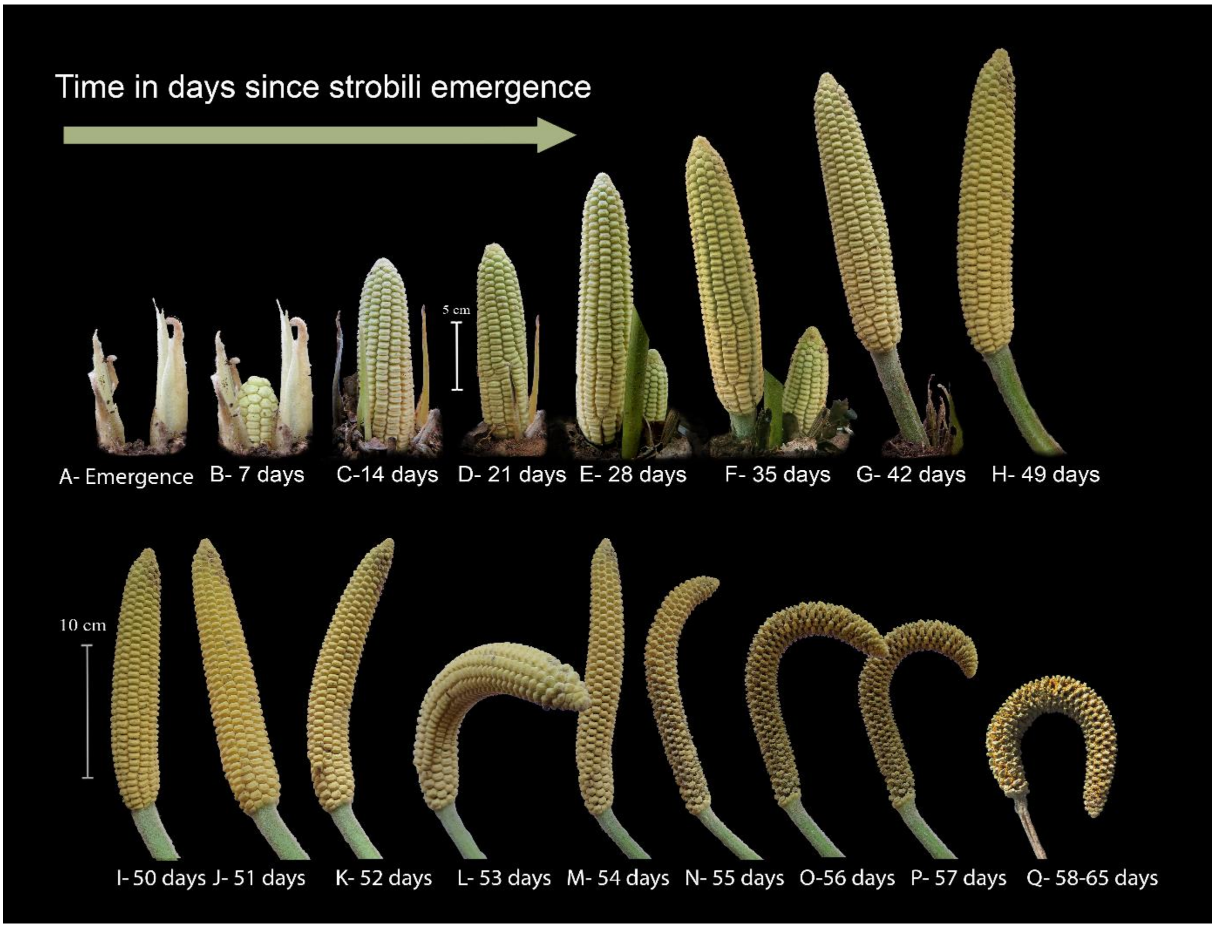

Figure 2A. Biology of *Z. boliviana* strobili. A. Morphology and lifespan of microstrobili. Phases: A-B. Emergence (~ 7 days); C-F. Emergence from the ground (~ 14-35 days); G. Fully emerged/developed (~ 35-40 days); H. Pre-release (~ 40-50 days); J. Distension of sporophylls (~ 50-51 days); K-N. Pollen release (~ 52-55 days); O-P. Post-release (~ 56-57 days) and Q. Dehydration/senescence and disintegration (~ 58-65 days).

On average, the lifespan of polleniferous strobili is 60 days, and that of ovuliferous strobili is 330 days (Fig. 3A-F). Both organs can show differences in the growth dynamics of their structures and, therefore, variations in the sizes of peduncles and in the structure of the strobilus itself. In polleniferous strobili, the process that precedes pollen release begins around the 45th day. After, followed by a short phase of distension of sporophylls (1-2 days), sometimes concomitant with the pollen release phase (~ 2-4 days). Finally, the post-release phase (~ 2 days) and the senescence phase occur in one day or more, depending on climatic conditions and on the activity of the organisms interacting with the organ (Fig. 2A). In ovuliferous strobili, from their emergence from the soil to the receptive phase, the organ requires an average time of 60-70 days of development and may have open cracks (receptive phase) for 7-15 days or a little longer. The post-receptive phase requires about 270-300 days and is followed by the seed dehiscence phase, which starts ~ 330 days after the organ emerges (Fig. 2B) and can last for up to 30 days, depending on the biotic and abiotic factors involved.

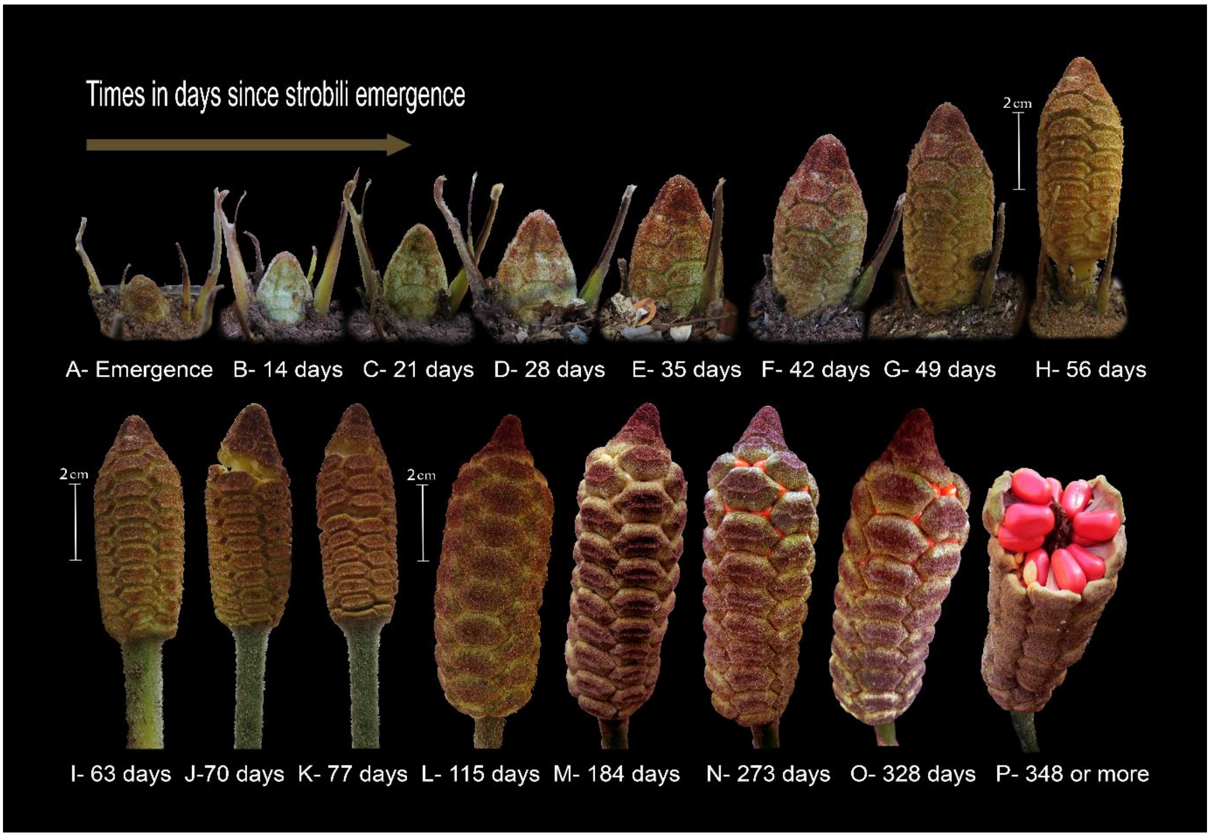

Figure 2B. A-B. Emergence/presence of sterile apex (~ 7-14 days); C-H. Emergence from the ground (~ 15-60 days); I. Fully emerged; Pre-receptive (~ 60-70 days); J-K. Open and receptive (~ 70-80 days); L. Closed post-receptive (~ 80-90 days); M-O. Seed development (~ 90-330 days) and P. Seed dehiscence (~ 330 days or more). Credits: Rosane Segalla, personal collection.

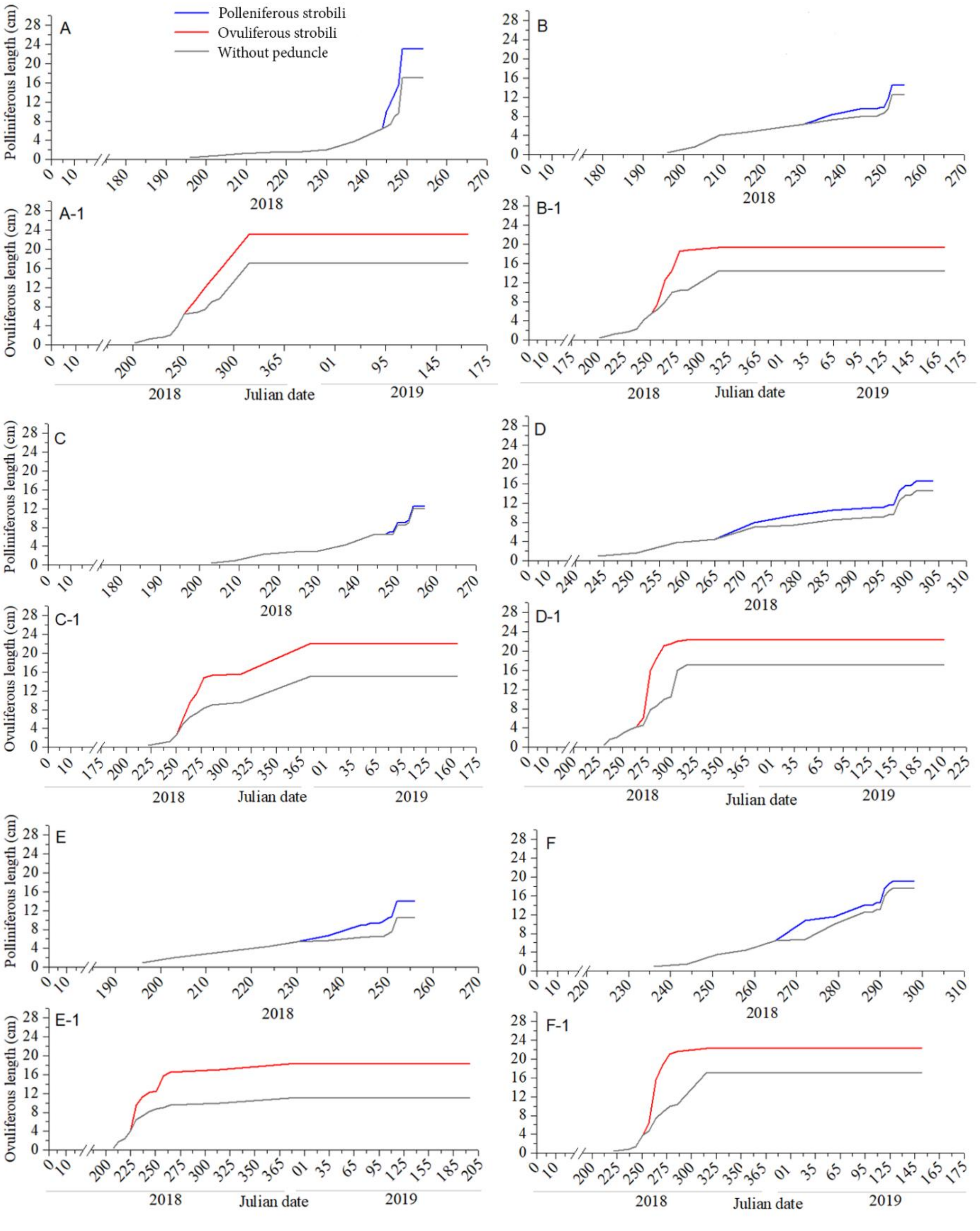

Figure 3. Growth dynamics of *Z. boliviana* strobili. A-F. Growth dynamics of polleniferous strobili; A1-F1. Growth dynamics of ovuliferous strobili.

Frequency of reproductive cycles

Over the 36 months of phenological observations, of the 413 individuals selected for study from both populations, 232 were reproductive individuals. Of the 105 plants sampled in VB, all male plants and 57.77% of the 45 female plants identified reproduced over the three reproductive seasons (2017-2019). Of the 60 male plants, 28.33% reproduced in more than one reproductive cohort, but the female plants produced strobili only once in the period. In VC, of the 216 individuals sampled, 70 (88.60%) of the 79 male plants, reproduced in the period and 34 (70.83%) of the 48 female plants were reproductive during the three years of monitoring. However, while 27.84% of the reproductive male plants reproduced in two or three uninterrupted cohorts, none of the reproductive female plants produced strobili in more than one breeding season. Figure 4AB illustrates the frequency of reproductive cycles of microsporangiate (male) and megasporangiate (female) individuals in the VB and VC populations.

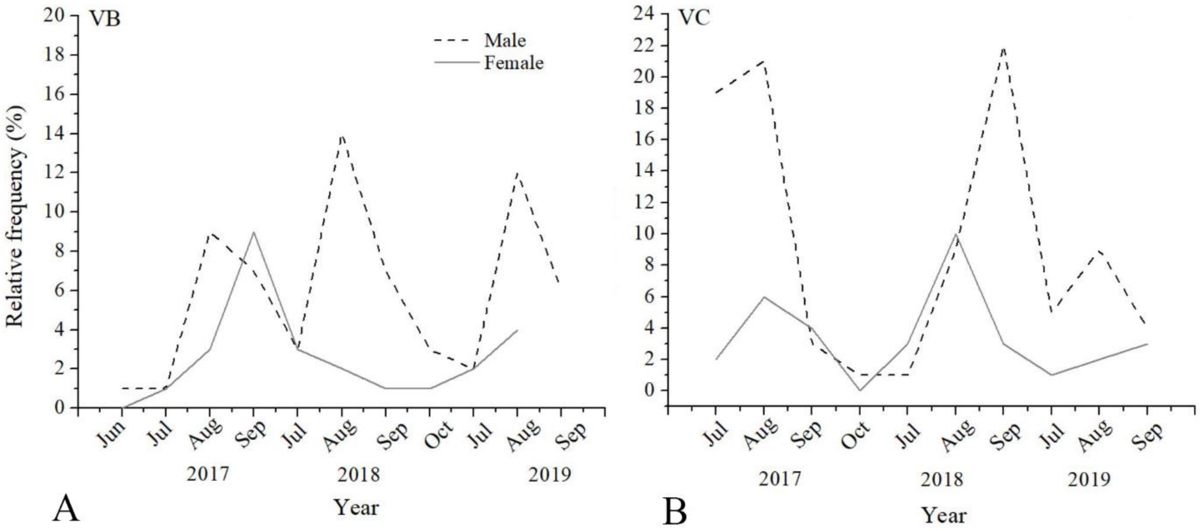

Figure 4. Frequency of reproductive cycles of microsporangiate (male) and megasporangiate (female) of *Zamia boliviana* individuals in two populations (January 2017-December 2019). (A) VB = Vale Bonito, (B) VC = Vale of Chapadão, Glória do Oeste and Cáceres, respectively, State of Mato Grosso, Brazil.

Synchrony of phenological intensity and activity of the populations

In general, our analyses indicated seasonality in the production of strobili within populations and for both sexes, and emergency activity starting in June and lasting until October (Fig. 4AB). Tables 2 and 3 (Supplement Material – SM) show the significance of the Open/receptive (OR) phase in relation to the Pollen release (PR) phase, and synchronism of these events is noted between sexes, based on phenological data obtained over three years of observation for the two populations studied. The peak activity of the OR and PR phases was slightly variable between the years of study and in the two populations studied, with the highest activity rates concentrated between the months of August and October (Table 2 and 3, Figure 5A (A-D)) shows the phenographs of the reproductive phases for both sexes, and illustrates the overlap between phenophases and the similar angular distance for the two populations studied.

When comparing the intensity index found for both male and female individuals in the PR and OR phases over the three years of observation, we noted that the average intensity of the PR phase in male individuals occurred from August to November in the VB population, according to the mean results of Fournier's intensity index: VB: august: 3.00, september = 2.95, october: 2.94, november: 2.00; VC: august = 1.00, september: 2.95, october: 3.07 and november: 2.17. On the other hand, female individuals from both populations showed medium intensity peaks for the OR phenophase over the evaluated period, between September and November, as follows: VB: september = 2.00, october: 2.72, november: 3.83; VC: september: 2.17, october: 3.00 and november: 4.00. In general, while in male individuals the maximum intensity (peak intensity) of the PR phase occurred between August and October in both populations studied, in female individuals, it was concentrated between October and November (Table 5 and 6; Fig. 5A (A-D) and 5B (E-H)).

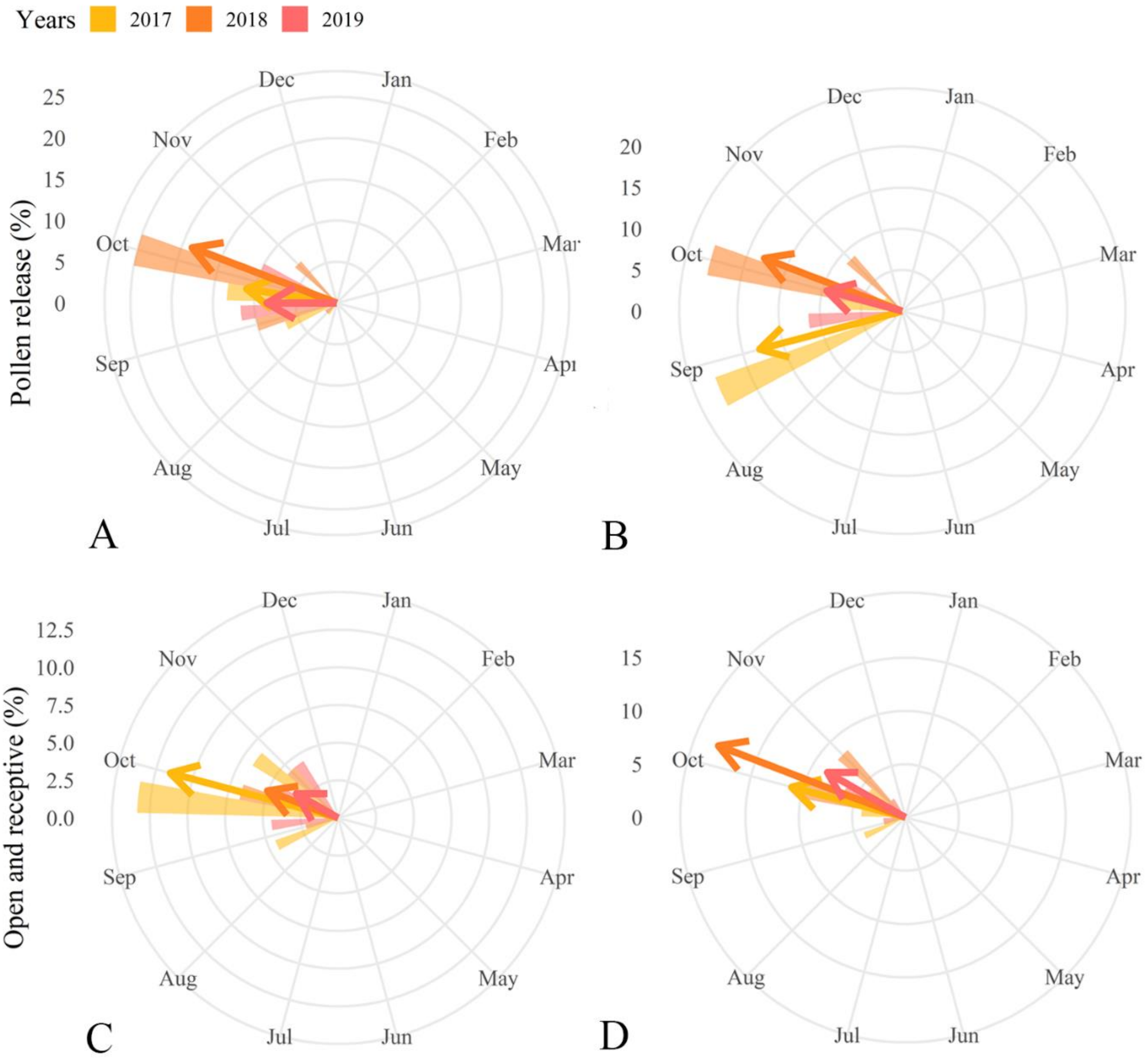

Figure 5A. Phenographs of the reproductive phenology of two *Z. boliviana* populations (VB and VC) in the state of Mato Grosso, Brazil. Polleniferous strobili – Pollen release: A = VB and B = VC; Ovuliferous strobili – Open and receptive: C = VB and D = VC. The arrows indicate the mean angles of phenophases, while the arrow length indicated the r value of phenophase concentration.

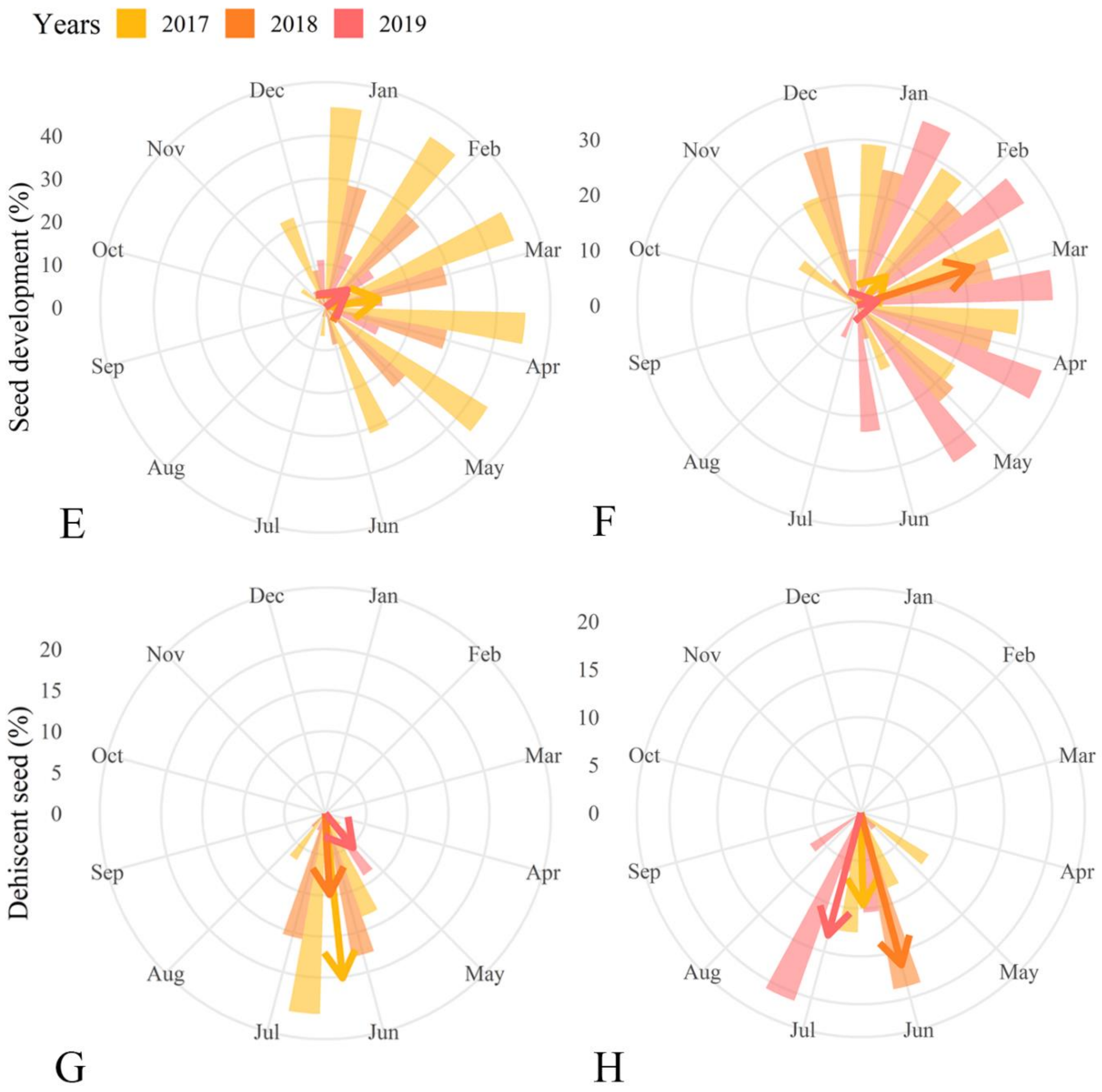

Figure 5B. Ovuliferous strobili – Seed in development: E = VB and F = VC; Seed dehiscent: G = VB and H = VC. The arrows indicate the mean angles of phenophases, while the arrow length indicated the r value of phenophase concentration.

Regarding the phenological activity of the populations, of the 60 and 79 male individuals that produced strobili in the populations, 13.33%, 25.00% and 11.66% of the VB population and 24.05%, 24.05% and 11.39% of the VC population were in the pollen release phase between August and October of 2017, 2018 and 2019, respectively (Table 2 and 3 – SM).

No statistical differences in the intensity index found for the strobili emergence phase were observed when comparing the three years of follow-up in both locations studied, both for the male plants (VB: K=2.35; p=0.67, and VC: K=2.63; p=0.62) and for the female plants (VB: K=1.12; p=0.89, and VC: K=0.78; p=0.94).

On the other hand, the phenological activity index found for the strobili emergence phase showed differences when comparing the different years in one of the locations studied (VC), both for the male plants (VB: F=0.25; df=2, 102; p=0.78, and VC: F=33.99; df= 2, 116; p<0.001) and for the female plants (VB: F=0.49; df= 2, 51; p=0.614 , and VC: F=7.32; df= 2, 65; p<0.001). Therefore, in the studied populations, the time of emergence of strobili varied in the three, but not its intensity. The values in the concentration of vector r were similar for phenophase IE, for both male and female individuals and in all years evaluated (Table 2 and 6 – SM).

In general, we found no statistical differences in the production of strobili between sexes and populations over the years of phenological assessments (Tables 4-6 in SM). We also found no significant differences between the PR and OR phases for phenological intensity and activity in 2017-2019 when applying the Wallraff rank sum test of angular distance to populations VB and VC. Where: Phenological intensity – PR x OR/VB – 2017, 2018, 2019, *p* value = 0.24; 0.24; 0.67 and VC – *p* value = 0.92; 0.89; 0.89, respectively; Phenological activity – PR x OR/VB – 2017, 2018, 2019, *p* value = 0.21; 0.07; 0.17 and VC – *p* value = 0.16; 0.00; 0.27, respectively. Exceptionally in the VC population in 2018, and almost similarly in the VB population, although not statistically significant (p value = 0.07), there was statistical difference between the angular dispersion of the activity of phenophases PR and OR. Table 6 (SM) summarizes the intersexual differences and associated statistics of two populations of *Z. boliviana*. Therefore, the intensity and activity of pollen release of microsporangiated strobili is synchronous with the intensity and activity of receptivity to pollen of megasporangiated strobili, in both populations studied.

DISCUSSION

Our study represents one of the largest series of systematic phenological data for Zamiaceae in South America. Along with other studies related to the phenology and population structure of cycads (e.g.: Clark and Clark 1987; Ornduff, 1987; Clark and Clark 1988; Negrón-Ortiz and Breckon 1989; Grobbelaar et al., 1989; Ornduff 1992; Pérez-Farrera and Vovides 2004; Lopez-Gallego and O'Neil 2010; Griffith et al., 2012; Clugston et al., 2016; Martínez-Domínguez et al., 2018), we clarified the magnitude of the synchrony between male and female plants, and its potential association with specific seasons, especially in highly seasonal ecoregions such as the Cerrado. The understanding of reproductive biology in a phenological context can help conserve ex situ cycads, which play a fundamental role in conservation by keeping genetic reserves of wild populations (Calonje et al., 2011; Terry et al., 2012; Clugston et al., 2016; Segalla et al., 2019). Furthermore, it would guide targeted pollen collection and assisted pollination during said reproductive period (Terry et al., 2012; Calonje et al., 2011; Clugston et al., 2016).

Strobili's biology: morphology, lifespan and growth dynamics

The monitoring of reproductive cohorts and the description of the strobili's phenophases confirmed that the events in the reproductive biology of this cycad follow the Cerrado's seasonality. The emergence of reproductive structures occurs in the dry period of the year, with the typical temporal distinction between the lifespan of polleniferous and ovuliferous strobili. The absence of perfect equivalence in the duration of the PR and OR phases between reproductive individuals in the period reinforces the concept of the different costs of the sexes in dioic systems. This differential characteristic leads to more frequent and abundant reproduction of male plants in comparison to female plants, as reported by Jones (2002). Polleniferous and ovuliferous *Z. boliviana* strobili are dissimilar in terms of morphology, with differences in color and appearance, a characteristic that is frequently found in *Zamia* spp. (Jones 2002). The post-receptive phase (late egg) is markedly longer in ovuliferous strobili (270-300) (Table 1; Fig. 3A-F), and was a characteristic also noted for *Zamia splendens* Schutzman (Haynes 2004) and *Zamia integrifolia* L.f. The latter requires 9-12 months for their seeds to mature (Tang 1990). According to Jones (2002), the life of ovuliferous strobili is much longer than that of polleniferous strobili, and both sexes have a similar period of development until pollination, after which the lifespan of polleniferous strobili ends, whereas ovuliferous strobili continue developing for a few more months. The disparity between the sexes is logical due to female plants producing larger strobili with longer lifespans, the formation of which causes a considerable depletion of their storage reserves, and, as a consequence, the intervals between the formation of strobili are longer in female than in male plants (Tang 1990; Jones 2002).

Differences in the growth dynamics of pollen and ovulate structures, such as variations in the sizes of peduncles and strobili (Fig. 3A-F), seem to be characteristics inherent to cycads., which possibly reflect the micro-conditions of these plants' habitats (e.g., variation in soil fertility, shading). Our results corroborate general descriptions of polleniferous strobili (Jones 2002), such as *Lepidozamia peroffskyana* Regel (Hall et al., 2004) and *M. macleayi* (Roemer et al., 2017). In these species, pollen dehiscence occurs after the elongation of the strobili's axis, followed by the opening of gaps between the sporophylls and exposure of the sporangia to the atmosphere. In *Z. boliviana*, the pollen release phase per strobilus is relatively short, between 2 and 4 days, but the dehiscence period can extend for more than a week, depending on the number of strobili per plant and its growth and maturation dynamics. Whereas the dehiscence of seeds can last for almost 30 days, depending on the size of the ovuliferous strobilus and its consumption by animals. Suinyuy et al. (2009) reported that in *Encephalartos friderici-guilielmi* Lehm, after pollen release, the microstrobilus quickly dried up, but different species of insects were associated with the organ's decomposition. In general, morphological variations in *Zamia* spp. appear to be sustained by and correlated with long-term climatic conditions and biogeography, as suggested by Calonje et al. (2019) for the genus, but also intrinsic to the Zamiaceae family, as discussed in Gutiérrez-Ortega et al. (2017); Vovides et al. (2018) and Martínez-Domínguez et al. (2018).

Frequency of reproductive cycles

The low reproductive frequency, mainly of female plants, over the three reproductive seasons in the populations is consistent with the dynamics of the cycle's frequency for other Zamiaceae species. Notably, in long-lived dioecious populations such as cycads, it is common for females to produce only one strobilus per season per stem, although some species can produce more than one strobilus per season per stem, especially male plants (Grobbelaar 2002). Generally, polleniferous strobili are produced more frequently and in greater abundance than in females, because they drain less of the plant's reserves (Tang 1990; Jones 2002). This characteristic is particularly important to address a range of unanswered questions in investigations about the correlation of ecological and life history with sexual dimorphism (Barrett and Hough 2013), as plants of ancestral lineages are still present in the contemporary world.

Synchrony of phenological intensity and activity of the populations

We identified a similar seasonally synchronous pattern in the reproductive phenology of *Z. boliviana*, supported by the application of the activity index and of Fournier's percentage of intensity, combined with statistical tests and graphical representation of the data. The analyses revealed an evident overlap in the period of the strobili's activities of emergency, pollen release and receptivity, equally compatible between sexes, populations and subsequent years (Table 2-5 (see SM)). Despite the tests showing differences regarding the angular dispersion of PR and OR activity in the VC population in 2018, the pattern is quite similar even if not significant, and is probably not a reason for reproductive failures in balanced populations. This slight difference may be associated with the variation in the time of receptivity of female individuals, as the process of opening and closing of the cracks is slower in megasporangiated individuals compared to dehiscence time of microsporangiated individuals.

In general, the synchronic phenological pattern observed for *Z. boliviana* is in line with what has been described for other cycad species, at least in the tropical region. Martínez-Domínguez et al. (2018) found a similar pattern for *Ceratozamia tenuis* (Dyer) D.W.Stev. and Vovides, endemic to Mexico, with congruence between the ontogenesis of both sexes and slightly disjunctive pollen production, preceding or extending beyond the peak of ovuliferous plants. In addition, in male plants, when more than one strobilus is produced per stem in a given season, they emerge either simultaneously or in succession, and therefore, pollen shedding may take place over a protracted period (Grobbelaar 2002), which can to contribute to slight differences in the plants' intensity and activity indexes. Therefore, this appears to be a recurring pattern among Zamiaceae species. Although the emergence of strobili occurred over four months, there was a peak of activity in the PR and OR phenophases, confirming that the reproductive process occurs synchronously, as expected, and as has been reported for most of the other *Zamia* species (Clugston et al., 2016; Martínez-Domínguez et al., 2018). In dioecious populations, male individuals tend to slightly anticipate and postpone their reproductive events (Forero-Montaña and Zimmerman 2010). This is possibly a strategy to ensure greater success in the pollination of female plants according to discussed by Escobedo-Sarti and Mondragón (2016) on study of *Catopsis compacta* Mez (Bromeliaceae). As long as pollinators are present, even with low perfect synchronies, it is still possible that successful pollination will be achieved, as highlighted by Escobedo-Sarti and Mondragón (2016). The shorter flowering period of females could reduce competition between them – if fewer female flowers emerged simultaneously, most of them would be more likely to be visited by pollinators (Escobedo-Sarti and Mondragón 2016).

Our study provides relevant and expected biological data for populations in equilibrium and showed the common temporal distinction between the development of polleniferous and ovuliferous strobili. The reproductive events of *Z. boliviana* follow the Cerrado's seasonality, with sex-to-sex synchronization of strobili production, peak intensity, activity and dehiscence of seeds coinciding with the dry season. The reproductive success of this cycad undoubtedly depends not only on reproductive synchronization, but on other requirements of the set of multiple biotic and abiotic factors (e. g.: population contingency, number of reproductive adults, pollen availability, effective pollination, seed dispersal) (Pérez-Farrera and Vovides 2004; Mora et al., 2013; Velasco-García et al., 2016; Octavio-Aguilar et al., 2017a). When deficient, all or any of these functional attributes can compromise the health of populations and lead to severe threats to cycads, species that are already among the most threatened globally (Okubamichael et al., 2016; Mankga and Yessoufou 2017). The data available so far suggest that endemic Cerrado lineages, such as that of *Z. boliviana*, emerged from the late Miocene onward, derived from numerous ancestral lineages native to the surrounding ecoregions (Simon and Pennington 2012), being therefore adapted to a highly seasonal system. This hypothesis corroborates Calonje et al. (2019), who studied the history of the *Zamia* genus in the New World and suggest that most extant *Zamia* species appear to have originated during the Pleistocene, indicating an effect of the climatic oscillations of this period on the genus' diversification. Jones

(2002) argues that perhaps the most adapted of the cycads are to be found in the xeric habitats where rainfall is low, extremely seasonal and often irregular, long periods of hot climate are common, and burning may take place annually. Such habitats include grassland and savanna (Brazilian Cerrado), where *Z. boliviana* can be found, and which can be positive for this species' survival. The acquisition and integration of phenological, genealogical, taxonomic and ecological information about cycads is important for science, as well as for conservationist approaches, as argued by Gutiérrez-Ortega et al. (2018), Vovides et al. (2018) and Martínez-Domínguez et al. (2018). Whether the phenological performance observed in *Z. boliviana* is long-lasting, adapting to changes and new environments and surviving drastic environmental changes, is debatable.

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SUPPLEMENTARY MATERIAL

Table 2. Intersex comparisons and statistics associated with phenological activity of the VB and VC population of *Z. boliviana* in 2017-2019 by phenophases.

Strobili	**Year**	**Sitio**	**Phenophases**	**Mean (a)**	**sd**	**vector length (*r*)**	**Rayleigh test**	**Rayleigh *p*-value**	**Watson test**	**Watson *p*-value**	**Watson-Williams test (*F*-value)**	**df**	**Watson Wheeler *p*-value**
Polleniferous	2017	VB	Immature.emerging	-141.03	21.42	0.93	0.9325	<0.0001	0.4403	<0.01	-	-	-
	2018	VB		-140.18	24.21	0.91	0.9146	<0.0001	0.7743	<0.01	-	-	-
	2019	VB		-143.61	17.75	0.95	0.9531	<0.0001	0.7546	<0.01	0.2475	2;102	0.7812
	2017	VB	Immature.fully.emerging	-124.83	21.23	0.93	0.9335	<0.0001	0.3653	<0.01	-	-	-
	2018	VB		-116.11	18.72	0.94	0.948	<0.0001	0.8295	<0.01	-	-	-
	2019	VB		-120	12.17	0.97	0.9777	<0.0001	0.5822	<0.01	0.91	2;49	0.4092
	2017	VB	Pollen.release	-99.76	19.23	0.94	0.9452	<0.0001	0.4992	<0.01	-	-	-
	2018	VB		-94.16	20.23	0.94	0.9395	<0.0001	0.8303	<0.01	-	-	-
	2019	VB		-107.63	17.35	0.95	0.9552	<0.0001	0.4958	<0.01	3.4786	2;82	0.03548
	2017	VB	Senescent.dry	-69.99	22.51	0.92	0.9257	<0.0001	0.478	<0.01	-	-	-
	2018	VB		-63.15	20.97	0.93	0.9352	<0.0001	0.7992	<0.01	-	-	-
	2019	VB		-80.65	15.83	0.96	0.9625	<0.0001	0.9079	<0.01	7.2156	2;103	0.00116
Ovuliferous	2017	VB	Immature.emerging	-130.24	18.21	0.95	0.9507	<0.0001	0.9453	<0.01	-	-	-
	2018	VB		-130.37	22.49	0.92	0.9258	<0.0001	0.1908	<0.01	-	-	-
	2019	VB		-136.66	21.93	0,93	0.9293	<0.0001	0.2789	<0.01	0.4917	2;51	0.6144
	2017	VB	Immature.fully.emerged	-106.66	21.93	0.92	0.9293	<0.0001	0.2789	<0.01	-	-	-
	2018	VB		-110.11	14.19	0.97	0.9698	0.0001	0.2102	<0.01	-	-	-
	2019	VB		-107.37	24.15	0.91	0.9149	<0.0001	0.2208	<0.01	0.0514	2;27	0.9499
	2017	VB	Open.and.receptive	-87.36	19.67	0.94	0.9428	<0.0001	0.4123	<0.01	-	-	-
	2018	VB		-84.89	20.71	0.93	0.9367	<0.0001	0.1825	<0.01	-	-	-
	2019	VB		-90	24.78	0.91	0.9107	<0.0001	0.1366	<0.01	0.1611	2;44	0.8517
	2017	VB	Close.post.receptive	-60	20.07	0.94	0.9405	<0.0001	0.4588	<0.01	-	-	-
	2018	VB		-54.89	20.71	0.94	0.9367	<0.0001	0.1825	<0.01	-	-	-
	2019	VB		-60	22.86	0.92	0.9234	<0.0001	0.1622	<0.01	0.2625	2;50	0.7702

Continuation …

Strobili	**Year**	**Sitio**	**Phenophases**	**Mean (a)**	**sd**	**vector length (*r*)**	**Rayleigh test**	**Rayleigh *p*-value**	**Watson test**	**Watson *p*-value**	**Watson-Williams test (*F*-value)**	**df**	**Watson Wheeler *p*-value**
	2017	VB	Seeds.in.development	-62.52	61.45	0.56	0.5626	<0.0001	0.797	<0.01	-	-	-
Ovuliferous	2018	VB		58.02	53.69	0.64	0.6446	<0.0001	0.5148	<0.01	-	-	-
	2019	VB		34.25	54.22	0.63	0.639	<0.0001	0.2354	<0.01	6.842	2;519	0.0011
	2017	VB	Dehiscent.seed	172.81	22.03	0.93	0.9285	<0.0001	0.7134	<0.01	-	-	-
	2018	VB		161.85	22.39	0.93	0.9265	<0.0001	0.5341	<0.01	-	-	-
	2019	VB		134.28	22.95	0.92	0.9229	<0.0001	0.3367	<0.01	13.955	2;92	<0.0001
	2017	VB	Senescent.dry	-157.19	22.06	0.93	0.9285	<0.0001	0.7134	<0.01	-	-	-
	2018	VB		-160.86	21.87	0.93	0.93	<0.0001	0.4835	<0.01	-	-	-
	2019	VB		164.28	22.5	0.92	0.9229	<0.0001	0.3367	<0.01	14.227	2;81	<0.0001
Polleniferous	2017	VC	Immature.emerging	-160.72	19.88	0.94	0.9416	<0.0001	1.0105	<0.01	-	-	-
	2018	VC		-129.26	15.41	0.96	0.9644	<0.0001	1.4603	<0.01	-	-	-
	2019	VC		-151.33	20.81	0.94	0.9362	<0.0001	0.3491	<0.01	33.998	2;116	<0.0001
	2017	VC	Immature.fully.emerging	-132.58	17.19	0.955	0.956	<0.0001	1.1022	<0.01	-	-	-
	2018	VC		-107.02	17.59	0.954	0.9542	<0.0001	0.4662	<0.01	-	-	-
	2019	VC		-133.61	15.02	0.966	0.9662	<0.0001	0.3259	<0.01	17.337	2;77	<0.0001
	2017	VC	Pollen.release	-110.43	17.48	0.95	0.9545	<0.0001	1.1738	<0.01	-	-	-
	2018	VC		-83.72	14.31	0.96	0.9693	<0.0001	1.2107	<0.01	-	-	-
	2019	VC		-110.5	17.05	0.95	0.9567	<0.0001	0.4551	<0.01	27.114	2;82	<0.0001
	2017	VC	Senescent.dry	-87.05	18.37	0.95	0.9499	<0.0001	1.7518	<0.01	-	-	-
	2018	VC		-57.16	19.84	0.94	0.9418	<0.0001	0.8768	<0.01	-	-	-
	2019	VC		-83.14	17.97	0.95	0.952	<0.0001	0.4834	<0.01	31.08	2;115	<0.0001
Ovuliferous	2017	VC	Immature.emerging	-144.89	20.72	0.93	0.9367	<0.0001	0.3651	<0.01	-	-	-
	2018	VC		-123.16	22.67	0.92	0.9247	<0.0001	0.6268	<0.01	-	-	-
	2019	VC		-131.93	14.77	0.97	0.9673	<0.0001	0.3131	<0.01	7.3298	2;65	0.00013
	2017	VC	Immature.fully.emerged	-114.89	20.71	0.93	0.9367	<0.0001	0.3651	<0.1	-	-	-

Continuation …

Strobili	**Year**	**Sitio**	**Phenophases**	**Mean (a)**	**sd**	**vector length (*r*)**	**Rayleigh test**	**Rayleigh *p*-value**	**Watson test**	**Watson *p*-value**	**Watson-Williams test (*F*-value)**	**df**	**Watson Wheeler *p*-value**
Ovuliferous	2018	VC	Immature.fully.emerged	-103.18	19.65	0.94	0.9429	<0.0001	0.5663	<0.1	-	-	-
	2019	VC		-105	15.08	0.96	0.9659	<0.0001	0.1167	<0.1	1.983	2;47	0.149
	2017	VC	open.and.receptive	-90	24.78	0.91	0.9107	<0.0001	0.1366	<0.05	-	-	-
	2018	VC		-80.86	19.27	0.94	0.945	<0.0001	0.3611	<0.01	-	-	-
	2019	VC		-90	19.01	0.94	0.9464	<0.0001	0.2008	<0.01	0.9407	2;39	0.399
	2017	VC	close.post.receptive	-70.37	22.49	0.925	0.9258	<0.0001	0.3816	<0.01	-	-	-
	2018	VC		-58	17.2	0.955	0.9559	<0.0001	0.7888	<0.01	-	-	-
	2019	VC		-60	19.01	0.946	0.9464	<0.0001	0.2008	<0.01	2.6749	2;61	0.07699
	2017	VC	seeds.in.development	45	61.5	0.56	0.5621	<0.0001	0.449	<0.01	-	-	-
	2018	VC		40.87	60.12	0.57	0.5766	<0.0001	0.5363	<0.01	-	-	-
	2019	VC		67,14	57,91	0,6	0,6	<0,0001	0,6389	<0,01	10,405	2;551	<0,0001
	2017	VC	dehiscent.seed	154,51	25,29	0,91	0,9071	<0,0001	0,3629	<0,01	-	-	-
	2018	VC		154,58	15,97	0,96	0,9619	<0,0001	0,789	<0,01	-	-	-
	2019	VC		173,88	23,01	0,92	0,9225	<0,0001	0,5634	<0,01	8,4757	2;89	0,00004
	2017	VC	senescent.dry	-172,48	25,29	0,91	0,9071	<0,0001	0,3629	<0,01	-	-	-
	2018	VC		-172,63	13	0,97	0,9746	<0,0001	1,0122	<0,01	-	-	-
	2019	VC		-153,16	22,67	0,92	0,9247	<0,0001	0,6268	<0,01	9,8216	2;83	0,00014

Table 3. Intersex comparisons and statistics associated with phenological intensity of the VB and VC population of *Z. boliviana* in 2017-2019 by phenophases.

Strobili	**Year**	**Sitio**	**Phenophase**	**Mean (a)**	**sd**	**Vector length (*r*)**	**Rayleigh test**	**Rayleigh *p*-value**	**Watson test**	**Watson *p*-value**	**Watson Wheeler test**	**Watson Wheeler *p*-value**
Polleniferous	2017	VB	Immature.emerging	-165	34.23	0.83	0.8365	0.0504	0.0287	>0.1	-	-
	2018	VB		-135	34.23	0.83	0.8365	0.0504	0.0287	>0.1	-	-
	2019	VB		-150	24.78	0.91	0.9107	0.0725	0.0341	>0.1	2.3492	0.6718
	2017	VB	Immature.fully.emerging	-135	34.23	0.83	0.8365	0.0013	0.0573	>0.1	-	-
	2018	VB		-105	34.23	0.83	0.8365	0.0013	0.0573	>0.1	-	-
	2019	VB		-115.48	25.29	0.91	0.9071	<0.0001	0.0907	>0.1	0.9275	0.9206
	2017	VB	Pollen.release	-106.63	35.52	0.82	0.8251	<0.0001	0.0881	>0.1	-	-
	2018	VB		-106.63	35.52	0.82	0.8251	<0.0001	0.0881	>0.1	-	-
	2019	VB		-120	26.36	0.89	0.8995	<0.0001	0.105	>0.05	1.7529	0.7811
	2017	VB	Senescent.dry	-60	45.15	0.73	0.7331	<0.0001	0.1163	<0.05	-	-
	2018	VB		-75	36.31	0.81	0.818	<0.0001	0.1138	<0.05	-	-
	2019	VB		-90	24.78	0.91	0.9107	<0.0001	0.1366	<0.05	2.2206	0.6953
Ovuliferous	2017	VB	Immature.emerging	-139,62	22,49	0,92	0,9258	0,0015	0,0954	>0,1	-	-
	2018	VB		-120	22,86	0,92	0,9234	<0,0001	0,083	>0,1	-	-
	2019	VB		-143,79	22,61	0,92	0,925	0,0059	0,0649	>0,1	1,1207	0,891
	2017	VB	Immature.fully.emerged	-115,48	25,29	0,91	0,9071	<0,0001	0,0907	>0,1	-	-
	2018	VB		-101,93	14,77	0,96	0,9673	0,0026	0,1565	<0,05	-	-
	2019	VB		-116,09	23,64	0,92	0,9184	<0,0001	0,0949	>0,1	4,5743	0,3338
	2017	VB	Open.and.receptive	-82.08	25.14	0.91	0.9082	<0.0001	0.1258	<0.05	-	-
	2018	VB		-82.08	25.14	0.91	0.9082	<0.0001	0.1258	<0.05	-	-
	2019	VB		-86.09	23.64	0.92	0.9184	<0.0001	0.0949	>0.1	1.62	0.8052
	2017	VB	Close.post.receptive	-64.52	25.29	0.91	0.9071	<0.0001	0.0907	>0.1	-	-
	2018	VB		-49.62	22.49	0.93	0.9258	0.0015	0.0954	>0.1	-	-
	2019	VB		-49.62	22.49	0.93	0.9258	0.0015	0.0954	>0.1	1.8147	0.7698
	2017	VB	Seeds.in.development	93.76	89.79	0.29	0.2929	0.107	0.512	>0.1	-	-

Continuation ...

Strobili	**Year**	**Sitio**	**Phenophase**	**Mean (a)**	**sd**	**Vector length (*r*)**	**Rayleigh test**	**Rayleigh *p*-value**	**Watson test**	**Watson *p*-value**	**Watson Wheeler test**	**Watson Wheeler *p*-value**
Ovuliferous	2018	VB	Seeds.in.development	83.79	83.21	0.34	0.3483	0.0599	0.393	>0.1	-	-
	2019	VB		75	73.51	0.43	0.4391	0.0128	0.0638	>0.1	1.0612	0.9004
	2017	VB	Dehiscent.seed	176.93	32.07	0.85	0.855	<0.0001	0.0723	>0.1	-	-
	2018	VB		175.45	33.98	0.84	0.8387	<0.0001	0.0958	>0.05	-	-
	2019	VB		160.37	22.49	0.92	0.9258	0.0015	0.0954	>0.1	1.7606	0.7797
	2017	VB	Senescent.dry	-165	34.23	0.83	0.8365	<0.0001	0.1147	>0.05	-	-
	2018	VB		-165	34.23	0.83	0.8365	<0.0001	0.1147	>0.05	-	-
	2019	VB		-180	24.78	0.91	0.9107	<0.0001	0.1366	<0.05	1.6681	0.7965
Polleniferous	2017	VC	Immature.emerging	-118.71	32.4	0.85	0.8522	0.0024	0.075	>0.1	-	-
	2018	VC		-142.09	25.15	0.9	0.9082	0.0246	0.0629	>0.1	-	-
	2019	VC		-150	24.78	0.91	0.9107	0.0725	0.0341	>0.1	2.6331	0.621
	2017	VC	Immature.fully.emerging	-120	24.78	0.91	0.9107	0.0022	0.0683	>0.1	-	-
	2018	VC		-120	24.78	0.91	0.9107	0.0022	0.0683	>0.1	-	-
	2019	VC		-135	15.08	0.96	0.9659	0.0119	0.1167	>0.05	1.7239	0.7864
	2017	VC	Pollen.release	-86.08	23.64	0.91	0.9184	<0.0001	0.0949	>0.1	-	-
	2018	VC		-90	24.78	0.91	0.9107	<0.0002	0.1024	>0.1	-	-
	2019	VC		-120	24.78	0.91	0.9107	<0.0003	0.1024	>0.1	5.0221	0.285
	2017	VC	Senescent.dry	-75	34.23	0.83	0.8365	<0.0001	0.1147	>0.05	-	-
	2018	VC		-45	34.23	0.83	0.8365	<0.0001	0.1147	>0.05	-	-
	2019	VC		-90	24.78	0.91	0.9107	<0.0001	0.1366	>0.05	3.9004	0.4197
Ovuliferous	2017	VC	Immature.emerging	-143.79	22.61	0.92	0.925	0.0059	0.0649	>0.1	-	-
	2018	VC		-119.11	30.27	0.86	0.8697	<0.0001	0.0976	>0.05	-	-
	2019	VC		-135	15.08	0.96	0.9659	0.0119	0.1167	>0.05	0.7802	0.9411
	2017	VC	Immature.fully.emerged	-116.09	23.64	0.91	0.9184	<0.0001	0.0949	>0.1	-	-
	2018	VC		-120	24.78	0.91	0.9107	<0.0001	0.1024	>0.05	-	-

Continuation ...

Strobili	**Year**	**Sitio**	**Phenophase**	**Mean (a)**	**sd**	**Vector length (*r*)**	**Rayleigh test**	**Rayleigh *p*-value**	**Watson test**	**Watson *p*-value**	**Watson Wheeler test**	**Watson Wheeler *p*-value**
Ovuliferous	2019	VC	Immature.fully.emerged	-102.81	14.92	0.96	0.9666	<0.0001	0.2119	>0.1	2.7851	0.5944
	2017	VC	Open.and.receptive	-83.04	23.78	0.91	0.9174	<0.0001	0.12	>0.05	-	-
	2018	VC		-83.04	23.78	0.91	0.9174	<0.0001	0.12	>0.05	-	-
	2019	VC		-83.04	23.78	0.91	0.9174	<0.0001	0.12	>0.05	2.0761	0.7218
	2017	VC	Close.post.receptive	-53.04	23.78	0.917	0.9174	<0.0001	0.12	>0.05	-	-
	2018	VC		-56.84	25.23	0.907	0.9076	<0.0001	0.1366	>0.05	-	-
	2019	VC		-60	24.78	0.91	0.9107	<0.0001	0.1125	>0.05	2.4271	0.6577
	2017	VC	Seeds.in.development	70.21	70.38	0.47	0.4702	0.0165	0.0521	>0.1	-	-
	2018	VC		75	73.51	0.43	0.4391	0.0128	0.0638	>0.1	-	-
	2019	VC		101.09	75.92	0.41	0.4156	0.0119	0.0566	>0.1	0.6811	0.9536
	2017	VC	Dehiscent.seed	160.37	22.49	0.92	0.9258	0.0015	0.0954	>0.1	-	-
	2018	VC		150	26.36	0.89	0.8995	<0.0001	0.105	>0.05	-	-
	2019	VC		-175.37	29.87	0.87	0.8729	<0.0001	0.1064	>0.05	4.9574	0.2917
	2017	VC	Senescent.dry	-180	24.78	0.91	0.9107	<0.0001	0.1366	>0.05	-	-
	2018	VC		-165	15.08	0.96	0.9659	<0.0001	0.2335	<0.01	-	-
	2019	VC		-165	34.23	0.83	0.8365	<0.0001	0.1147	>0.05	2.6717	0.6142

Table 4. Intersexual reference values[1] for intensity and phenological activity in the period 2017-2019 for the Pollen Release and Open/Receptive phases in the VB population of *Z. boliviana*.

Phenological intensity								Phenological activity		
		Pollen Release			Open/Receptive			Open/Receptive		
Month	Angle	2017	2018	2019	2017	2018	2019	2017	2018	2019
Jan	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Feb	30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mar	60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Apr	90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
May	120	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jun	150	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	180	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aug	210	3.00	3.00	3.00	0.00	0.00	0.00	0.00	0.00	0.00
Sep	240	3.00	3.00	2.85	2.00	2.00	2.00	4.44	2.22	4.44
Oct	270	2.62	2.86	3.33	2.83	2.33	3.00	13.33	6.66	4.44
Nov	300	3.00	3.00	0.00	4.00	4.00	3.50	6.66	4.44	4.44
Dec	330	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[1] Reference values for intensity can vary from 0 to 4 and activity from 0 to 100%.

Table 5. Intersexual reference values[1] for intensity and phenological activity in the period 2017-2019 for the Pollen Release and Open/Receptive phases in the VC population of *Z. boliviana*.

Phenological intensity								Phenological activity		
		Pollen Release			Open/Receptive			Open/Receptive		
Month	Angle	2017	2018	2019	2017	2018	2019	2017	2018	2019
Jan	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Feb	30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mar	60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Apr	90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
May	120	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jun	150	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	180	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aug	210	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sep	240	2.84	3.00	3.00	2.5	2.0	2.0	4.16	2.08	2.08
Oct	270	3.16	3.05	3.00	3.0	3.0	3.0	4.16	10.41	6.25
Nov	300	3.50	3.00	3.00	4.0	4.0	4.0	4.16	8.33	2.08
Dec	330	0.00	0.00	0.00	0.0	0.0	0.0	0.00	0.00	0.00

[1] Reference values for intensity can vary from 0 to 4 and activity from 0 to 100%.

Table 6. Statistical summary of the intersex comparisons and Wallraff's angular distance tests associated with phenological intensity and activity for the Pollen Release (PR) and Open/Receptive (OR) phases of the VB and VC populations of *Z. boliviana* in 2017-2019.

Phenological intensity – PR x OR					Phenological activity – PR x OR		
	Year	Kruskal-Wallis chi-squared (χ)	df	p-value	Kruskal-Wallis chi-squared (χ)	df	p-value
VB	2017	1.36	1	0.24	1.55	1	0.21
	2018	1.36	1	0.24	3.10	1	0.07
	2019	0.18	1	0.67	1.81	1	0.17
VC	2017	0.00	1	0.92	1.96	1	0.16
	2018	0.01	1	0.89	10.63	1	0.00
	2019	0.01	1	0.89	1.20	1	0.27

CHAPTER 3

> We too would like to see sex used for its oldest purpose, which we assert is self sex as a conservative variation-reducing form of rejuvenescence.
> Too often, we forget that self sex can be for much more than recreation.
> (Gorelick, R., and Carpinone, J. (2009). Origin and maintenance of sex: the evolutionary joys of self sex. Biological Journal of the Linnean Society, 98(4), 707–728.)

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Male biased sex ratio across populations of *Zamia boliviana* (Zamiaceae)

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Background and Aims: Populations of dioecious plants commonly exhibit dissimilarities in sex ratios. Differential expenditure for reproduction between genders is cited as the primary mechanism responsible for the occurrence of male bias sex-ratio, with enhanced effects on long-lived species, but these mechanisms are still poor understood. We explore the sex ratio in the endemic gymnosperm *Zamia boliviana* (Zamiaceae) populations from Brazilian savanna (Cerrado). We aim to investigate what is the *Z. boliviana* sex ratio, and whether population density and ecological correlates lead to variation in the sex proportion along Cerrado habitats.

Methods: The study was conducted with ten *in situ* populations of *Z. boliviana*, in which we estimated the populations' sex ratio and performed a redundancy analysis to assess the relationship between biotic populations' traits, such as sex ratio, and associated environmental features. Soil texture classes were used to classify the cycad's habitats and were expressed in a ternary phase diagram.

Key Results: The results show a male-biased sex ratio in most populations surveyed. A possible explanation is the differential energy expenditure of sexes in reproduction. Environmental factors did not explain the redundancy in the reproductive characteristics. The cycad can occur in different habitats in their endemism zone.

Conclusions: Our study provides indications that reproductive expenditure of sexes are the most plausible factor, in tertiary sex ratio assessments, governing the mechanisms of variation compared to secondary factors. However, long-term data supported by improved methods may clarify the strength of each factor driving biased sex ratios in older lineages of tropical systems.

Keywords: Environmental factors, Reproduction cost, Resource allocation, Sex bias, Sexual dimorphism

INTRODUCTION

Dioecy is characterized by the presence of male and female reproductive structures in different individuals (Bawa and Beach 1981). Although relatively uncommon, this reproductive system occurs predominantly in gymnosperms and approximately 6% of angiosperms (Renner and Ricklefs 1995). The dioic system is mostly found on isolated clades and rarely associated with species-rich clades (Renner 2014; Käfer et al., 2016). Although almost half of all families and the majority of orders contain dioecious species, dioecy is rare within these higher taxa, as shown from its phylogenetic distribution, and mostly occurs in long-lived species (Käfer et al., 2016; Barrett et al., 2010). The obligate outcrossing mechanism imposed by dioecy means the absence of potential mates of the opposite sex renders a effectively sterile individual (Käfer et al., 2016). Dioecy is commonly associated with a suite of life historys and reproductive traits (Barrett et al., 2010). The dioecious system is there for complex, because it involves sexual specialization, incurring genetic, demographic, and ecological costs (Renner 2014).

Populations of dioecious species show a nearly continuous variation in sex ratios from a strong male to strong female bias, especially in habitats affected by abiotic stresses such as poor soils or low humidity (reviewed in Barrett et al., 2010). The variations are usually related to specialization and competitive ability in resource allocation between genders. This is an expected result of trade-offs in allocation, associated with sex-differential reproductive costs (Freeman et al. 1997; Zhang et al., 2014). Investigation of environmental influences on the sex ratio commonly reports it as a result of variation in site quality or local resources (e.g., climate, soil moisture, and altitude), as well as male and female proximity (Freeman et al., 1997; Stehlik et al., 2008). Particularly for cycads, Krieg et al. (2017) found several patterns of physiological and morphological differences between sexes, but their relationship to ecological factors needs to be investigated further.

In the tropics, the unisexual system is strongly correlated to local environments (Matallana et al., 2005). Studies in tropical habitats show that the frequency of dioecy reaches 35% of the dominant woody species (Matallana et al., 2005). Indeed, tropical ecosystems are unique environments for many endemic dioecious species such as those of the genus *Zamia* (Lopez-Gallego 2015; Segalla et al., 2019). Particularly in Cerrado, woodland areas show a percentage of dioecy which was consistently higher than those of their sympatric Cerrado areas (Oliveira 1996). Tropical dioecious species present a biological pattern of general characteristics found in that dioic system, such as sex ratio variation, sex-based differentiation in life history traits and differential allocation of resources between genders (see Bullock 1982; Nicotra 1998; Morellato 2004; Amorim et al. 2011; Leal et al. 2013; Rangel and Silva 2013; Riba-Hernández et al. 2014; Zorzanelli et al. 2015).

Cycads are dioecious plants, with 356 taxa found around the world (Calonje et al., 2020). They typically take long to reach sexual maturity and exhibit asynchronous reproductive activity within single or multiple reproduction cohorts (Norstog and Nicholls 1997). Deviations from the expected 1:1 sex ratio were reported for species of the families Cycadaceae and Zamiaceae, tending to be male-biased in most cases (Grobbelaar 2002). At the biological level, the male bias in Zamiaceae can possibly be caused by the following factors or a combination thereof, according to Calonje et al. (2011) and the *Z. boliviana* phenology study (Segalla, R. – unpublished data): 1) male individuals emerge and develop strobili earlier than females; 2) male individuals have longer periods of emergence and development of strobili than females; 3) male individuals produce strobili more regularly than females; 4) male individuals can produce more strobili per reproduction cohort than females; 5) microstrobili have a shorter lifespan (~ two months) than megastrobili (~ ten months or more). Although the cumulative sex ratio approached equality in the few cases where populations were monitored long term, it is thought that the results may vary widely in wild populations (Calonje et al., 2011). Very little, however, is known about conditions in habitat.

In a general sense, the term "sex ratio" can be defined as the number of males to females in a given group of organisms (Calonje et al., 2011). However, the sex ratio of a group can change over time and can be formally split into three categories: primary sex ratio, secondary sex ratio, and tertiary sex ratio (Majerus 2003; Calonje et al. 2011). Primary sex ratio is the ratio of males to females after fertilization, secondary sex ratio is the ratio at birth, and tertiary sex ratio is the ratio at sexual maturity (Majerus 2003; Calonje et al. 2011). The sex ratio or sexual rate equilibrium can indicate that there is no competitive advantage for males, despite a lower reproductive cost (Octavio-Aguilar et al., 2017). It is recognized that, if each year, males and females produced strobili with equal frequency and, the larger the seedling group in a population, the smaller the chances become of it consisting exclusively of either males or females (Grobbelaar 2002). Therefore, chances increase that the recruitment of this population will contain both sexes (Grobbelaar 2002). These proportions are generally assumed for healthy populations (Ackleh and Zhang 2009; Barrett 2015). However, most cycads occur in nature as small scattered populations where their continued existence is in grave danger, due largely to the anthropic activity (Grobbelaar, 2002, Segalla et al., 2019). Thus, apart from deterministic biological factors, other forces such as ecological and stochastic factors and founding effects (Pérez-Farrera et al., 2017; Mankga and Yessoufou 2017), subject populations to sex ratio variations. The knowledge of the sex ratio of dioecious plants is useful to inform about the health of their populations.

Here we investigate the sex ratio of the cycad *Zamia boliviana* (Brongn.) A. DC. (Cycadales, Zamiaceae) (Fig. 1), a species occurring in Cerrado vegetation (Fig. 2). We were interested in determining the sex ratios of these cycad populations *in situ* and how this ratio varies in different Cerrado habitats (Fig. 2). Thus, we tested the hypothesis that the sex ratio in *Z. boliviana* may be affected by environmental conditions found in different populations (light incidence and soil properties). Our study addressed the following specific questions: (1) What is the sex ratio across different populations of *Z. boliviana*? (2) What are the predominant characteristics of *Z. boliviana* habitats regarding soil physical properties and light intensity? (3) Can physical or chemical soil variables and light levels in habitats affect the sex ratio of *Z. boliviana*?

MATERIAL AND METHODS

Study species

Zamia boliviana is a small perennial plant (up to 0.80 m tall), with a subterranean stem (xylopodium) and up to 1 to 3 leaves in each crown (Fig. 2). According to phenology records (Segalla, R. – pers. obs.), the vegetative and reproductive structures are formed in the soil during the dry season. At times the plants can become deciduous or brevideciduous for an undetermined period. Male plants can develop from one to five microstrobili per stem or branch (apical meristem) of xylopodium, whereas female plants produce only one megastrobilus per stem or branch (Fig. 2B-C-F-G-H). Starting from soil emergence, the lifespan of the microstrobili lasts more or less two months while megastrobili can last up to ten months (Segalla, R. 'Chapter 2', unpublished data). We have adopted the name "xylopodium" in this study to denote the perennial thickened woody axis of the underground system of *Z. boliviana* (Fig. 2F-G-H). This xylopodium has a complex structure and includes the base of the hypocotyl, the root–stem transition region and the proximal portion of the main root, as described for different species from the Cerrado (Hayashi and Appezzato-da-Glória 2007). A fire event was recorded in DES area, in November 2016. This happened within the reproductive period of the cycad and seeds and emerging or newly emerged strobili were burned. This prevented us from identifying the sex of many individuals during our sampling in 2017 and was exacerbated by the fact that megastrobili are not necessarily produced annually.

Study region

Populations of *Z. boliviana* are distributed within the intertropical zone in the central portion of South America (Fig. 1) (Segalla et al., 2019). It is characterized by high total solar radiation incidence throughout the year (SEPLAN 2000) and equatorial and tropical hot climates, with little seasonal and annual temperature variation (Köppen 1918; Geiger 1954; Kottek et al., 2006; Deblauwe et al., 2016). The region has two distinct seasons: a dry season (May to September), with an average rainfall of 42.3 mm, and a wet season (October to April), with an average rainfall of 186 mm.Mean annual temperatures range from 22 ºC to 26 °C, low temperatures from 18 ºC to 23 ºC and high temperatures from 25 ºC to 30 ºC (Karger et al., 2016).

This cycad is restricted to an area between Bolivia (Beni, Cochabamba, La Paz, and Santa Cruz) and Brazil, in the state of Mato Grosso, within an elevation range of 130 m to 450 m above sea level, and different types of Cerrado (Brazilian savanna) (Table 1, Fig. 1) (Segalla et al., 2019). Areas where the cycad occurs are relatively flat or exhibit minor changes in topography (SEPLAN 2000). Slopes predominate in the region of Chapada dos Guimarães, in the State of Mato Grosso, with the formation of deep valleys (SEPLAN 2000). *Z. boliviana* frequently forms dense clumps of individuals of both sexes in different successional stages or different reproductive ages (Segalla, R. – pers. obs.). Most of the populations have been decimated or severely fragmented by the clearing of land for agriculture in both countries (Table 1, Fig. 1) and (Segalla et al., 2019). During this study, a fire event occurred in the region containing the DES population one year before sex ratio evaluation (Table 2).

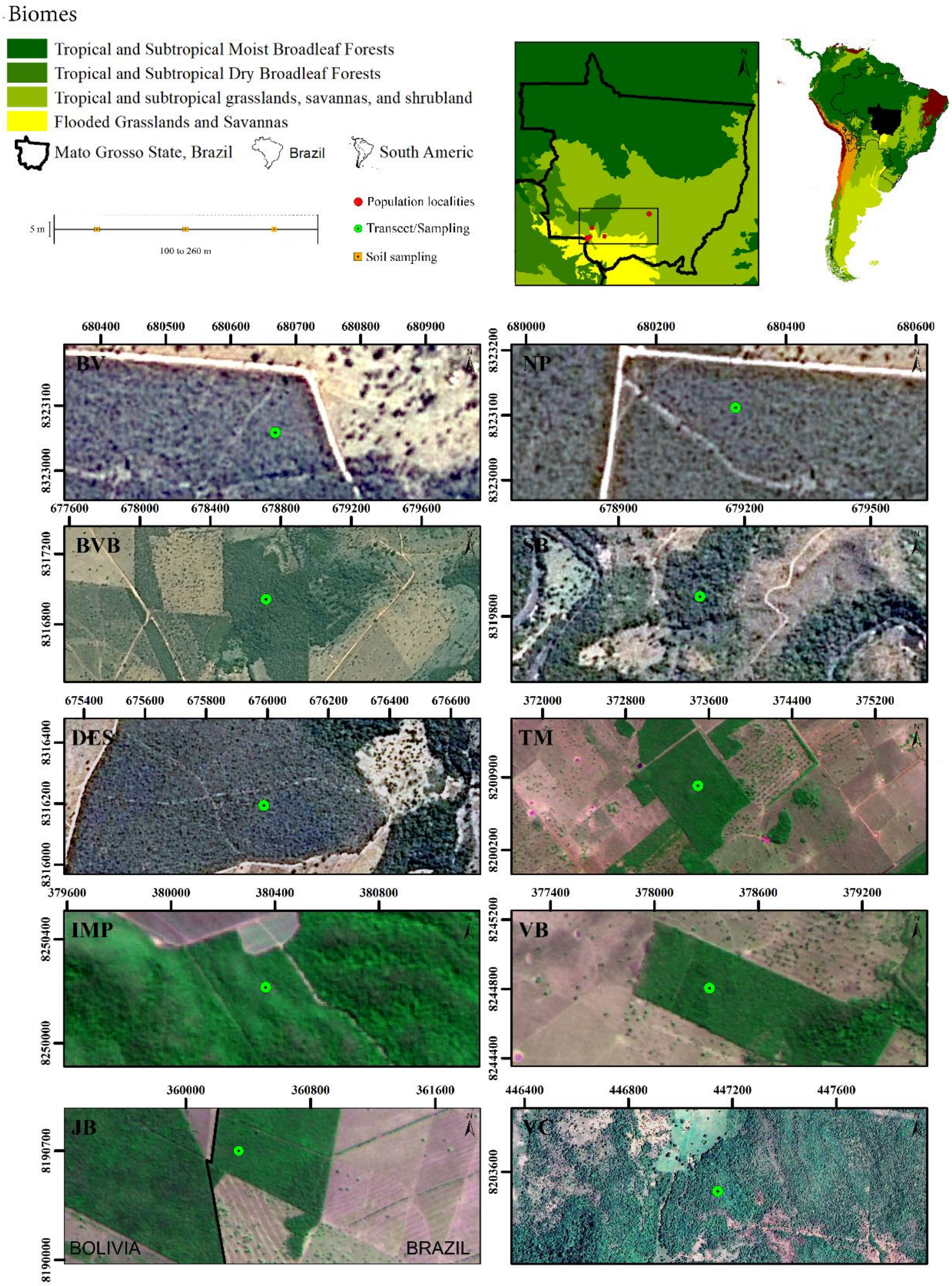

Figure 1. Geographic distribution and locality of occurrence according to ecoregions types and sampling of *Zamia boliviana* in South America, state of Mato Grosso (Brazil), and Brazil-Bolivia border region. Areas of the studied populations with schematic view of the sampling method and locations marked with green points indicate the studied populations. Note: Specific geographical coordinates of the areas with populations are omitted from this chapter to minimize the risk of illegal extraction of this species from the wild.

Tabela 1. Ecological information on the areas of sex ratio sampling in *Zamia boliviana* populations.

Locality/ site in MT^1 and site code	**Area (ha)**	**Elev. (m)**	**Soil type***	**Habitat types**	**Light condition****	**History of anthropogenic events** (≥ 50 years ago) Pass (PS) Present (PR)			
						1 - Fire occurrence 2 - Acess farming animals 3 - Mining of precious metals 4 - Regenerated area after cultivation 5 - Wood extraction			
Chapada dos Guimarães (BV)	3.27	423	Haplic Cambisols	Cerrado *stricto sensu*	Partial shade (~ 65.51 light)	1PS	2 PS/PR		
Chapada dos Guimarães (BVB)	33.88	431	Haplic Cambisols	Riparian forest transition cerradão	Partial shade (~ 67.75 light)	2 PS/PR	5 PS/PR		
Chapada dos Guimarães (DES)	15.44	420	Haplic Cambisols	Cerrado *stricto sensu*	Open/Partial shade (~ 55.06 light)	1 PS/PR	2 PS/PR	4 PS/PR	5 PS/PR
Mirassol do Oeste (IMP)	10.58	367	Petric Plinthosols	Cerrado *stricto sensu* transition semideciduous forest	Partial shade (~ 66.34 light)	1PS/PR			
Fontier Brazil/Bolivia (JB)	30.71	193	Plinthosols	Cerrado *stricto sensu*	Partial shade (~ 65.95 light)	1 PS/PR	2 PS/PR		
Chapada dos Guimarães (NP)	3.84	495	Haplic Cambisols	Cerrado *stricto sensu*	Partial shade (~ 56.72 light)	1 PS/PR	2 PS/PR	4 PS/PR	
Chapada dos Guimarães (SB)	6.97	398	Petric Plinthosols	Cerrado *stricto sensu*	Open/Partial shade (~ 71.4 light)	1PS/PR	2 PS/PR	3 PS	
Cáceres (TM)	65.15	285	Red Latosols	Cerrado *stricto sensu*	Open/Partial shade (~72% light)	1PS/PR	2 PS/PR	5 PS/PR	
Glória do Oeste (VB)	39.60	300	Red Yellow Latosols	Cerrado *stricto sensu* transition cerradão	Partial shade (~ 68% light)	1 PS	5 PS		
Cáceres (VC)	15.37	411	Litholic Neosols	Cerrado *stricto sensu*	Partial shade (~57.62 light)	1PS	2 PS/PR		

* Predominant soil type;
** Obtained in the dry period. MT^1 = Mato Grosso State

Sampling sex ratio variation

To investigate sex ratio variation in different habitats of *Z. boliviana*, we sampled populations from distinct areas of the Cerrado in the Brazil-Bolivia border region and other localities of the state of Mato Grosso (MT), as described in Table 1. For the purpose of this study, we determined the tertiary sex ratio (ratio at sexual maturity) according to Majerus (2003). We also define an individual as the set of leaves (1-3) belonging to a single ramet, regardless of the number of xylopodium ramets (Fig. 2B-C-G-H). This criterion was chosen due to the subterranean habit of the xylopodium in this cycad and, in some cases, the proximity of clonal and non-clonal individuals, which is not always distinguishable above the ground. The surveyed area covered the municipalities of Cáceres (JB 30.720 ha; TM 65.155 ha; VC 15.376 ha), Glória do Oeste (VB 39.605 ha), Mirassol do Oeste

(IMP 10.581 ha) and Chapada dos Guimarães (BV 3.270 ha; BVB 33.884 ha; SB 6.977 ha; NP 3.843 ha and DES 15.440 ha) (Fig. 2). Groups or clusters of at least 1,000 male and female individuals within a radius of approximately 10 thousand square meters or one hectare were considered as populations. Initially, transects of 5 x 100 meters were established, containing a minimum of 100 individuals of both sexes for each sampling unit. The transect area was extended as necessary to meet the established inclusion criteria (Fig. 2). The visual census was performed by means of active searching for, and marking of, adult plants within transects. The sex ratio was determined based on the identification and counting of plants with mega- and microstrobili between August and October 2017, which is the reproductive peak. Plants were classified as male, female, and unidentified plants (adult plants, but without strobili). This determination was made according to plant size (≥ to 0.80 m tall) and the number of leaflets per leaf (≥ 12). The sampled material was deposited in the Central Herbarium of the Federal University of Mato Grosso. In each transect we recorded the population density, calculated as the number of males, females and undetermined plants per sampling unit (transect). Other ecological information collected for cycad populations are shown in Table 1.

Habitat characterization. The general characteristics of habitats over the distribution of populations were investigated by using soil texture classes. Soil texture was classified according to the results of the physical analysis of the clay, silt and sand components for each sample. It is useful to indicate variation in clay content of the soil texture classes. Our results were compared with field estimates and analytical results according to FAO (2006).

Sex ratio and its correlation with environmental factors

Physical and chemical variables. To investigate whether physical and/or chemical soil variables in the habitat of *Z. boliviana* can affect the sex ratio, we sampled soils along the transect for each surveyed area at equidistant points (Fig. 2). Surface soil samples (0-30 cm) were obtained along each transect using a Dutch soil auger and then combined into composite samples. The soil samples obtained in all surveyed areas were air-dried and sieved (0.5 mm mesh). The samples were analyzed for physical and chemical properties according to the procedures adopted in Vourlitis et al. (2011). The physical properties of the soil tested, include stoniness, moisture and organic matter (SOM) content and particle size composition (percent sand, silt, and clay). The physical parameters tested were classified by particle-size diameter in mm as follows: (A) Five sand fractions: very coarse sand (VC); coarse sand (C); medium sand (M); fine sand (F); very fine sand (VF); total sand (TS); total clay and water-dispersible clay. The chemical properties measured were (as assessed colorimetrically) boron (B), copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and aluminum (Al) content, as well as pH.. The analyses were carried out in the Soil Laboratory of the "Luiz de Queiroz" School of Agriculture, University of São Paulo (USP-ESALQ), São Paulo, Brazil.

Light variables. To investigate whether the incident light in the habitat of *Z. boliviana* interacts with the sex ratio of this cycad, we estimated the percentage of canopy openness light for each locality, using a hemispherical image taken with a NIKON F-501 camera, coupled with a NIKON 8mm fisheye lens with a 180° angle of view. Images were taken every 25 m along the transection of all areas and according to recommendations of Galvani and Lima (2014).

Figure 2. Habitats and morphology of *Z. boliviana*: (A-B) In rocky calcareous soil. (C-D-G) In sandy Haplic Cambisols (D) In Cerrado *stricto sensu*, with occurrence of fire and clonal xylopodium and single xylopodium (D1-2) clonal adult plant with brevideciduous bud, (D-3) adult plant. (E) In Oxisols (ironstone outcrops). (F) Female plant with single xylopodium in Red-Yellow Latosols. (G) Underground organ (xylopodium): (1) buds in development (2) apical bud growth region (3) ground level. (H) Underground organ (xylopodium): (1) buds in development (2) apical bud growth region (3) ground level — Credits: Rosane Segalla, personal collection.

Statistical analysis

Sampling sex ratio variation. The mean and overall frequency of males and females in the ten populations of *Z. boliviana* were calculated. To test whether the male to female plant ratio intra and inter the ten populations sex ratio patterns showed a deviation from the expected isoplethic equilibrium (1:1:1), we used the *G*-test for goodness-of-fit (Sokal and Rohlf 2011).

Habitat characterization. To detect possible differences among the ten habitats of *Z. boliviana* in terms of soil composition and structure, the habitats were ordered according to texture classes for percentages of clay, silt, and sand, and illustrated in a ternary phase diagram.

Sex ratio and its correlation with environmental factors

Physical, chemical and light variables. Solar radiation in habitat was estimated according to the percentage of canopy openness (canopy ground cover) on each transect and the images were processed using Gap Light Analyzer (GLA) software version 2.0 (Lang et al., 2010). We used a redundancy analysis (RDA) to assess whether there were redundancies between summaries of cycad populations according to the biotic terms (considering the total area, total plant abundance and abundance according to sex, abundance of reproductive structures (number of strobili according to sex), sex proportion and the environmental conditions (soil parameters and incident light). Out of 25 physical and chemical variables, the following were chosen: light, coarse sand, boron (B), copper (Cu), manganese (Mn), aluminum (Al) and V% (base saturation). These variables were selected based on the lowest Pearson's correlation coefficients and variance inflation factor (VIF<2) between variables using *vif* function from *usdm* package (Wei and Simko 2017) in R (TeamCore-R 2017). To perform the RDA analysis, the parameters for soil and incident light were standardized with a mean of zero using the "standardize" method of the *decostand* function in *Vegan* package. Environmental factors were standardized using the Hellinger method (Borcard *et al.* 2018). The RDA analysis was performed using the *rda* function in *Vegan* (Oksanen et al. 2012), and the significance of environmental variables was tested using the function anova.cca (Legendre et al. 2011) with argument by = "terms".

RESULTS

Sex ratio pattern

Estimates of the sex ratio were obtained from 1572 individuals sampled from 10 populations. Table 2 summarizes the results of the sex ratio for all populations studied. Seven out of ten populations were male-biased (Table 2; Fig. 3).

Tabela 2. Ecological information of the ten sampled populations of *Zamia boliviana*. Number of males, number of females and unidentified individuals, sex ratio (female/male), and the Chi-squared (χ^2) and *P* value for a deviation from a 2 ♀: 1 ♂ sex ratio, expressed as the proportion of males.

SS	Area m^2	*N*/IP	*N*/UP	PD/ha	*N G*ender		Sex bias	Sex ratio	χ^2	*P-value*
					♂	♀				
VB	1300	176	82	135.38	59	35	1.69	1.69:1	6.13	0.01
TM	1300	106	46	81.73	37	23	1.61	1.61:1	3.27	0.07
VC	500	216	136	0.43	49	31	1.58	1.58:1	4.05	0.04
IMP	500	231	145	0.46	65	21	3.1	3.1:1	22.512	<0.001
JB	500	71	25	0.14	38	8	4.75	4.75:1	19.56	<0.001
NP	500	154	55	0.3	53	46	1.15	1.15:1	0.49	0.48
SB	500	127	48	0.03	45	34	1.32	1.32:1	1.53	0.22
BV	750	105	62	0.14	32	11	2.91	2.91:1	10.25	0.001
BVB	650	151	81	0.23	47	23	2.04	2.04:1	8.23	0.004
DES	500	235	99	0.47	119	17	7	7:01	76.50	<0.001

Sampling sites (SS), Individuals by population (*N*/IP), Number unidentified plant (UP), Population density per hectare (PD/ha).

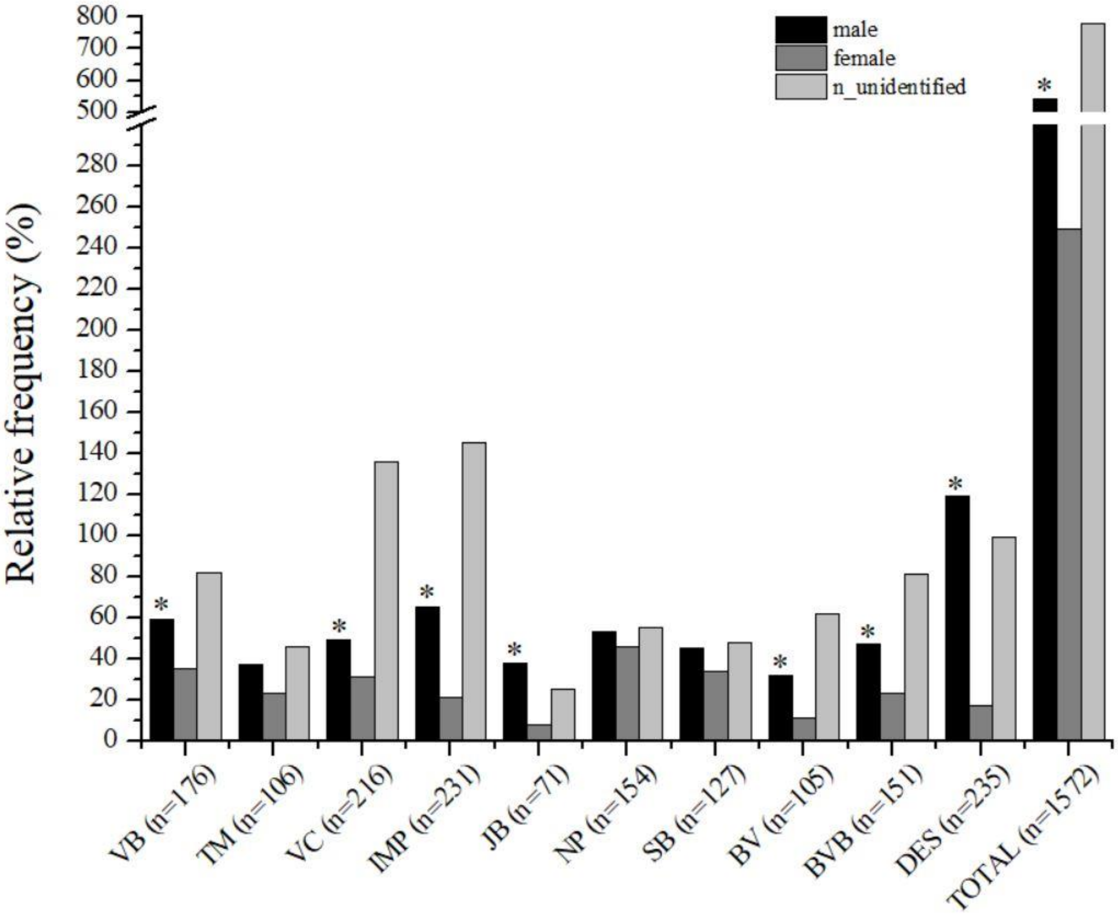

Figure 3. Distribution of male and female individuals in ten populations of *Zamia boliviana*. Bars show the sex ratios (male: female), suggesting significant deviation from the expected 1:1 ratio as determined by the *Chi-square* test.

Habitat features

Populations of *Z. boliviana* were categorized into three distinct habitat types according to the soil texture classes (Table 1, Fig. 4). Four of ten populations (BV, BVB, NP and DES) are found in Chapada dos Guimarães, MT, a region of wavy to strong wavy reliefs and soils primarily composed of sandy Cambisols (Fig. 4 – Habitat 1). Three (TM, JB and VB) occur in transition areas of vegetation refuges of the Paraguay River depression, where flat to smooth-wavy relief is prevalent, flat areas or in lower thirds of slopes, with potential movement or outcrops of the water table. These areas are formed, predominantly, by Latosols, Plinthosols, and Neosols with a Loamy Sand texture (LS) (Fig. 4 – Habitat 2). The other three (IMP, SB and VC) populations are distributed among the elevations of the rugged relief of the Província Serrana, and the valleys and sloped relief of Chapada dos Guimarães. Its main soils are Plinthosols and Neosols, with Sandy Clay Loam (SCL) transitioning to Sandy Loam (SL) textures (Fig. 4 – Habitat 3). To summerize, the soils of the three habitats have distinct morphological, physical and chemical characteristics, but all have a sandy to rocky texture and medium to high drainage. The distribution of Cerrado phytophysiognomies are all related to the presence of medium to high levels of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn) and aluminum (Al), whether associated with the lithology of soils or not.

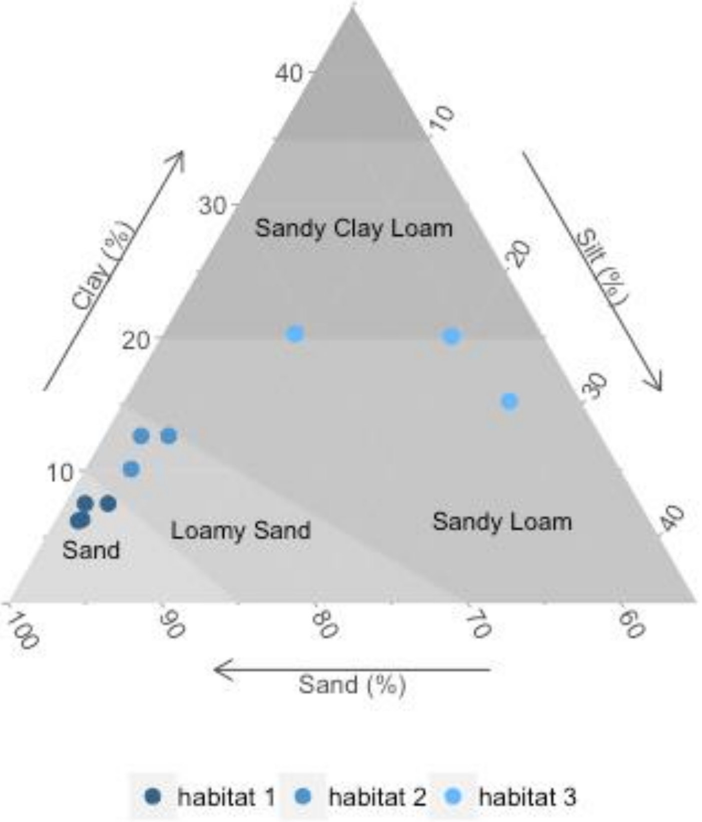

Figure 4. Soil texture triangle of the upper 0-30 cm soil layer in 10 types habitats of *Z. boliviana* in localities from Mato Grosso state.

The relationship between, or percentages of clay, silt, and sand, for soil texture classes was indicated by the ternary phase diagram and the coarse sand gradient is indicated by the biplot (Fig. 4; Fig. 5). Generally, this cycad was found in sandy soils of low fertility, intermediate levels of incident light and marked climatic seasonality in the Cerrado. The individuals form clumps mainly in the open Cerrado physiognomies sensu stricto (Fig. 2-5). In these physiognomies, the percentage of light reaching habitat may exceed 70% during the dry period (Table 1). Morphologically, *Z. boliviana* exhibits a subterranean habit with organs containing a considerable reserve (Fig. 2G-H). Cambisols are more permeable and in this study, were associated with the development of plants with an extensive root system and branched or clonal xylopods (Table 1; Fig 2G-H). Contrary to this, cycad populations associated with Red-Yellow Latosol have, predominantly, single (non-branched) and shallower xylopods (Table 1; Fig 2F).

Sex ratio and its correlation with environmental factors

The correlation of biotic variables (sex ratio) with abiotic variables (physical and chemical parameters of soil and incident light), of each of the ten populations of *Z. boliviana* revealed that the environmental variables do not explain redundancy in the reproductive characteristics of the populations (RDA model, $F_{7.2} = 2.366$; $p = 0.256$), even with an accumulation of 85% of the variation of these reproductive characteristics (see supplementary material) in the first two axes of the ordination. However, the coarse sand exhibited significance in the composition of the model, demonstrating a gradient of this propriety in the studied populations ($F_{1.2}$= 8.900; p = 0.037; Fig. 5). The sandy texture was present in all populations, but in decreasing order, the populations (number = population in RDA figure) NP'6, BV'8, BVB'9, and DES'10 presented the highest percentage of sand in their soils; followed by the TM'2, JB'5, VB'1 populations, with intermediate values and IMP'4, SB'7 and VC'3 with lower values.

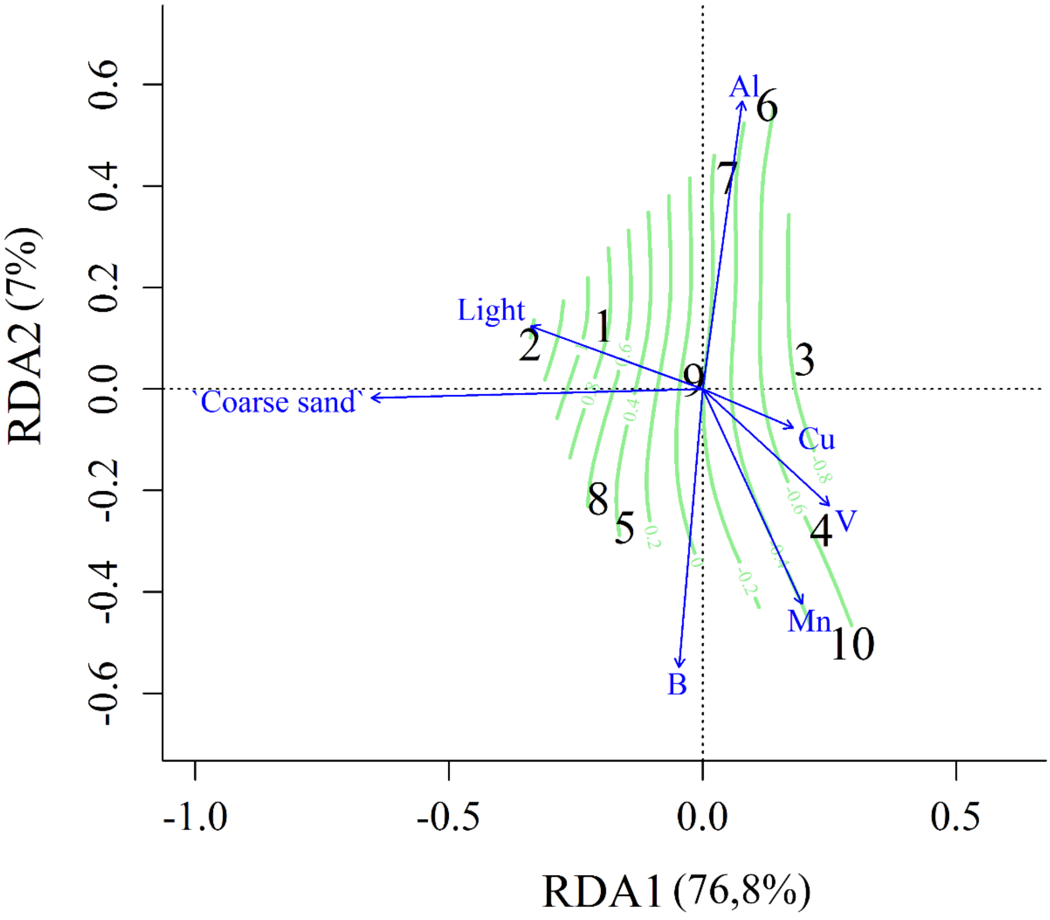

Figure 5. Biplot of the relationship between physicochemical parameters and sex ratio of 10 populations of *Zamia boliviana*. The numbers represent the populations on the ordination axes 1-2 extracted from redundancy analysis. The arrows indicate the orientation of populations based on the physicochemical parameters of soil and incident light. According to the significance of the variable coarse sand, we include its surface (in green) that shows how the populations are distributed according to this characteristic. The numbers (1-10) represent the ten populations studied (1=VB; 2=TM; 3=VC; 4=IMP; 5=JB; 6=NP; 7=SB; 8=BV; 9=BVB; 10=DES).

DISCUSSION

Our investigations on sex ratio and habitat type of *Z. boliviana* populations in the Cerrado (Brazilian Savanna) revealed the following findings: (1) most populations present male bias (70%) (Table 2; Fig. 3); and (2) as expected, *Z. boliviana* occupies different habitats within its area of distribution, with good adaptability to sandy and well-drained soils (Table 1; Fig. 4-5). (3) we did not find any relation that physical or chemical soil variables or light levels in the habitat might somehow affect the sex ratio of this cycad (Table 2; Fig. 1; Fig. 5); however, we found an environmental gradient among population related to the soil properties. From these results, we discussed presumed causes, as well as, similar studies.

Sex ratio pattern

This discrepancy in sex ratio in *Z. boliviana* appears to be related to the differential expenditure to produce ovulate strobili compared to pollen strobili, leading consequently to a male bias, as argued by Calonje et al. (2011). Presumably the production of pollen strobili requires less energy expenditure than ovulate strobili and are therefore produced more often during consecutive episodes of reproduction. This causes deviations in favour of male sex, especially in short-term sampling (Tang 1990; Calonje et al., 2011). In accordance with others studies, we assumed that the differential energy expenditure to reproduce the sexes, is preponderant in sex ratio variation. It is an important contributor to sex bias and can to explain a deviation from a sex ratio of 1:1 in dioecious populations (Ornduff 1987; Barrett et al., 2010)

According to Barrett et al. (2010) at least three factors related to the costs of reproduction can contribute to male bias: (i) earlier onset of male flowering, (ii) more frequent flowering in males, and (iii) greater gender-specific mortality in females. However, contrary to the last-mentioned factor, and although it needs to be further investigated, there is no biological evidence that differential mortality may be a factor sufficient to cause male deviations due to mortality in female plants of *Z. boliviana*. We attribute the morphology and ecological adaptations of this perennial cycad from the Cerrado, such as the presence of a xylopodium, with a large nutrient reserve and brevideciduous events, as the main reason to refute this cause. Oliveira (1996) suggested that of the ca. 30%, of dioecious species of Cerrado, 12% are in open areas, with predominant brevideciduous habit. The biased sex ratio as observed in Myrsinaceae, of open Cerrado, was associated with vegetative regeneration (Oliveira 1996).

Male-biased sex ratio in cycads was reported in species of the genera *Ceratozamia* Brongniart, *Dioon* Lindley, *Encephalartos* Lehmann, *Lepidozamia*, *Macrozamia* Miquel, *Microcycas* (Miq.) A. DC. and *Zamia* L. (reviewed in Calonje et al., 2011). Deviation from the 1:1 sex ratio in cycads appears to be the norm in relatively small populations or when sampling adult-sized individuals only to determine the sex ratio (Clark and Clark 1987). In these cases, the bias towards males is usually higher (Calonje et al., 2011). However, most cumulative studies indicate no significant differences in sex ratio, either for living collections (Ornduff 1987; Calonje et al., 2011) or *in situ* populations (Grobbelaar 2002; Octavio-Aguilar et al., 2017), tending to equality in sex ratio. Grobbelaar (2002) describe that large and vigorous populations of *Encephalartos* have a sex ratio of approximately 1:1, but small populations of this genus appear to be dominated by males, such as in *Encephalartos* latifrons Lehm (4:1).

About 50 years ago, the DES and NP areas were used for crops, with frequent weeding and cutting of vegetation at ground level. These activities have probably stimulated individuals to branch and form clonal xylopods and this tendency was intensified by Cambisols characteristics. These conditions, together with the fire that occurred in DES, combined to increase the frequency and reproductive asynchronicity of the sexes, compounded by our difficulty to distinguish clonal from non-clonal branches above the ground, consequently may have contributed to sex ratio variation and male bias in the DES population. Thus, the sex ratio results of this study seem to be related to a set of deterministic biological and ecological factors and stochastic events, which affect, in some way, the cycad populations. This explains the fact that not all variation in sex ratio in this study occurred in a specific type of environment (e.g., male bias versus soil type). In addition, many unknown stochastic events occurred or are presumed to have occurred in these areas, making it impossible to correlate them to biological data of the populations.

We also highlight that although data on mortality have not been measured in this cycad, our field observation and the morphological characteristics of this cycad are according to findings of Octavio-Aguilar et al. (2017) for *Z. furfuracea* L.f.. For them, this parameter is not a crucial factor involved in the differential performance of the sexes in this genus (Octavio-Aguilar et al., 2017). Instead the decline in cycad populations (e.g., anthropogenic activity) is one of the most recurrent and probable factors, mainly in South America (Segalla et al., 2019), to trigger effects on the sex ratios of cycads. In summary, factors related to differential expenditure, ecophysiological characteristics such as brevideciduos events (xylopod latency in female plants) possibly lasting for more than one season (Segalla, R. – pers. obs.), anthropogenic events leading to removal of individuals (Donaldson 2003, Lopez-Gallego 2015, Mankga and Yessoufou 2017; Segalla et al., 2019) and analysis from non-cumulative study (Calonje el al., 2011), possibly drive sex bias in *Z. boliviana*. The effect of the factors above can therefor determine the structure and dynamics in the long-term of cycad populations (Octavio-Aguilar et al., 2017b), and also their reproductive viability. This is supported since the biased sex ratio may impact the dioecious species' viability by reducing the effective reproductive population, influencing female fecundity and/or decrease pollen availability through an increase in the distance between reproductive individuals (Riba-Hernández et al., 2014; Octavio-Aguilar et al., 2017; Lazcano-Lara and Ackerman 2018).

Habitat features

In biological terms, it is assumed that the present distribution of *Z. boliviana* in different physiognomies of the Cerrado (Skelley and Segalla 2019) probably reflects the diversification history of the genus in South America (Calonje et al., 2019). The dispersal strategy used, the adequate and safe sites for its colonization and concomitant use and occupation of soil (Segalla and Calonje 2019; Segalla et al., 2019), suggest that *Z. boliviana* presents morphological diversity compatible with the prerequisites to survive the seasonal climatic Cerrado conditions until now (Calonje et al., 2019b). The morphological and physiological characters acquired by this cycad, probably allowed it to improve its ability to tolerate abiotic limitations (e.g., soils rich in Al, low fertility, rocky outcrops) and situations of environmental stress (e.g., high temperatures, long dry season, fire). Indeed, *Z. boliviana* seems well adapted to these habitat conditions because the energy reserves in the underground organs allow it to develop new shoots when the aerial parts are lost to fire or after brevideciduous events. This corroborates the complexity of ecological and demographic factors affecting the sexual reproduction system (Pannell and Dorken 2006). These characteristics are suggested to be more involved in ensuring the persistence of the species and improving strategies for reproductive success of populations (Vázquez-Torres et al., 2001; Octavio-Aguilar et al., 2017a; Lazcano-Lara and Ackerman 2018).

Regarding cycad environmental conditions, our records have shown that this cycad can occur in a considerable diversity of soil types and their physical and chemical compositions. Cerrado's soils among habitats of cycad are characterized, in general, as alkaline (Reatto et al., 2008), with low natural fertility, but good structure and predominantly sandy and well-drained soils (SEPLAN 2000). This cycad also seems well adapted to intermediate levels of incident light, inherent to climatic seasonality of the Cerrado. The pedological zone where this cycad also occurs include: Oxisols, mostly dystrophic and acidic but which have good water permeability; Cambisols, chemically dystrophic with low chemical fertility and predominant sandy texture; Plinthossols, generally compact but hydromorphic with great variability in their chemical properties and Neossols, shallow soils associated with rock outcrops (e.g., in this study, limestone rocks), high levels of primary minerals, morphologically quite heterogeneous, little evolved and with an arbitrary depth to less than 50 cm, according to descriptions of Reatto et al. (2008). The presence of the cycad in different environmental contexts, suggests occupation and growth in a wide variety of habitats throughout its distribution. It seems reveals fitness for, and ecological plasticity to adapt to different micro-conditions. From a conservation point of view, this is an important point for species conservation strategies.

Sex ratio and its correlation with environmental factors

The absence of an association between incident light and the sex ratio, probably reflect the adaptability of this cycad to the habitat where it occurs. This was also found in dioic *Mercurialis perennis* EU., (Vandepitte et al., 2010), where no correlation was found between light and the sex ratio. The study of Nicotra (1998), with *Siparuna grandiflora* (Kunthin Hub. & Bonpl., A. DC.,) Siparunaceae, showed that the light availability affected reproductive activity in both sexes. Although light may play an important role in specie's biological development, it needs to be better studied in *Zamia* spp..

It is noticeable that the populations did not exhibit any biotic characters that grouped with the characteristics of the soil. The coarse sand component was the only attribute that separated them along a gradient and seems to reflect the soil typology of the cycad habitats. Particularly, the populations BV, BVB, NP and DES occur in habitats composed predominantly of sandy soils and this characteristic, typical to Cambisols, probably influences the fitness of this cycad. Given the assumed differences in energy expenditure of the sexes and the occurrence of these populations in characteristically sandy habitats (Habitat 1), it is possible that this factor drives differences in the allocation of resources between the sexes. This suggests that it is easier for male plants in these areas to meet their energy requirements for vegetative and reproductive functions and can maintain more frequent reproduction without interruption. In contrast, female plants have additional requirements, which can lead to irregular reproduction. These factors, associated with other life history events and non-cumulative sampling, may have accentuated the male bias in those populations.

In environmental context, physiological differences between males and females can influence the sex ratio within a population when environmental conditions are heterogeneous (reviewed in Krieg et a., 2017). The classic study of Ornduff (1987) within populations of *Zamia pumila* L., was the first to record that soil quality affects the sex ratio. This researcher found in shallow, poor soils, populations were male biased, and females reproduced less frequently. In sites with more favorable soils, the proportion of males to females was equal, and females reproduced more frequently. Together with these factors, we presume that in an ecological scale, stochastic forces and founders effects also can act on natural habitats and cause a biased sex ratio. Variation in population density, spatial distribution of sexes, resource availability, structure of founder population, pollinator visits, anthropic actions; spontaneous colonization, genetic and demographic characteristics are examples of such forces we want to highlight. These factors were also pointed out as relevant in others plants (Cunha et al., 2014), and cycad studies (Pérez-Farrera et al. (2017); Mankga and Yessoufou (2017) and Octavio-Aguilar et al., (2017).

All the above forces are characteristic of a population's history (Dorken and Pannell (2008); Field et al., 2013; Lazcano-Lara and Ackerman (2018), and it are thought to reinforce sex ratio patterns (Field et al., 2013; Calonje et al. 2011; Segalla et al., 2019). These acting factors, whether associated or not, probably co-occur concomitant to reproductive costs and non-cumulative sampling to potentially cause the sex-ratio variation, especially in small and scattered cycad populations (Grobbelaar 2002; Segalla et al., 2019). Our understanding, however, of the many underlying mechanisms that can cause a variation in the sex ratio of plant populations, is still limited and more work needs to be done before it can be fully understood (Field et al. 2012; Juvany and Munné-Bosch 2015).

It should be noted that the small number of populations sampled in our study (N = 10) is common for cycads. However, according to Yu and Lu (2011) and Vandepitte et al., (2010), male-biased sex ratio may be quite pronounced in small populations (≤ 1.000 individuals) and corroborate this study, because *Z. boliviana* populations are mostly small and fragmented. Small endemic populations, such as in this cycad are especially vulnerable to changes in land use and habitat loss (Lopez-Gallego 2015; Segalla et al., 2019). This fact further reduces the probability of finding a superior N and is an obstacle for future study. This factor limits opportunities to detect robust patterns and to distinguish which forces act most strongly to cause sex ratio deviations in long-lived tropical plants. Despite the challenges, long-term studies, that consider each species separately as a unique case study, combining biological, ecological and functional approaches, are promising and will help to improve our knowledge where it is lacking, as emphasized by Juvany and Munné-Bosch (2015). The data of this case study therefor supports the current arguments for the biased sex ratio, as found in other long-lived dioecious species, including cycads.

CONCLUSIONS

We want to highlight four general considerations to guide future research and focus to improve our understanding of the mechanisms causing biased sex ratios in cycads and other dioecious species of tropical systems: (i) The results show a male-biased sex ratio in most populations surveyed. A possible explanation for this is the differential energy expenditure of sexes in reproduction. In biological terms, this seems be the most plausible factor governing the mechanisms of variation found, compared to secondary factors and more precisely, in tertiary and short-term sex ratio assessments. Our evidence supports this scenario since the environmental variables did not explain the biased sex ratios of *Z. boliviana* populations. However, the possibility remains that future research, with more extensive data sets and potentially improved methods, will clarify the impact of costs of reproductions that we believe drives the biased sex ratios in this cycad, and others factors pointed out in this study. A first step is likely to come from precise determination of the stages at which the bias occurs and the factors influencing the survival, growth, and flowering of females and males of *in situ* populations (e.g., Barrett 2002; Stehlik and Barrett 2005; Calonje et al., 2011; Fild et al., 2012; Khorsand Rosa et al., 2014); (ii) Genomic approaches should provide accurate methods for the rapid identification of large numbers of sex-specific markers (e.g., Prakash and Van

Staden 2006; Fild et al., 2012). In fact, future studies should take advantage of the different genomic techniques currently available, to help identify several ecological processes within cycad populations, including sex ratio determination (On primary, secondary and tertiary sex ratio level), patterns of gene exchange by seeds and pollen, population structure and species cohesion (e.g., Barrett 2002; Sharma et al., 1998; 2004; Leal et al., 2016, Pinheiro et al., 2018); (iii) It is imperative to increase efforts related to extensive and cumulative sampling in long-lived species of *in situ* populations, and perhaps cumulative studies will dilute biased results for most cases; (iv) Finally, the mapping and understanding of habitats of cycads and other dioecious species in South America must be improved (e.g., Lopez-Gallego 2015; Segalla et al. 2019). It is essential to assess the health of populations and study their reproductive dynamics over time, especially, in small populations of endemic micro-refugia and fragmented landscapes. The evaluation of micro-environmental factors and spatial structure (e.g., Yu and Lu 2011), to predict the degree and direction of sexual dimorphism development in these older dioecious lineages, will also benefit for these studies.

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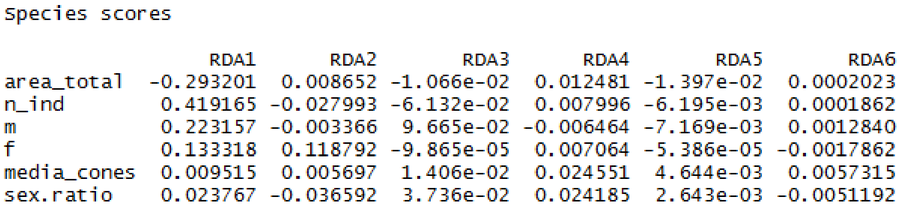
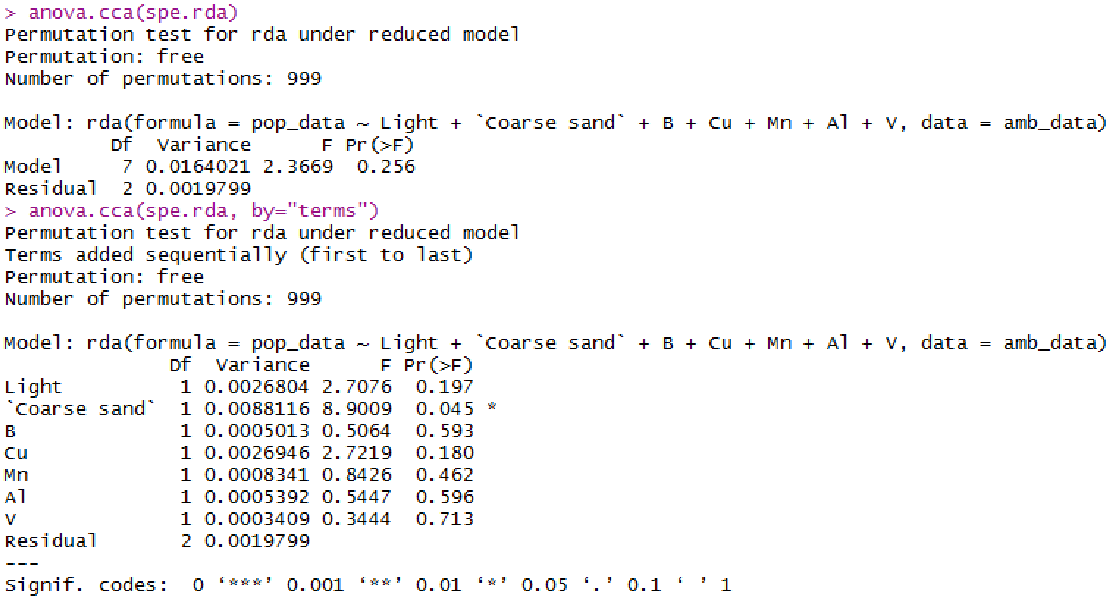
SUPPLEMENTARY MATERIAL

Redundancy analysis (RDA)



```
Species scores

                  RDA1      RDA2       RDA3      RDA4       RDA5       RDA6
area_total   -0.293201  0.008652 -1.066e-02  0.012481 -1.397e-02  0.0002023
n_ind         0.419165 -0.027993 -6.132e-02  0.007996 -6.195e-03  0.0001862
m             0.223157 -0.003366  9.665e-02 -0.006464 -7.169e-03  0.0012840
f             0.133318  0.118792 -9.865e-05  0.007064 -5.386e-05 -0.0017862
media_cones   0.009515  0.005697  1.406e-02  0.024551  4.644e-03  0.0057315
sex.ratio     0.023767 -0.036592  3.736e-02  0.024185  2.643e-03 -0.0051192
```



```
> anova.cca(spe.rda)
Permutation test for rda under reduced model
Permutation: free
Number of permutations: 999

Model: rda(formula = pop_data ~ Light + `Coarse sand` + B + Cu + Mn + Al + V, data = amb_data)
         Df  Variance      F Pr(>F)
Model     7 0.0164021 2.3669  0.256
Residual  2 0.0019799
> anova.cca(spe.rda, by="terms")
Permutation test for rda under reduced model
Terms added sequentially (first to last)
Permutation: free
Number of permutations: 999

Model: rda(formula = pop_data ~ Light + `Coarse sand` + B + Cu + Mn + Al + V, data = amb_data)
              Df  Variance      F Pr(>F)
Light          1 0.0026804 2.7076  0.197
`Coarse sand`  1 0.0088116 8.9009  0.045 *
B              1 0.0005013 0.5064  0.593
Cu             1 0.0026946 2.7219  0.180
Mn             1 0.0008341 0.8426  0.462
Al             1 0.0005392 0.5447  0.596
V              1 0.0003409 0.3444  0.713
Residual       2 0.0019799
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

CHAPTER 4

> *Borboletas me convidaram a elas.*
> *O privilégio insetal de ser uma borboleta me atraiu.*
> *Por certo eu iria ter uma visão diferente dos humanos*
> *e das coisas.*
> *Eu imaginava que o mundo visto de uma borboleta -*
> *Seria, com certeza, um mundo livre aos poemas.*
> *Daquele ponto de vista:*
> *Vi que as árvores são mais competentes em auroras*
> *do que os humanos.*
> *Vi que as tardes são mais aproveitadas pelas garças*
> *do que pelos humanos.*
> *Vi que as águas têm mais qualidades para a paz do*
> *que os humanos.*
> *Vi que as andorinhas sabem mais das chuvas do que*
> *os cientistas.*
> *Poderia narrar muitas coisas ainda que pude ver do*
> *ponto de vista de uma borboleta.*
> *Ali até o meu fascínio era azul.*
> (Modificado de Barros, Manoel de. Ensaios fotográficos. Rio de Janeiro: Ed. Record, 2000.)

Photo credits: Rosane Segalla, personal collection.

New report of *Eumaeus* (Lepidoptera: Lycaenidae) associated with *Zamia boliviana*, a cycad from Brazil and Bolivia

Rosane Segalla and Patrícia Morellato

Scientific note published October, 2019 in the journal *Cycad Newsletter*.

Reference: Segalla, R. and Morellato, P. (2019). New report of *Eumaeus* (Lepidoptera: Lycaenidae) associated with *Zamia boliviana*, a cycad from Brazil and Bolivia. *Cycad Newsletter* – Feature article. 4(1).

New report of *Eumaeus* (Lepidoptera: Lycaenidae) associated with *Zamia boliviana*, a cycad from Brazil and Bolivia

The species of the genus *Eumaeus* Hübner, 1819 (Lepidoptera: Lycaenidae) are obligate-herbivores, in the larval stage, of many Neotropical Zamiaceae (Clark et al. 1992; González, 2004; Castillo-Guevara, 2007; Cascante-Marín and Araya, 2012; Prado et al., 2014; Ruiz-García et al., 2015). They exhibit gregarious behavior and their bright colors are thought to be aposematic, serving as a warning signal to predators (DeVries 1977; Bowers and Larin, 1989; Nash et al., 1992). Lepidoptera, generally, are commonly studied for their interactions with their plant hosts (Castillo-Guevara 2007), however, many interactions between plants and animals, especially those involving cycads, are poorly documented in South America.

Eumaeus minyas Hübn. is distributed from Mexico to Colombia, in South America and is exclusively involved in herbivory of *Zamia* feeding on of fronds and female reproductive strobilus of *Zamia furfuracea* L.f., *Zamia skinneri* Warsz. exA.Dietr., *Zamia neurophyllidia* D.W.Stev., *Zamia loddigesii* Miq. (DeVries 1976, 1983; Clark and Clark,1991; Clark et al. 1992; Castillo-Guevara and Rico-Gray, 2002), *Zamia encephalartoides* D.W.Stev. (González, 2004) and olso of *Dioon edule* Lindl. (Castillo-Guevara, 2007).

Zamia boliviana (Brongn.) A.DC. (Cycadales, Zamiaceae) occupies a distribution range between Bolivia (Beni, Cochabamba, La Paz and Santa Cruz) and Brazil, in the state of Mato Grosso (Jørgensen et al. 2014; Skelley and Segalla 2019; Segalla and Calonje 2019; Segalla et al. 2019). The species is a small plant, up to 0.80 m tall, with a subterranean stem, and 1 to 3 leaves per crown. It inhabits different Cerrado (the Brazilian savanna) phytosiognomies, from scrubland to woodlands, across different types of soils, and also on rocky outcrops (Skelley and Segalla 2019). Many populations have been decimated or severely fragmented due to the expansion of extensive agriculture and ranching activities in brazil but also Bolivia (Segalla et al., 2019; Skelley and Segalla 2019, Segalla et al. 2019).

The occurrence of *E. minyas* in populations of *Z. boliviana* has been recorded *in situ* in the state of Mato Grosso, Brazil, over the last four years and results suggest a stable co-occurrence in cycad populations of different cerrado areas. Voucher material has been deposited in the entomological collections of Museu de Zoologia of Universidade of São Paulo (MZUSP) and at the Laboratório de Scarabaeoidologia (Setor de Entomologia da Coleção Zoológica, UFMT), Instituto de Biociências, Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil. Eggs, adults and different instars of larvae were observed in *Z. boliviana* plants (Fig. 1). Oviposition occurred on vegetative and reproductive parts of the cycad, with eggs being present on cataphylls, petioles, leaflets and rachis, pollen strobilus and ovulate strobilus (Fig. 1).

Figure 1 – *Eumaeus minyas* in vegetative and reproductive parts of *Zamia boliviana*. A-B. eggs on cataphylls and a recent emerged leaf, C-D. larvae on petioles and leaflet, E-F. larvae on pollen strobilus and ovulate strobilus G. pupae in the abaxial side of a leaf and H. adult butterfly on *Z. boliviana* leaf. All photos from Cáceres, Mato Grosso State, Brazil. (Photos: Rosane Segalla).

Despite the few studies on the ecology of this lycaenid butterfly in South America, we can infer that its geographical distribution occurs concomitantly with the distribution of the populations of species of *Zamia*, their host plants species. The populations of *E. minyas* appeared healthy, with all life stages observed and an uninterrupted life cycle as expected for natural populations (Fig. 1). However, the destruction of cycad habitats threatens the survival of this endemic butterfly species, as it is an obligate herbivore which depends on these plants for the completion of its life cycle. Our observations suggest that the butterfly prefers to oviposit on immature expanding leaves or young reproductive parts. We have evidence that the vegetative and reproductive phenology of *Z. boliviana* is adapted to the Cerrado seasonality and that *E. minyas* life cycle is in synchronicity with the emergence and development of *Z. boliviana* leaves and strobilus.

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CHAPTER 5

> *O que os cientistas aprenderam sobre Deus, a partir dos estudos sobre suas criações?*
> *"Sua extrema afeição por besouros"*
> (J.B.S. Haldane by Strong, D.,R., Lawton, J.H., Southwood, R. 1984. Insects on plants: Community patterns and mechanisms. Blackwell scientific Publication. London: Acad. Publ. p. 581-594.).

And thus…

In the end,
We will conserve only what we love,
We will love only what we understand,
We will understand only what we are taught.
(Baba Dioum, from a speech made in 1968, New Delhi, India,
general assembly of the International Union for Conservation of Nature.)

Reproductive biology of a South American cycad involving brood-site pollination mutualism

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Background and Aims: Brood-site mutualisms represent extreme levels of the reciprocal specialization between plants and insect of which beetles, generally, are taxa with restricted distributions and sole pollinators of many cycads. However, the reproductive biology of South America cycads remains little known experimentally. Here, a *Zamia* (Cycadales, Zamiaceae) was use to investigate plant-pollinator interactions from the perspective of dioecy and obligatory mutualism. Our aim was to examine strobili, their visitors, and the nature of their involvement with the plant to identify potential pollinators.

Methods: We monitored reproductive cohorts and registered the strobili's visitors. Afterwards, we classified the activities of these visitors and focused on describing the behavior of the most active, reliable and recurrent agents in both reproductive structures, to ascertain the potential pollinators and their relationship with the cycad. *In situ* experiments were used to demonstrate the strength and efficiency of plant-pollinator interactions.

Results: Although other insects visit the strobili, only a Coleoptera (Erotylidae: *Pharaxonotha*) was present seasonally. It was the only species capable of transporting pollen and developing life cycle associated to polleniferous strobili, while concomitantly visiting ovuliferous strobili. Experimental tests associated with observational evidence and the seasonal frequency of this Erotylidae in the cycad's reproductive organs indicate brood-site mutualism with this beetle, a potential pollinator of *Zamia boliviana*.

Conclusions: Our research confirms previous studies on obligatory mutualism involving cycads and *curculionidae* in the New World. Future research on dioecious species from South America should consider these interactions for better understanding codependent biological systems and formulating predictions applicable to these species.

Keywords: Beetle life cycle; Biotic vectors; Dioecious plants; Ecological interaction; Host plants; Plant-insect interactions

INTRODUCTION

The reciprocally specialized plant–pollinator interactions are among the most important ecological interactions (Solga et al., 2014; Suinyuy et al., 2015). The degree of ecological dependence of plants on pollen vectors to produce seeds depends on their reproductive systems (Richards, 1997; Johnson et al., 2009). These interactions and may range from completely obligatory, as in species that use particular flowers as brood sites or sources of food, to facultative, as in species that have generalist diets including some food from flowers (Richards, 1997; Faegri andVan der Pijl 2016; Johnson et al., 2009). Dioecious plants such as cycads or genetically self-incompatible species are wholly dependent on cross-pollination to produce seeds (Norstog and Fawcett 1989; Richards 1997). In the sense, the brood-site mutualisms represent not only an extreme level of reciprocal specialization between plants and insect pollinators (Suinyuy et al., 2015), but a necessary process in plant biology systems.

The morphological and physiological features associated with the reproductive system of dioecious plants have a genetic base (Dellaporta and Calderon-Urrea 1993; Khosla and Kumar 2015, Krieg et al., 2017) and are considered barriers to self-fertilization, playing an essential role in the plants' evolution (Khosla and Kumar 2015). In members of Zamiaceae, there is a striking sexual dimorphism in the reproductive structures of the two sexes (Jones 2002; Grobbelaar 2002). A close relationship between habitat colonization and reliance on specialized and obligatory biotic vectors for reproduction increases the risks of successful reproduction for both plant and pollinator. The reproductive biology of the Zamiaceae family (Cycadales) is characterized by cross-pollination and predominantly obligatory and specialized mutualistic interactions between pollinators and their host plants (Norstog et al. 1986; Tang 1987; Norstog and Fawcett 1989; Vovides 1991; Terry 2001; 2005; Hall et al., 2004; Suinyuy et al., 2009; Valencia-Montoya et al., 2017; Segalla et al. 2019).

Despite their rarity and fascinating biological history, cycads are among the most threatened groups of plants worldwide (Stevenson et al., 2018; IUCN 2019). The codependency on mutualism may render both insect and cycad especially sensitive to the properties associated with cycad population declines (Terry et al. 2005; Terry et al., 2012; Lopez-Gallego 2015; Segalla et al., 2019). The ancient origins of cycads provide an opportunity to study older insect-plant interactions, which may reveal much about the early development of insect pollination systems (Terry et al., 2005). Understanding the obligatory and specialized mutualistic pollination system that involves plants of an ancient lineage and the associated entomofauna is mandatory to evaluate the evolution and pathways that led to this unusual interaction (Roemer et al., 2008). In addition, it may provide new insights into the evolutionary history of New World cycads, predict vulnerabilities and to apply this knowledge mainly to the conservation of these ''flagship'' species (Terry et al., 2005; Roemer et al., 2008; Terry et al., 2016; Tang et al., 2018ab).

The biological knowledge about the *Zamia* species in South America is still notably scarce, and *in situ* conservation efforts will need to conserve not only the cycads, but also the permanence and health of their interactions with pollinators and herbivores (Schneider et al., 2002; Terry et al., 2005; González 2004; Solga et al., 2014; Lopez-Gallego 2015; Valencia-Montoya et al., 2017; Segalla et al., 2019). Even with some advances, little systematic testing including exclusion studies to evaluate the effectiveness of wind and insects as pollen vectors has been conducted on the precise role of the pollinators of South America cycads. The presence of insects at receptive strobilus (e. g.: Skelley and Segalla 2019) raise questions as to their effectiveness as pollinators.

In this work, we follow the phenophases of *Z. boliviana* polleniferous strobili and ovuliferous strobili, observing the activities of their visitors to (i) ascertain the plant-pollinator reproductive systems of cycad and their reproductive biology; (ii) identify common visitors of the strobili and evaluate the type of relationship they have with the cycad; and (iii) demonstrate the efficiency of pollination vectors (insects and winds) in the reproductive biology of *Z. boliviana*, based on vector exclusion studies. Our study complements the knowledge of the natural history of *Z. boliviana* and its likely pollinator *Pharaxonotha cerradensis* Skelley and Segalla (Skelley and Segalla 2019), instigating new study hypotheses deemed as fundamental for predicting extinction risks and developing strategies for the conservation of cycads and associated interactions.

MATERIALS AND METHODS

Study species and study site

Climatic factors of the habitat – *Zamia boliviana* populations can be found within the intertropical region of the central portion of the South American continent (Fig. 1-A), characterized by high total solar radiation, falling upon the surface of the soil practically throughout the whole year (SEPLAN 2000; Deblauwe et al. 2016). Equatorial and Tropical Hot climates prevail, with little seasonal and annual thermal variation (SEPLAN 2000; Deblauwe et al. 2016). The average minimum daily temperature is 20.3°C, and the average maximum daily temperature is 32.7°C (SENAMHI 2019). The region experiences two seasons, a dry season (May to September) with an average rainfall of 31.0 mm, and a rainy season (October to April), with an average rainfall of 178.4 mm (SENAMHI 2019). This cycad, which occurs in the Cerrado, is a small perennial plant (up to 0.80 m tall) with a subterranean stem (xylopodium) and from 1 to 3 leaves in each crown (Fig. 1-B). The reproductive structures are formed in the soil, from June to November. Male plants can develop from one to six pollen strobili per stem or branch of xylopodium, whereas female plants produce only one megastrobilus. Phenological records indicate this cycad has a life cycle intrinsic to the seasonality of its habitats. *Z. boliviana* strobili are visited by *P. cerradensis*, but this and other interactions involved in the reproductive biology of *Z. boliviana* have yet to be confirmed.

Strobili visitors: diversity, frequency and behaviors

The occasional (non-systematic) registration of visitors, their diversity, frequency and behavior in the polleniferous strobili and ovuliferous strobili was performed *in situ* in 2016-2019, in reproductive cohorts of cycad populations of MT. In 2017, systematic records were made in a population of Cáceres – MT, and in 2018 and 2019, in populations of Chapada dos Guimarães – MT. Focal observations were carried out in the morning (06:00 – 12:00), afternoon (14:00 – 18:00), and evening (20:00 – 23:00). Insect samples found in or on the strobili were collected in 70% ethanol and deposited in the biological collections of the Laboratório de Scarabaeoidologia at Universidade Federal de Mato Grosso, Instituto de Biociências, Cuiabá, Mato Grosso, Brazil (UFMI), and of the Museu de Entomologia Pe. Jesus Santiago Moure, Universidade Federal do Paraná, Curitiba, Paraná, Brazil (DZUP), for being identified or kept as testimonial material. Additionally, we haphazardly collected strobili from populations of the visited sites, and recorded the presence of insects, number of specimens and life cycle stage (larva, pupa or adult).

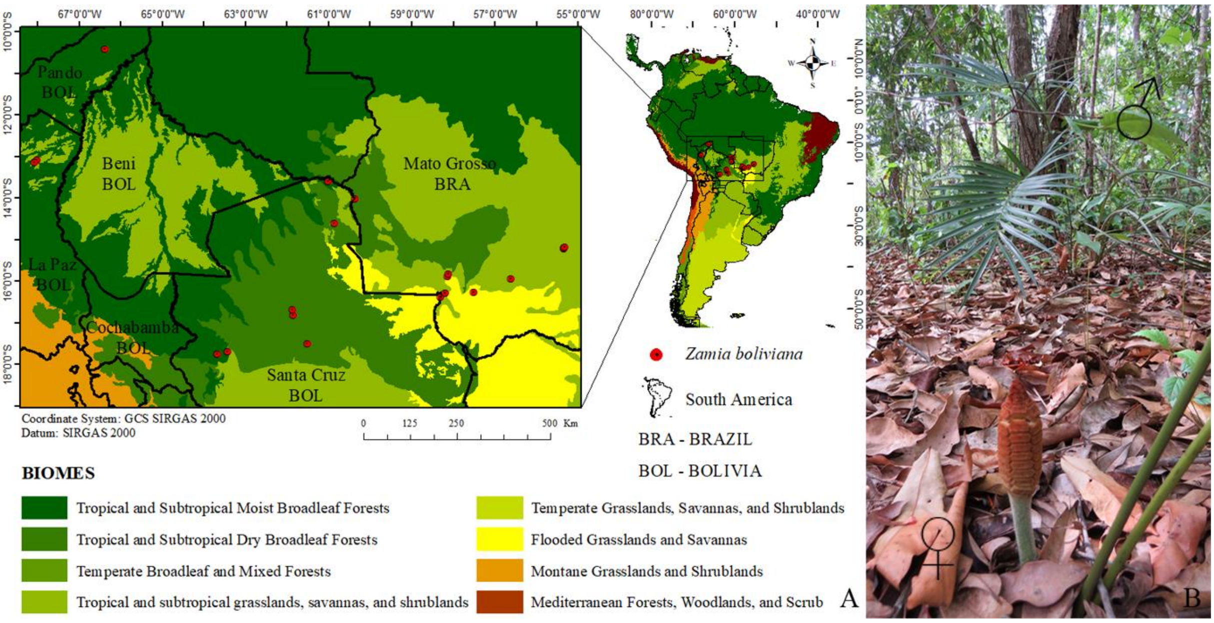

Figure 1. A. Geographic distribution and place of occurrence, according to type of biome, of *Zamia boliviana* in South America, state of Mato Grosso (Brazil), and region of the Brazil-Bolivia border. B. Habit of *Z. boliviana* at place of occurrence. Credit: Rosane Segalla, personal collection.

Efficiency of vectors and cycad-pollinators relationship

Pollination vector tests

The efficacy of vectors (wind and insects) in the pollination of *Z. boliviana* was tested and compared in experimental vector exclusion treatments (modified from Norstog et al. 1986; Tang 1987; Terry 2001) to: (i) show whether pollen was transported by insects and evaluate their effectiveness in pollination. For this test, we used a cloth to cover the ovulate strobili, placing it ~ 3 cm above the ground to block the entry of pollen delivered by wind while allowing the access of insects from below (*n* = 10); (ii) show whether the wind acted as a subsidiary in the pollination of *Z. boliviana*. We used a mesh cloth (mesh diameter = 0.15 mm) placed over the strobili and sealed at the peduncle with a cord, blocking the access of insects but allowing the entry of pollen delivered by wind. A lanolin ring was placed on the stalk before tying the base of the bag to discourage the entry of insects (*n* = 10); (iii) negative control – the action of both vectors (wind and insects) of the ovulate strobili was excluded to test for self-pollination or agamospermy. We used the same type of protection as the wind exclusion treatment, but sealed at the peduncle with a cord (*n* = 10); (iv) positive control − the ovuliferous strobili were left unprotected (natural state) (*n* = 21). The plants were located by actively searching for populations in Chapada dos Guimarães. The treatments were applied to the ovuliferous strobili prior to the pollen receptivity phase (Fig. 1B). Beetles (Coleoptera, Erotylidae) collected from polleniferous strobili and ovuliferous strobili were examined under a magnifying glass to identify the presence of pollen and obtain information on their pollination capacity. After the receptive period, the protections were removed so that the strobili could develop normally. The effectiveness of pollination for each treatment was determined by counting the number of seeds produced by the ovuliferous strobili (pollinated ovules) and after 300-350 days of their emergence. The pollinated and non-pollinated ovules were distinguished by their morphological aspects after the ovuliferous strobili's development. Pollinated ovules increase in size and develop a hard sclerotesta and red sarcotesta. Ovules that have not been pollinated remain small, opaque in color, and with undeveloped sclerotesta and sarcotesta (Fig.2B). The effect between treatments was evaluated based on t-test comparisons (Zar 2010), and analysis of variance with significance inferred by the Monte Carlo permutation algorithm (Olivier Bousquet et al., 2004) and estimation of the confidence interval.

RESULTS

Insect visitors, behavior and efficiency of vectors in pollination

Pollination vector exclusion tests

The reproductive success of *Z. boliviana* measured in the pollination control tests revealed that excluding winds does not affect the number of seeds developed (t = 0.6306; P = 0.5358 (Monte Carlo Permutation) (Table 1). On the other hand, seed yield was significantly affected (P = 0.000001; (Table 1; Fig. 2AB), with complete failure of the seeds developed when only beetles or beetles and winds are excluded from the pollination process (Table 1; Fig.3)

Table 1. Average percentage seed set in beetle-exclusion and wind-exclusion experiments.

Treatment	*N*	Mean	SE	IL 95% CI	SL 95% CI
Control strobili	21	16.5	3.7	8.9	24.1
Wind excluded strobili	10	20.9	5.4	9.9	31.8
Insect excluded strobili	10	92.7	5.4	81.7	103.6
Wind and insects excluded	10	93.1	5.4	82.1	104.0

Table 1.1. Seed formation

t=0.6306 P (Monte Carlo Permutation) = 0.5358

Group	Mean	SD	IL CI 95%	SL IC DE 95%
Control strobili	77.2	26.8	65.0	89.4
Wind excluded strobili	71.2	19.0	57.5	84.8

Mean = Média, SD = Desvio Padrão, IL = Inferior Limit, CI = Confidence Interval, SL= Superior Limit.

Table 1.2. Ovules no developed seed

	Sum of sqrs	df	Mean square	F	P
Between groups	68386.6	3	22795.5	76.3	
Within groups	14039	47	298.703		
Total	82425.6	50			Permutation P = 0.000001

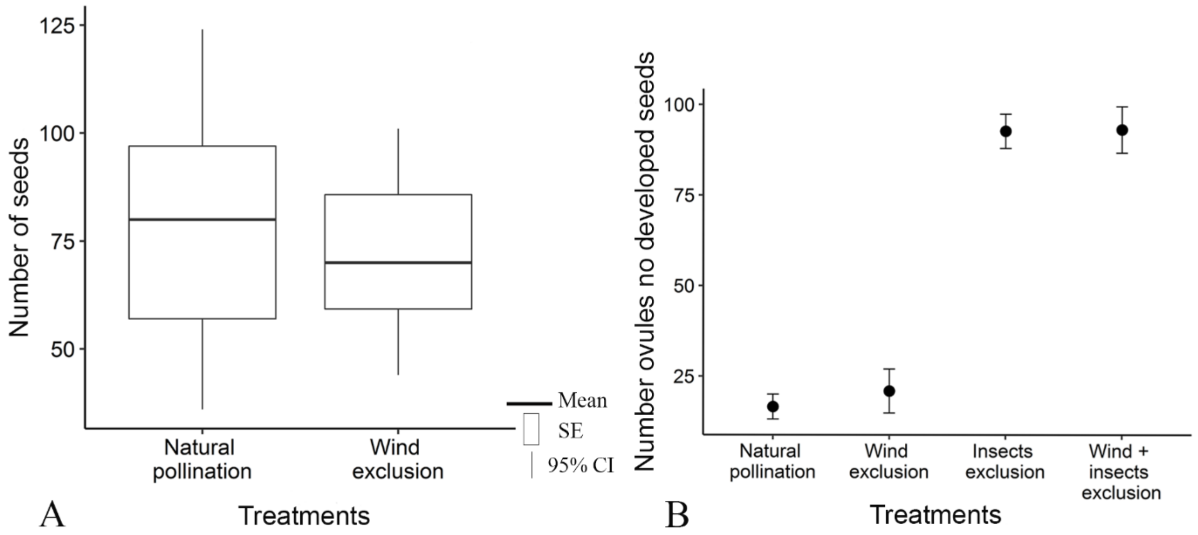

Figure 2. A. T-test and analysis of variance with significance inferred by the Monte Carlo permutation algorithm, and estimation of the confidence interval. Treatments: Control treatment (ovuliferous strobili kept under natural conditions, without excluding pollination agents) (n = 21); Wind excluded strobili (allowing the access of insects) (n = 10); Insect excluded (allowing the entry of wind) (n = 10); Wind and insect excluded (n = 10). The error bars indicate 95% confidence intervals. The exclusion of insect and wind and of insect only are not included in the model and their error bars are not displayed because all their replicates equaled zero. B. No developed ovules. The bars indicate a 95% confidence interval around the means. The results for natural pollination and exclusion of wind do not differ from each other, but are much lower than the results for exclusion of insects and of wind and insects. The results for exclusion of insects and of wind and insects not differ from each other.

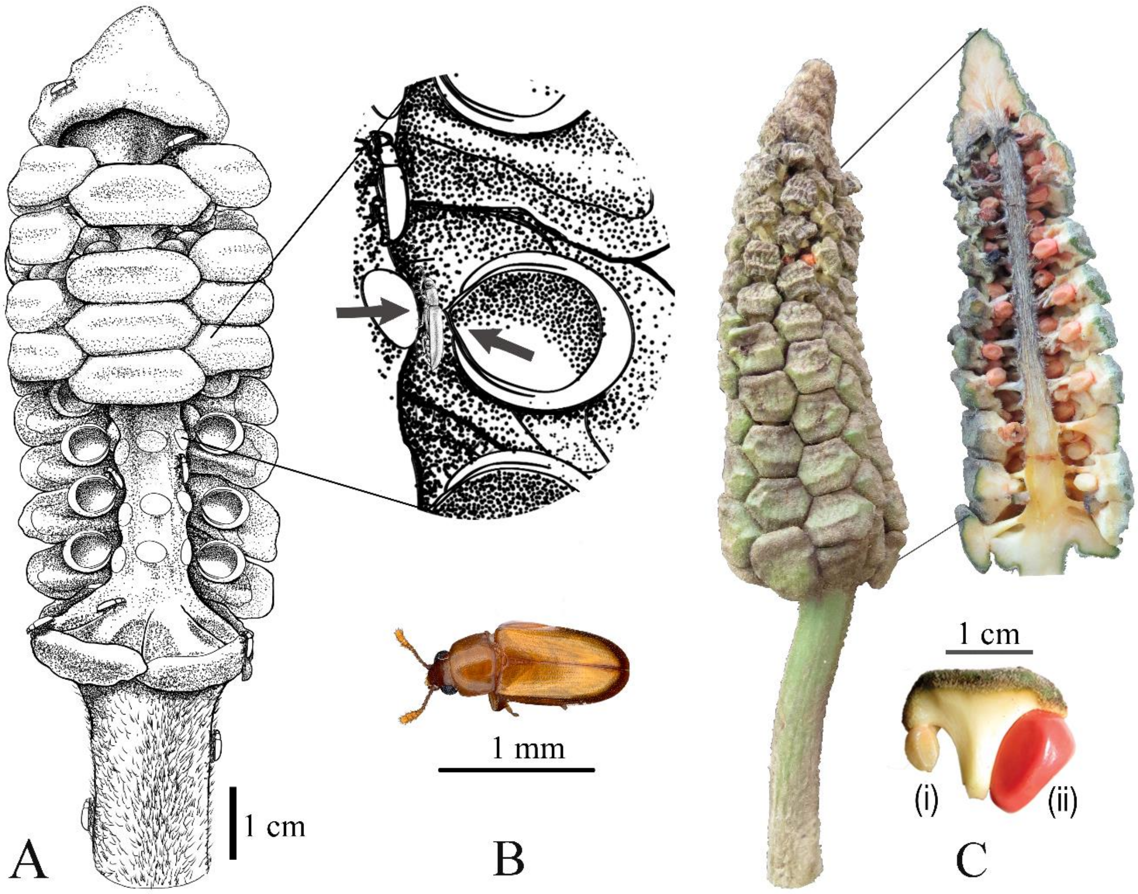

Figure 3. External and internal morphological structure of ovuliferous strobili of *Z. boliviana*. A. Strobili open with receptive ovules. The arrows indicate the thickness between the central axis of the megastrobili and the walls of the Megasporophylls. B. Strobili with non-pollinated ovules. C. Megasporophyll detail with non-pollinated ovules (i) and pollinated ovules or seed (ii).

Visitors and their behavior in the strobili

Visitors were classified into two groups: reliable (potential pollinator and herbivores) and unreliable. This second group of visitors was considered seasonal or sporadic in some of the reproductive cohorts between 2016 and 2019, and developed their activity at some point of the strobili’s phenophases. Figure 4A-I presents the occurrences of visitors in the strobili and summarizes their main behaviors during the visits.

Figure 4. Visitors of *Z. boliviana* strobili. A. *Janbechynea* aff. *paradoxa* on polleniferous strobili. B and E. *Plebeia* aff. *minima* on polleniferous strobili. C. *Eumaeus minyas* on ovuliferous strobili. D. *Trigonisca* sp. F. Chrysomelidae. G. *Camponotus crassus*. H. *Pheidole* sp$_1$. I. *Pheidole* sp$_2$. Scale bars = 1 mm. Credit: Rosane Segalla, personal collection.

We found *P. cerradensis* (Coleoptera: Erotylidae) consistently present in the successive reproductive cohorts (2016 to 2019) of *Z. boliviana*. *Pharaxonotha cerradensis* was found in the soil, near male and female reproductive plants, and in the polleniferous strobili and ovuliferous strobili (Fig.5), as described below. In the polleniferous strobili, the beetle is attracted when the organ is about to be distended from the microsporophyll (Fig.5-2 ~ 50 days), which is followed in one or two days by the onset of pollen dehiscence, with average duration of three to four days. The frequency of individuals in the pollen structure increases gradually, concomitantly with the distension of microsporophylls and opening of microsporangia. The polleniferous strobili's physiological state, the plant's pollen availability and its density with their maturing determine the amount of *P. cerradensis* individuals in these structures. The estimation of the number of beetles per microstrobilus showed an average of 60 *P. cerradensis* individuals in the pollen release phase. In the pre-pollen dehiscence phase, these beetles begin patrolling the soil structure for the polleniferous strobili. As the microsporophylls distend and the microsporangia open, *P. cerradensis* repeatedly visits and forages the external region and the microsporophylls' pores, usually from the base to the apex of the pollen structure. As it finds open microsporangia, it consumes the parenchymatic content (pollen), a process that results in its body structure becoming immersed in pollen (Fig.5-3). Generally, the patrolling and foraging of the outer surface alternate with the accommodation of individuals in the microsporophylls' pores. The colonization of the pollen structure occurs until the microsporangia completely opens, and declines with the decrease in the resource, when the individuals gradually move to other polleniferous strobili undergoing a similar maturation process.

This species often demonstrates poor light tolerance and commonly focuses its foraging on the side opposite to the source of light, or on the parts of the strobili that are in contact with the leaf litter. Although it visits the strobili day and night, it seems more active at night, preferably at dusk, on cloudy days and in plants that grow in full shade. It moves rapidly on the ground and seems to prefer land to air, but can take flight frequently. When it is foraging or housed in the strobili, it commonly exhibits elusive behavior when sensing touch and vibration of the pollen or ovulate structures. In these cases, it tends to move immediately through the peduncle of the organs to the ground, falling or lifting flights. The beetle lodges itself in the bracts, next to the cycad's xylopodium, but was not found in the flowers of associated angiosperms. The microstrobili section demonstrates that the beetle reproduces within the polleniferous strobili (Fig.5-5). Over the whole process of colonization, mating, oviposition, larval development and emergence of the adult, the microstrobilus serves as a place of copulation, incubation, shelter and food for beetles and their offspring (Fig.5). *Pharaxonotha cerradensis* was the only visitor with occurrence in the ovuliferous strobili, remarkable body pollen load over its stay and foraging activity in the polleniferous strobili (Fig. 5-2.1;3).

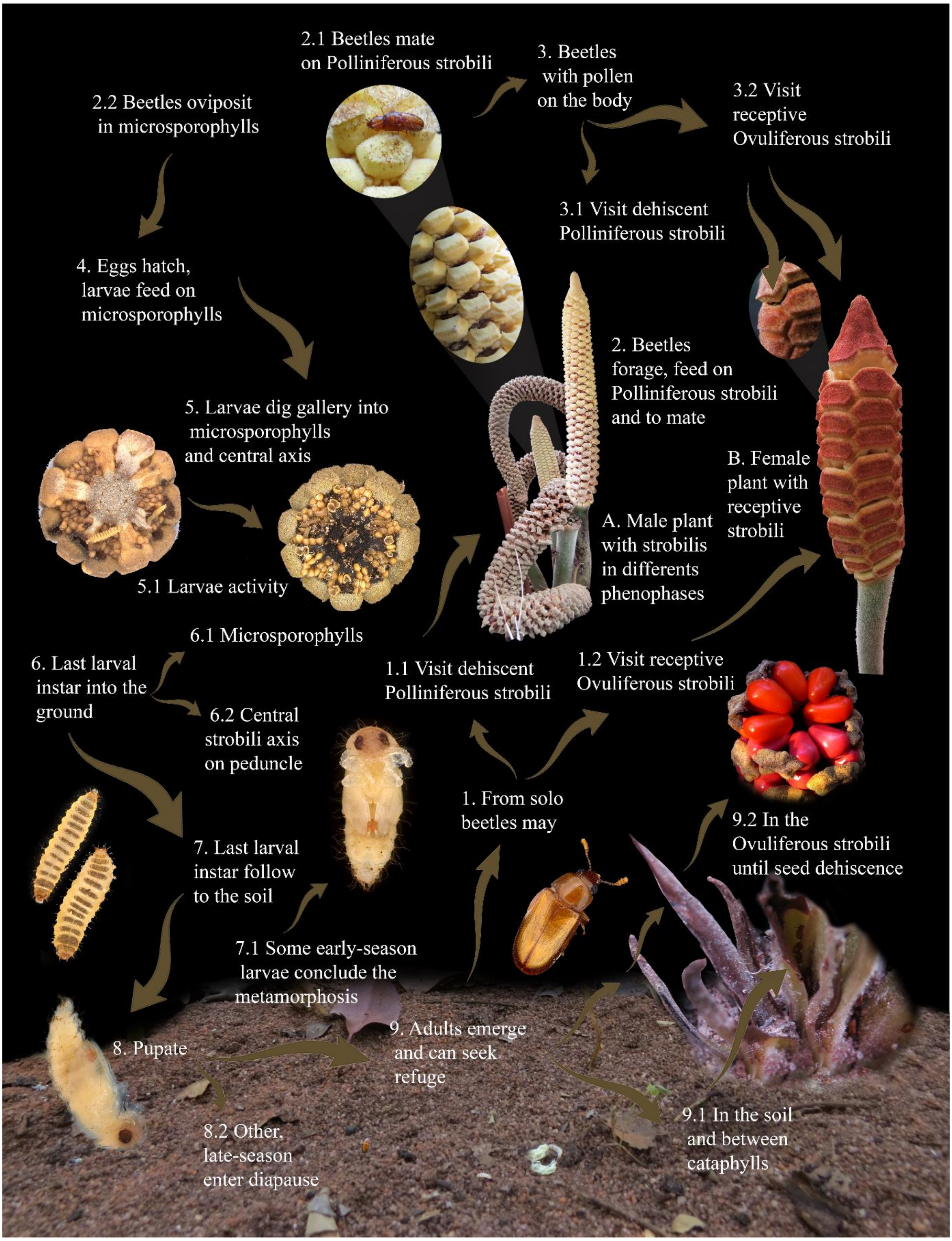

Figure 5. Reproductive biology of *Zamia boliviana* with brood-site pollination by *Pharaxonotha cerradensis* Skelley and Segalla (Coleoptera: Erotylidae) in brood-site mutualism. A. Male plant with strobili in different phenophases. B. Female plant with receptive strobili. Behavior of beetles in polleniferous strobili (1.1; 2; 2.1; 2.2; 3.1) and ovuliferous strobili (1.2; 3.2; 9.2). Cross-section of a polleniferous strobili and details on the feeding, mating, oviposition, and larval activity of beetles. 7.1 Some early-season larvae conclude the metamorphosis. 8.2 Other individuals enter late-season diapause and await the emergence of new strobili in the next reproductive season (according to Norstog and Fawcett 1989). 9. Emergence of new adults. The entire cycle is completed in about 8-10 days. Credit: Rosane Segalla, personal collection.

In the ovuliferous strobili, the beetle can be found day and night. The convex morphology of the ovuliferous strobili (Fig. 3A) makes it difficult to record their internal activities and the delivery of pollen to the ovules' micropyles. The attempts to record its activity using an inspection camera were unsuccessful, mainly due to its agility and elusive behavior under light, its sensitivity when sensing touch, and our difficulty accessing the pores of the ~ 0.5 mm strobilus. However, we performed recurring observations of the beetle (day and night), usually in groups of individuals housed at the base of the receptacle, at the apex, and more rarely in pores of the mid-section (Fig.5-3.2). The number of individuals was estimated from samples of bagged and dissected ovuliferous strobili. We found an average of 6 individuals per receptive megastrobilus, which is lower than the estimates made for polleniferous strobili. Habitat observations and fluorescent dye tests indicated that *P. cerradensis* moves through the walls that support the megasporangia, possibly brushing the ovules and accidentally delivering pollen to their microphylls. The microphylls are strategically turned towards the inside of the megastrobilus' axis, and the spaces between the ovules and the central axis (~ 3 mm) correspond to *P. cerradensis*' body size (Fig.3A). We did not find pollination drops or any kind of reward on the ovuliferous strobili. We also found no predation of ovules by *P. cerradensis,* although they were observed to guard the ovuliferous strobili. The individuals positioned themselves in the pore while facing the outside of the organ, constantly moving their antennas. When feeling vibrations in the structure, one or two *P. cerradensis* individuals moved from their position or from inside the ovuliferous strobili and traversed the external surface of the organ to inspect it, after which they returned to the same niche.

We also observe *Eumaeus minyas* (Hübner, [1809]) on *Z. boliviana* in all reproductive cohorts of the cycad from 2017 to 2019. Phenological records of reproductive cohorts within this period revealed that the occurrence of *E. minyas* in polleniferous strobili and ovuliferous strobili is recurrent. The visits started happening since the strobili's emergence phase, preferably to plants that grew in full shade, in the early hours of the morning or at dusk. The individuals' landing on and probing the strobili were followed by oviposition on these structures (Fig. 4C). *Janbechynea* aff. *paradoxa* (Monros 1953), (Coleoptera: Polyphaga: Orsodacnidae) was the only seasonal and solitary phytophagous herbivore observed on polleniferous strobili in the pre-release phase (Fig. 4A). With low density (*n*=3) and acting in the morning and late afternoon, its activity was limited to the consumption of tissues of the microsporophylls.

Seasonally or opportunistically unreliable visitors as *Plebeia* aff. *minima* (Gribodo, 1893) (Fig. 4E) and *Trigonisca* Moure, 1950 (Fig. 4D) were observed visiting only polleniferous strobili in the pollen release phase, in sporadic and opportunistic occurrences and, therefore, with low density of visits, both among and within reproductive cohorts. Its activity was restricted to foraging and pollen collection. Ten individuals of a Chrysomelidae, Eumolpinae (Fig. 4F) species were observed, exclusively foraging in polleniferous strobili in the pollen release phase. We also registered three ant species (Fig. 4G-I), with predominantly patrolling and foraging behavior. *Pheidole* sp_1 and *Pheidole* sp_2 Westwood, 1839 were observed obtaining food in the pollen release phase, exclusively in polleniferous strobili. *Camponotus crassus* Mayr, 1862 (Fig. 4G) visited both structures at different lifespan phases, including the pollen release and receptivity phases, but was not observed entering the ovuliferous strobili's pores. All these species showed no remarkable body pollen load.

DISCUSSION

This study extends the knowledge of the biology and reproductive ecology of cycads, especially for this *Zamia* of the Cerrado, a South American ecosystem with high frequency of habitat loss. In fact, a high level of specialization was found between *Z. boliviana* and *P. cerradensis*, the most probable pollinator for this *Zamia* species. This insect was present during almost all reproductive phases of male and female plants, acting as the main pollinator. In addition, *P. cerradensis* also uses *Zamia* reproductive organs to reproduce, characterizing a brood site pollination mutualism. Previous studies have also found brood-site pollination in other *Zamia* species (Norstog et al. 1986; Tang 1987; Norstog and Fawcett 1989; Franz and Skelley 2008; Valencia-Montoya et al., 2017). Our research highlights the importance of describing intricate plant–pollinator interactions in order to understand the evolution of pollination syndromes in ancestral clades, and also providing crucial data for management of threatened plant groups.

Insect visitors, behavior and efficiency of vectors in pollination

The follow-up of four reproductive cohorts of *Z. boliviana* and performance of observational and experimental studies corroborate our hypothesis that the pollination of this cycad is mediated by a biotic vector. The results of the vector exclusion tests notoriously emphasize the effectiveness of curculionids in the pollination of *Z. boliviana*, and demonstrate that the effect of wind is not an effective pollen vector.

In line with previous studies, our results showed that wind plays no subsidiary role in pollinating *Z. boliviana*, according to the following lines of evidence: i) the pollinator exclusion experiment (Table 1; Figs. 2AB and Fig. 3) demonstrated that pollination was unfeasible in the absence of *P. cerradensis*. Comparatively, the exclusion of wind had a negligible effect on pollination, because the presence of beetles was sufficient to produce a result similar to natural pollination; ii) the morphological-architectural structure of *Z. boliviana* polleniferous strobili and ovuliferous strobili (Fig. 4 and 5), arranged at an average height of 12 cm from the ground during the strobili's pollen release and receptivity phase, result in: (a) most of the pollen mass being shed by gravity near the polleniferous strobili (~ 5 cm in circumference); (b) a natural barrier to the arrival of pollen to the micropyles by air being created by: the ~ 0.5 mm pores, usually only at the base and apex of the ovuliferous strobili; the arrangement of ovules with the micropyles facing inwards and with a ~ 2 mm space between the micropillar array and the wall of the shaft supporting the megasporophylls; the thickness of the megasporophylls' walls (~ 3 mm), and; the presence of a sterile apex on the distal end of the ovuliferous strobili, and iii) the phase of receptivity to the pollination of the ovuliferous strobili not always being synchronized with the polleniferous strobili's pollen release phase, near female plants. Although the reproductive events of *Z. boliviana* coincide with favorable weather conditions for wind pollination (dry period of the year), the plants mostly pollinated by this vector must have a morphology and physiology that is compatible with the mechanisms ensuring that the pollination process is complete (FaegriandVan der Pijl 2016; Proctor et al., 1996), conditions that seem incompatible for *Z. boliviana*.

On the other hand, we found that a single species of Erotylidae co-occurs with populations of this cycad and performs its life cycle in association with pollen strobili, in synchronous and seasonal reproductive events (Skelley and Segalla 2019), and, concomitantly with the reproductive period of both, also visits ovulate strobili (Fig. 5). Indeed, several studies have demonstrated that beetles (Coleoptera) in the family Erotylidae (Cucujoidea) are important pollinators of cycads (reviewed in Skelley et al. 2017). Previous pollination studies with Zamiaceae species (Norstog et al., 1986; Vovides 1991; Donaldson 1995; 1997; Terry 2001; Hall et al., 2004; Terry et al., 2005; Valencia-Montoya et al., 2017) found limited or no pollination of polleniferous strobili in tests from which insects were excluded and, therefore, weak or no presence of wind pollination in the cycads studied. Thus, the observations and experiments we conducted with *Z. boliviana* show that biotic pollination in this cycad substantially outweighs wind pollination and corroborates about 30 other biology and ecology studies with Zamiaceae (reviewed in Segalla et al. 2019), which show the predominance of biotic pollination for the family. The *Pharaxonotha* Reitter (Coleoptera: Erotylidae) genus is found in the strobili of all four genera of cycads (*Ceratozamia*, *Dioon*, *Microcycas*, and *Zamia*), endemic in the New World and predominantly Pantropical (Vovides 1991; Chaves and Genaro 2005; Franz and Skelley 2008; Terry et al., 2012; Tang et al., 2018), and is the first cycad pollinator recorded in Brazil.

Our findings reveal that *P. cerradensis* was the only visitor that demonstrated the compatible requirements of a potential pollinator of *Z. boliviana*. It's habits, when it is in direct interaction with the plant, are consistent with pollination in an obligatory and specialized process of brood-site mutualisms. The palynivore habit of *P. cerradensis*, together with its morphological characteristics (Skelley and Segalla 2019) and behavioral evidences, corroborate that this is the only known visitor capable of performing activities as an effective pollinator of *Z. boliviana*, for the main reasons of: a) not damaging the strobili's lifespan; b) reproducing in the polleniferous strobili without damaging the pollination process; c) being present with high frequency and density in the polleniferous strobili (mean n=60) and ovuliferous strobili (mean n=6) compared to other visitors; d) and having concomitant seasonal frequency with the emergence of strobili, both among and within reproductive cohorts of the cycad. The morphological characteristics of *P. cerradensis*, mainly the presence of hair on the dorsal region and on the antennas (Skelley and Segalla 2019), combined with the characteristics of the ovuliferous strobili, possibly favor the adherence of pollen grains to the beetle and their subsequent detachment from the ovule's micropyle upon contact with the insect as it walks along the central axis and, consequently, the set of megasporangia.

Generally, the morphological characteristics of the reproductive system of plants pollinated by beetle, as occurs in certain species of Arecaceae, as well as curculionid beetles, constitute an important part of the plant-pollinator interaction structure (Barfod et al. 2011; Skelley et al. 2017, de Medeiros et al., 2019). The natural history of specialized brood pollinators associated with a generalist plants is reported for other tropical species (e. eg.: Arecaceae species, Barfod et al. 2011; de Medeiros et al. 2019). A number of pollination mechanisms in cycads and palms have provided direct or indirect evidence for an framework of interaction, which combines morphology attributes involving beetle-pollinated species (Barfod et al., 2011). The most common beetle visitors of cycads strobilis and palm flowers are the weevils (family Curculionidae) (Barfod et al., 2011). Exclusive pollination by beetles (or more rarely thrips) that breed on plant tissues is a very common condition in palms (de Medeiros et al., 2019), as in Zamiaceae species (Segalla et al., 2019).

Although rewards that are nutritional (pollen grains, nectar, stigmatic exudates, oils, resins, gums, tissues), or related to, oviposition sites (shelter, sexual attractants, brood sites) are considered the major rewards to pollinators (Simpson and Neff 1983; Barfod et al., 2011), these rewards are known to be restricted to pollen strobili in cycads (e.g.: food – parenchymatic content 'pollen' and brood sites). Whether pollinators receive any significant reward from the ovuliferous strobili for their pollination services is not known (Terry et al., 2001). In this case study we assume that the beetle is probably mistakenly attracted to the ovuliferous strobili when volatiles are released from the plant, but may temporarily remain in the structure, induced by comfort and thermal shelter, which are useful for the maturation of their halteres. We assume that the sentinel-like behavior is inherent to their interaction with the host plant. Most likely, these rewards are insignificant compared with polleniferous strobili that provide warmth as well as a nourishing breeding and feeding environment (Terry et al., 2001). However, the costs of delivering pollen to the ovuliferous strobili may be small if the insects are only temporarily duped by ovuliferous strobili that use similar cues as polleniferous strobili (Terry et al., 2001). Consistent morphological relationships between plant and beetle seem to indicate adaptations of both towards a mutualistic interaction.

Studies on the ecology and behavioral evolution of brood-site mutualisms and associations may provide new insights into the evolutionary history (e. eg.: conditions on which these interactions are favoured, according to de Medeiros et al., 2019). In the current global scenario of species loss and vulnerability and their imbricate pollination systems as New World cycads, these studies are fundamental for applicability mainly to conservation (Roemer et al., 2008, Tang et al., 2018; Nunes et al., 2018; de Medeiros et al., 2019).

Seasonally reliable visitors – cycad-herbivore relationships

Although butterfly *E. minyas* visits strobili seasonally, their participation has no relationship with the pollination of this cycad. Its main interest is restricted to oviposition in the reproductive and vegetative structures of *Z. boliviana*, because it is an obligatory herbivore in the larval stage (Prado et al. 2011; Segalla and Morellato 2019). *Janbechynea* aff. *paradoxa* had low frequency in the cycad populations monitored, and were found in only one population, relatively isolated from the others. Prado et al. (2012; 2014) suggested that specialized Coleoptera are synchronized with the emergence of new foliage due to the smooth texture of their leaflets. Herbivorous Coleoptera, such as the *Janbechynea* genus, represent a problem for the cycads if their densities and frequencies are high because they can quickly decimate plants, as highlighted by Reyes-Ortiz et al. (2016). However, these episodes would be less likely *in situ* populations due to the distribution of cycads in biodiverse habitats, as is the case of *Z. boliviana*. Future studies about these relationships can provide new insights into their origins and behaviors (Prado 2011).

The ants observed on *Z. boliviana* strobili feeding on pollen were considered opportunistic in obtaining this resource. *Camponotus crassus* was not considered a pollinator due to its inconsistency or poor fidelity to the strobili. In general, ants have limitations to pollinate due to their lack of hairs or smooth integument and the frequency with which they clean their bodies, limiting the adhesion of pollen (Conceição et al., 2004). In addition, the presence of the metapleural gland in most species, producing lipophilic substances, may inactivate the pollen (Beattie 1985). These and other characteristics make the probability of pollination almost nil, and the low chance of success of cross-pollination (Conceição et al., 2004), a feasible condition for *Z. boliviana*. The knowledge about the reliability of plant-visiting ants is scarce, and the true pollination potential of these insects extends to tropical cycads. However, their role in pollination, if any, awaits further research.

Our research joins the evidence accumulated over the last three decades showing that insect pollination is widespread in New World cycads (Tang et al., 2018b), as well as that most angiosperm and many gymnosperm species depend on animal pollinators for production of seeds and maintenance of their populations, mainly in the neotropical region. The relatively high levels of specialization among plants-animals in tropical regions may suggest a relatively long period of climatic and ecological stability, necessary for such interactions evolve (Johnson et al., 2009), a legacy to be understood and preserved. Terry et al. (2001) comment that whatever the evolutionary reality, it is interesting that within one cycad genus, there are such finely tuned specialist pollinators, representing different insect orders that have many associations with basal angiosperms. Although the majority of cycads in the New World host more than one species of beetle associate in their strobili and some of them host as many as three species (Tang et al., 2018b), only *P. cerradensis* occurs in *Z. boliviana*. Independently of diversity, animal-mediated pollination usually leads to some degree of outcrossing and thus promotes and maintains genetic variation in populations, which in turn allows species to adapt to new and changing environments (BDC 2018). Our case study supports the understanding that mutualisms between plants and their floral visitors sustains not only plant diversity, but also the diversity of an estimated 350,000 animal species with different degrees of ecological dependence, mainly insects, birds and mammals according Ollerton (2017).

CONCLUSIONS

The follow-up of reproductive cohorts and description of visitors of *Z. boliviana* highlights the importance of knowledge of the natural history as a subsidy to other research questions. The knowledge of biology of the reproductive systems of cycads and their pollinators is fundamental to ecological studies, especially in tropical regions. Indeed, insect pollination is a critical facet of cycad biology and conservation (e.g.: Roemer et al., 2008; Terry et al., 2016; Tang et al., 2018ab). In this study, we showed that different insect species, such as Coleoptera, Hymenoptera and Lepidoptera, visit *Z. boliviana* strobili, but most of them are not characterized as pollinators, mainly due to their low frequency and density in the strobili, and their preference for polleniferous strobili. Additionally, the ovuliferous strobili's morphology and the exclusion of wind as a pollen vector, in addition to the high rate of seed pollination and the absence of known agamospermia for *Z. boliviana* reinforce the finding of this study that *P. cerradensis* predominantly meets the requirements of an effective pollinator compared to other visitors. However, it is still unknown whether there are other species acting in the transportation of this cycad's pollen. Our investigation revealed the existence of essential and specialized brood-site mutualism between insect-plant, in a process that allows the viability of the reproductive cycle of both *Z. boliviana* and *P. cerradensis*. In addition, it enriches the data for testing hypotheses about the taxonomy and biogeography of cycads, especially South American cycads, in more depth (e.g.: Tang et al., 2018b).

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GENERAL CONCLUSIONS

Fui criada no mato e aprendi a gostar das
coisinhas do chão – Antes que das coisas celestiais.
(Modificado de BARROS, Manoel de.
Retrato do artista quando coisa. Rio de Janeiro: Record, 1998.)
Photo credits: Rosane Segalla, personal collection.

This dissertation presents the current knowledge on Zamiaceae, with emphasis on *Zamia* from South America (Chapter I). It contains the results of phenological patterns (Chapter II) and sex ratio (Chapter III) from the case study with populations of *Z. boliviana* in the natural environment. It also gathers biotic interactions associated with this cycad. One case is the registration of *Eumaeus minyas* Hübn. (Lepidoptera: Lycaenidae), first for a species of *Zamia* in Brazil (Chapter IV). The other study case describes a species of *Pharaxonotha* (Chapter V) as the only potential pollinator of this cycad. These ecological interactions and other opportunistic or non-obligate visitors have been carefully described over approximately five years of observations.

It is promising that many of the genera and species of this family are already well-studied. However, after considering the species richness of the genus *Zamia* in South America, where many of them are still biologically, ecologically and phylogenetically little known according to the systematic review of Zamiaceae (Chapter I) and the case study with *Z. boliviana* (Chapter II-V), we conclude that:

i) *Zamia boliviana* was a good model for initial studies with dioecious species, especially *Zamia* from South American ecosystems;

ii) Phenological studies that identify recurrence patterns in biological events, such as the phenological study with *Z. boliviana* (Chapter II), are expensive in time and resources but are essential in the tropics. Our study with *Z. boliviana* consisted of populations in the natural environment and is unprecedented for *Zamia* spp. from South America. The results are promising as they showed a synchronous reproductive phenological pattern in the two populations studied, which is an essential requirement for the reproductive health of dioecious populations;

iii) Studies focused on the origin of patterns and processes involving dioecious systems, such as those that pointed out a predominantly male bias in *Z. boliviana* (Chapter III), are recommended for other dioecious species. Long-term monitoring of dioecious populations in their natural habitat may bring greater clarity to studies of this nature and lend support to the arguments that deterministic forces (e.g., environmental filtering) and stochastic processes (e.g., anthropic effects, variation in sex ratio and population density, resource availability) possibly regulate the dynamics of these ecological systems. These studies are fundamental to predict the likely responses of these systems to environmental changes;

iv) Data surveys on the natural history of tropical biodiversity, such as the antagonistic and obligate interaction between *E. minyas* and *Z. boliviana* (Chapter IV), are important, especially at present. Such studies allow identifying not only agents in interaction but the dependence level with their host plants, the areas of co-occurrences and evaluating the stability of these associations in times of constant habitat change, and finally,

v) Studies that demonstrate opportunistic or mutualistic ecological interactions, including our description of specialized and obligate mutualism between *Z. boliviana* and *P. cerradensis* (Chapter V), should be prioritized. This study model show plant-pollinator interactions from the perspective of dioecy, an obligate mutualism, and the consequences of imbalance or loss of these interactions for both. A good understanding of these interactions will bring more light to the accumulated knowledge and allow us to advance towards new concepts about reproductive systems and the exactitude of these contemporary biological interactions.

Overall, our research using the case study with *Z. boliviana* showed that the reproductive events (as shown in the phenological and sex ratio study) of this cycad are shaped by the bioclimatic and seasonal factors of the Cerrado. Also, the life cycles of *E. minyas* and *P. cerradensis* are synchronized with the lifespan of the organs of this host plant.

The biological and socio-cultural importance of cycads and other dioecious species makes studies on this group of rare, endemic and long-lived species, as well as their interactions, even more essential. Although the knowledge of the biological history of these species in their original habitats is challenging, they should be encouraged within the scientific community. The gains from these efforts are directed towards science and societies. The integration of results, including those from this case study, to other areas of science produces more complete responses with positive effects for different decision-making purposes.

New challenges include fostering and implementing actions with public or private organizations and society, in general, to continue studies similar to the current study. Furthermore, the application of results in conservation strategies of dioecious populations, especially *Zamia* spp., in public or private areas and biological collections is crucial. It should be a permanent attempt to safeguard integrating and sustaining elements of a region with marked biodiversity hotspots. Most of them are still little known and have been defined by historical processes under the influence of contemporary factors.