



# Male-biased effective sex ratio across populations of the threatened *Zamia boliviana* (Zamiaceae)

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**Abstract** Populations of dioecious plants commonly exhibit dissimilarities to the equilibrium expectation of a 1:1 sex ratio. Differential expenditure for reproduction between genders is cited as the primary mechanism responsible for a male-biased sex ratio, with increased effects on long-living species, but these mechanisms are still poorly understood. We explore the sex ratio in the endemic gymnosperm *Zamia boliviana* (Zamiaceae) populations from the Brazilian savanna (the Cerrado). We aim to investigate what the *Z. boliviana* sex ratio is, and whether population density and ecological correlates lead to variation in the sex proportion among Cerrado habitats. The study was conducted on ten in situ populations of *Z.*

*boliviana* at sexual maturity (tertiary sex ratio). We estimated the populations' sex ratio and performed a redundancy analysis to assess the relationship between biotic traits, such as sex ratio, and associated environmental features. Soil texture classes were used to classify the cycad habitats and were expressed in a ternary phase diagram. The results show a significant male-biased sex ratio in seven of the ten populations surveyed. Environmental factors did not explain the redundancy in the reproductive characteristics. However, the cycad occurs in different habitats in their endemic zone. Our study provides new biological data for *Z. boliviana*, suggesting that the differential reproductive expenditure of sexes in reproduction is governing the mechanisms of sex ratio

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variation, compared to local environmental factors in this cycad. The pattern of effective sex ratio found here improves our understanding of mechanisms causing biased sex ratios in cycads and other dioecious species from tropical ecosystems.

**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 commonly in gymnosperms and approximately 6% of angiosperms (Renner and Ricklefs 1995). 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-living species (Renner 2014; Käfer et al. 2016; Barrett et al. 2010). The obligate outcrossing mechanism imposed by dioecy means that the absence of potential mates of the opposite sex renders an effectively sterile individual (Käfer et al. 2016). Dioecy is commonly associated with a suite of life histories and reproductive traits (Barrett et al. 2010). The dioecious system is therefore complex, because it involves sexual specialization, incurring genetic, demographic, and ecological costs (Renner 2014).

The term “sex ratio” *latu senso* is defined as the number of males in relation to females in a given group of organisms (Calonje et al. 2011; Geodakyan 2012). However, the sex ratio of a population may change over time and can be formally split into three categories: primary sex ratio (ratio of males to females after fertilization); secondary sex ratio (ratio at birth); and tertiary sex ratio (ratio at sexual maturity) (Majerus 2003). 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). In Cycadaceae and Zamiaceae species it is recognized that if each year males and females produce strobili with equal frequency, then the larger the seedling group in a

population, the smaller the chances become of it consisting exclusively of either males or females (Grobelaar 2002). Therefore, chances increase that the recruitment of this population will contain both sexes (Grobelaar 2002). These proportions are generally assumed for healthy populations (Ackleh and Zhang 2009; Barrett 2015). Thus, there can be different factors that cause sex ratio variations among populations in the natural habitat.

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 (Delph 2019). 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. 2007; Morellato 2004). According to Allen and Antos (1988), the costs of reproduction are often higher in females, causing strong biases in the sex ratio due to earlier initiation of flowering, more frequent flowering, and higher mortality of females than of males (Allen and Antos 1993). Barrett et al. (2010) list at least three factors related to the costs of reproduction that 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. 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.

Cycads are dioecious plants, with 356 taxa found around the world (Calonje et al. 2021). These plants typically take a long time to reach sexual maturity, with plants in a population that do not produce strobili regularly, and therefore, the reproductive phenology is often asynchronous in a particular given season or between seasons (Grobelaar 2002; Jones 2002). In cycad populations, deviations from the expected 1:1 sex ratio were reported for species of the families Cycadaceae and Zamiaceae, with populations commonly showing a male bias in most assessments of

tertiary sex ratio (Grobbelaar 2002; Calonje et al. 2011). According to Calonje et al. (2011), the cumulative sex ratio may vary widely in wild populations, but little is known about local habitat conditions that can influence the balance of male and females (Grobbelaar 2002).

Here we investigate the tertiary (phenotypical) sex ratio of *Zamia boliviana* (Brongn.) A. DC. (Cycadales, Zamiaceae) (Fig. 1), a species occurring in woody Cerrado vegetation (Fig. 2). We were interested in determining the sex ratios of reproductive plants of these cycad populations in situ and how this ratio varies in different Cerrado habitats (Fig. 2). Thus, we tested the hypothesis that the tertiary sex ratio in *Z. boliviana* may be affected by local 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*? Does it differ from the expected 1:1 ratio? (2) What are the predominant characteristics of *Z. boliviana* habitats regarding soil physical properties and light intensity, and are those variables related to the sex ratio of local *Z. boliviana* populations?

## Methods

### Study species

*Zamia boliviana* is a small perennial plant (0.80 m average height above ground), with a subterranean stem (xylopodium) and between one and three leaves in each crown in adult plants and individuals of both sexes are randomly distributed at distances varying from 1 m or more (Fig. 2d, e, g). 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). *Zamia boliviana* can have a single xylopodium or multiple xylopodia. The multiple system comprises branched or clonal xylopodia, but with very limited spread and joined by portions of stem or roots at the base (Fig. 2h). This morphology, although there are exceptions,

have distinguishing clumps of plants of the same sex above the ground. According to phenology records (Soares 2020), the vegetative and reproductive structures are formed in the soil during the dry season. The plants can become deciduous or brevideciduous for undetermined periods of time. Male plants can develop from one to five microstrobili per stem or branch (apical meristem) of xylopodium (Fig. 2a, b), whereas female plants produce only one megastrobilus per stem or branch (Fig. 2c–f). Starting from soil emergence, the lifespan of the microstrobili lasts more or less two months, while megastrobili can last up to ten months (Soares 2020).

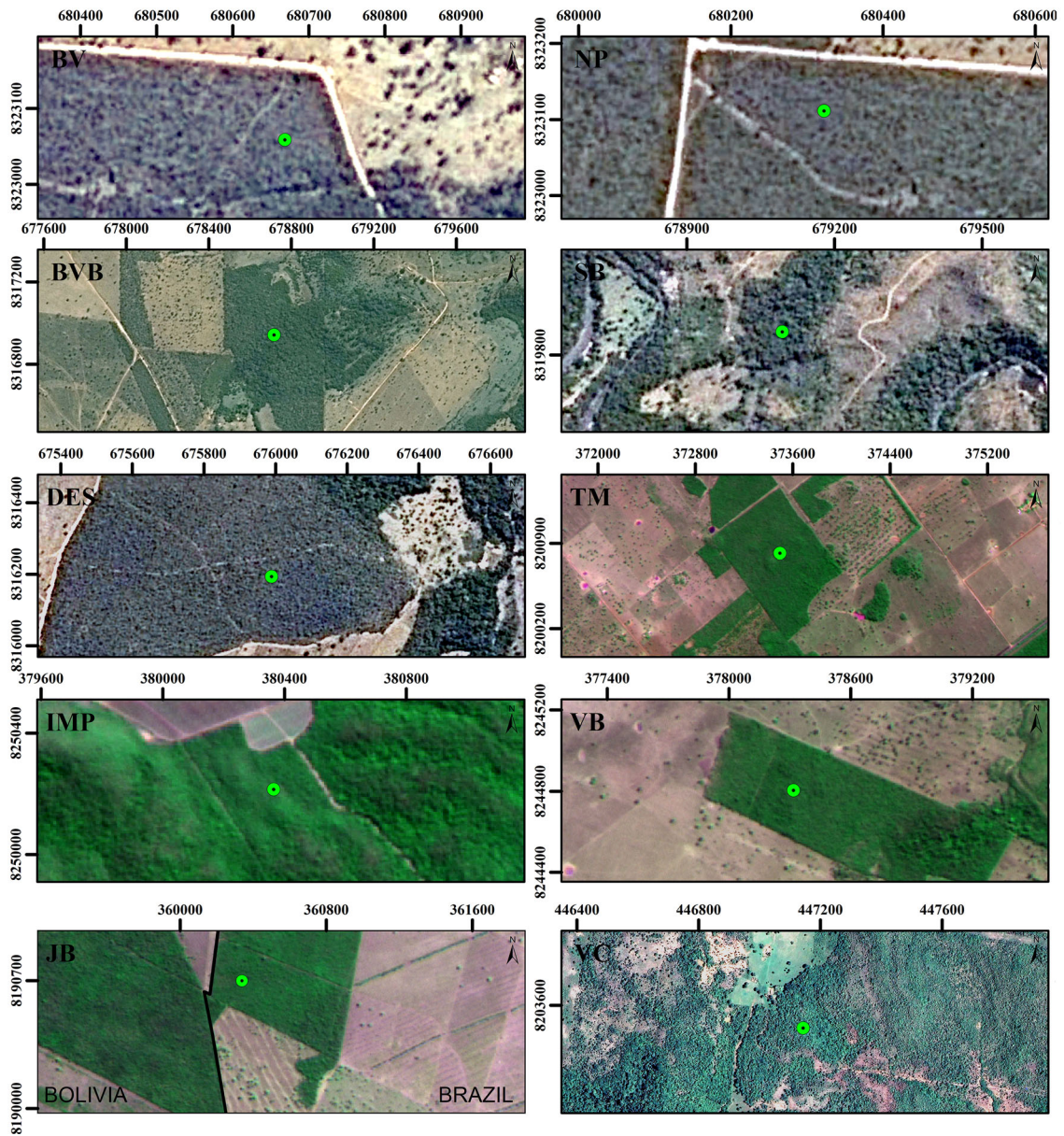
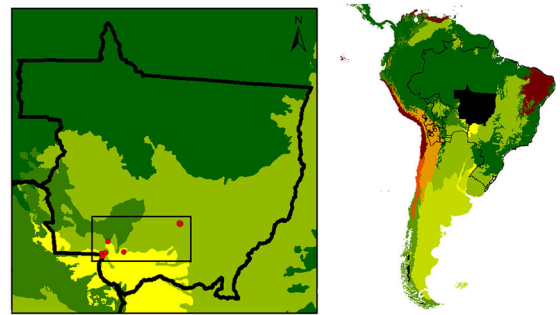
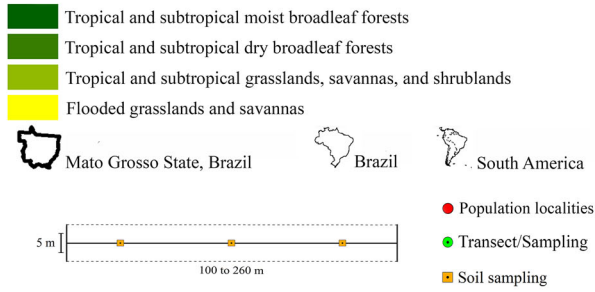
### Study region

Populations of *Z. boliviana* are distributed within the intertropical zone in the central portion of South America (Fig. 1) (Segalla et al. 2019). The habitats are characterized by high total solar radiation incidence throughout the year (SEPLAN 2000) and equatorial and tropical hot climates, with little seasonal or annual temperature variation (Köppen 1918; Geiger 1954; Kottek et al. 2006). 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 °C to 26 °C, low temperatures from 18 °C to 23 °C, and high temperatures from 25 °C to 30 °C (Karger et al. 2017).

*Zamia boliviana* distribution is restricted to parts of Bolivia (Beni, Cochabamba, La Paz, and Santa Cruz) and Brazil, in the state of Mato Grosso (MT), within an elevation range of 130 m to 450 m above sea level, and different vegetation types within the Cerrado biome (Oliveira-Filho and Ratter 2002; Ribeiro and Walter 2008), mainly the Cerrado sensu stricto (Brazilian woodland savanna), hereafter just Cerrado (Table 1, Fig. 1) (Segalla et al. 2019). Cycad occurs in relatively flat landscapes or terrain exhibiting minor changes in topography (Segalla et al. 2019; SEPLAN 2000). Slopes predominate in the region of Chapada dos Guimarães (MT), with the formation of deep valleys (SEPLAN 2000). *Zamia boliviana* frequently form dense clumps of individuals of both sexes in different successional stages or at different reproductive ages (Soares 2020). Most of the populations have been decimated or severely fragmented by the clearing



Biomes



◀ **Fig. 1** Geographic distribution and locality of occurrence according to ecoregion 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: vegetation types, according to the classification of Olson et al. (2001), and the manipulation of geospatial data was done using QGIS

of land for agriculture in both countries (Table 1, Fig. 1) (Segalla and Calonje 2019; Segalla et al. 2019). During this study, a fire event occurred in the region of the DES site population one year before sex ratio evaluation (Table 1). This happened within the reproductive period of the *Z. boliviana* and evidence showed that seeds and emerged (megastrobili of previous season) and emerging or newly emerged strobili were probably burned. This prevented us from identifying the sex of many individuals during our sampling in that area in 2017.

#### Sampling, sex determination, sex ratio variation, and habitat characterization

To investigate sex ratio variation in different habitats of *Z. boliviana*, we sampled populations in ten sites from four distinct areas of 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 sex ratio at sexual maturity, the tertiary sex ratio according to Majerus (2003). We 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 xylopodium and, in some cases, the proximity of potential clonal individuals, which is not always distinguishable from above ground (Fig. 2). 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) (Table 1). Groups or clusters of at least 1,000 male and female individuals within a radius of approximately 10,000 square meters were considered as populations. Initially, transects of 5 × 100 m 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. The visual census of *Z. boliviana* was performed by means of active searching for, and marking of, adult plants within the 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 *Z. boliviana* (Soares 2020). Plants were classified as male, female, or unidentified plants (adult plants, but without strobili). This determination was made according to plant size ( $\geq$  to 0.80 m tall) and the number of leaflets per leaf ( $\geq$  12). In this study we considered each observable above-ground branch as an independent plant due to the impossibility of observing the nature of xylopodia (clonal or separate individuals) below ground. This criterion was defined after a pilot project carried out from within the VB area to better understand the biological system of this species. 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 indeterminate plants per sampling unit (transect). Other ecological information collected for the *Z. boliviana* populations is shown in Table 1 and was obtained as follows: predominant soil type; habitat types and light condition—on-site sampling carried out by this study or reviews in SEPLAN publications; and history of anthropogenic disturbances—information provided by landowners and our observations of evidence of disturbances in sampled sites. The specific geographical coordinates of populations are omitted to minimize the risk of illegal extraction of this species from the wild.

#### 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







**Fig. 2** Habitats and morphology of *Z. boliviana*: **a, b** Male plant: a. In Red-Yellow Latosols and b. In rocky calcareous soil. **c, d, g** In sandy Haplic Cambisols in cerrado, with occurrence of fire, clonal xylopodium and single xylopodium (g1-2) clonal adult plant with brevideciduous bud, (g3) 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

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 (Dane and Hopmans 2002; FAO 2006) as follows: (A) Five sand fractions—very coarse sand (VC); coarse sand (C); medium sand (M); fine sand (F); and 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”

**Table 1** Localities, codes, areas, and elevation for the sites of sex ratio sampling in *Zamia boliviana* across Mato Grosso State (MT), Brazil, and associated ecological information

Locality	Site Code	Area (ha)	Elevation (m)	Soil type <sup>b</sup>	Habitat type	Light condition	History of anthropogenic disturbances <sup>c</sup>	
							Past	Current
Chapada dos Guimarães	BV	3.27	423	Haplic Cambisols	cerrado	Partial shade (~ 65.51 light)	1; 2	1; 2
	BVB	33.88	431	Haplic Cambisols	Riparian forest—cerradão	Partial shade (~ 67.75 light)	2;5	2; 5
	DES	15.44	420	Haplic Cambisols	cerrado	Open/Partial shade (~ 55.06 light)	1; 2; 4; 5	1; 2; 4; 5
	NP	3.84	495	Haplic Cambisols	cerrado	Partial shade (~ 56.72 light)	1; 2; 4	1; 2; 4
	SB	6.97	398	Petric Plinthosols	cerrado	Open/Partial shade (~ 71.4 light)	1; 2	1; 2; 3
Glória do Oeste	VB	39.60	300	Red Yellow Latosols	cerrado—cerradão	Partial shade (~ 68% light)	1; 5	–
Mirassol do Oeste	IMP	10.58	367	Petric Plinthosols	cerrado—semideciduous forest	Partial shade (~ 66.34 light)	1	1
Cáceres	JB <sup>a</sup>	30.71	193	Plinthosols	cerrado	Partial shade (~ 65.95 light)	1; 2	1; 2
Cáceres	TM	65.15	285	Red Latosols	cerrado	Open/Partial shade (~ 72% light)	1; 2; 5	1; 2; 5
Cáceres	VC	15.37	411	Litholic Neosols	cerrado	Partial shade (~ 57.62 light)	1; 2	2

<sup>a</sup>Site JB: Frontier Brazil/Bolivia

<sup>b</sup>Predominant soil type

<sup>c</sup>Past > 30 years ago; Current ≤ 5 years

Symbols: 1 = Fire occurrence, 2 = Access of farming animals, 3 = Mining, 4 = Regenerated area after cultivation, 5 = Wood extraction

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### 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 site, using a hemispherical image taken with a NIKON F-501 camera, coupled with a NIKON 8 mm fisheye lens with a 180° angle of view. Images were taken every 25 m along the transect, with the camera positioned at a height of 0.30 m above the ground, in all areas and according to the recommendations of Galvani and Lima (2014). Solar radiation at each site was estimated according to the percentage of canopy openness (canopy ground cover) in each transect and the images were processed using Gap Light Analyzer (GLA) software version 2.0 (Lang et al. 2010).

### Statistical analysis

#### Sex ratio variation

To test whether the male to female plant tertiary ratio intra and inter the ten populations' sex ratio patterns deviate from the expected isoplethic equilibrium (1:1), we used the *G*-test for goodness of fit (Sokal and Rohlf 2011).

#### Habitat characterization

To detect possible environmental differences among the ten sites of *Z. boliviana* in terms of soil composition and structure, the sites were ordered according to soil texture classes for percentages of clay, silt, and sand, and illustrated in a ternary phase diagram, according to the FAO (2006).

#### Sex ratio and its correlation with environmental factors

##### Physical, chemical, and light variables

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), 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 correlation coefficients and variance inflation factor ( $VIF < 2$ ) between variables using the *vif* function from the *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 the *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. 2019), 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 1,572 individuals sampled from ten populations. Table 2 summarizes the results of the sex ratio for each population studied. Seven out of ten populations were male biased (VB 1.69♂:1♀,  $X^2_{(61.277)}$ ,  $p = 0.013$ ; VC 1.58♂:1♀,  $X^2_{(4.05)}$ ,  $p = 0.044$ ; IMP 3.1♂:1♀,  $X^2_{(39.486)}$ ,  $p = 3.304$ ; JB 4.75♂:1♀,  $X^2_{(19.565)}$ ,  $p = 9.722$ ; BV 2.91♂:1♀,  $X^2_{(10.256)}$ ,  $p = 0.001$ ; BVB 2.04♂:1♀,  $X^2_{(82.286)}$ ,  $p = 0.004$ ; DES 07♂:01♀,  $X^2_{(76.5)}$ ,  $p < 2.2$ ) and three were close to the expected sex ratio of 1:1 (TM 1.61♂:1♀,  $X^2_{(3.266)}$ ,  $p = 0.070$ ; NP 1.15♂:1♀,  $X^2_{(0.494)}$ ,  $p = 0.481$  and SB 1.32♂:1♀,  $X^2_{(1.531)}$ ,  $p = 0.215$ ) (Table 2, Fig. 3).

### Habitat characterization

Populations of *Z. boliviana* were categorized into three distinct habitat types according to the soil texture classes (Table 1; Fig. 4). Four out of ten populations (BV, BVB, NP, and DES) are from Chapada dos Guimarães, a region of wavy to very wavy reliefs and soils primarily composed of sandy Cambisols (Fig. 4–Habitat 1). Three sites (TM, JB, and VB) occur on a

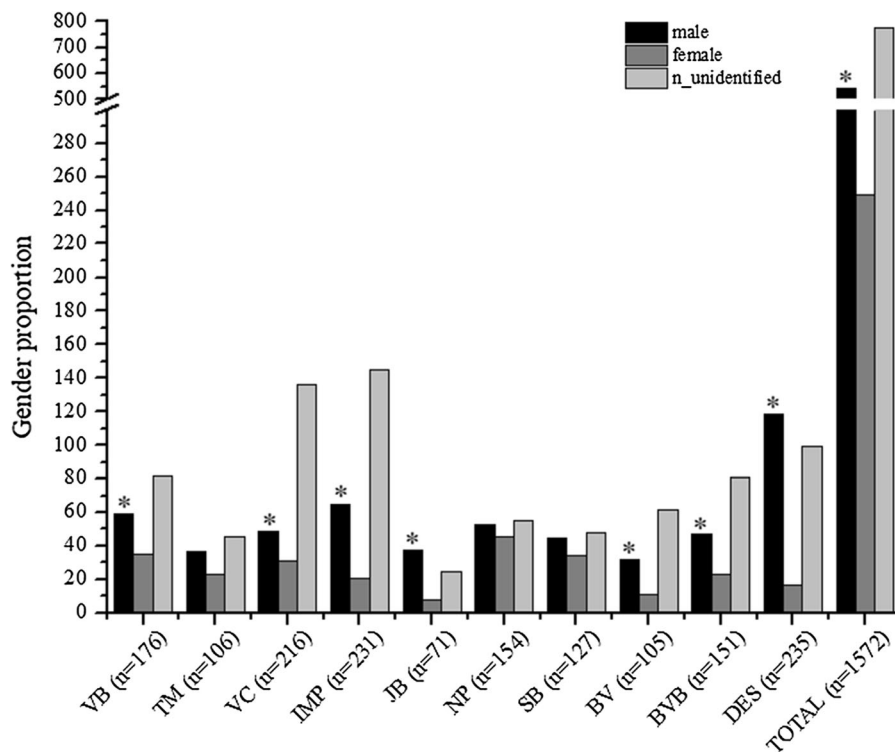


**Table 2** Ecological information on the ten sampled populations of *Zamia boliviana*, Mato Grosso State, Brazil. Number of males, number of females and unidentified individuals, sex

ratio (female/male), and the chi-squared ( $\chi^2$ ) and *P* value for a deviation from a 2 ♀: 1 ♂ sex ratio, expressed as the proportion of males

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

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



**Fig. 3** Distribution of sampled individuals (male, female, and unidentified individuals) in ten populations of *Zamia boliviana*. The black and dark gray bars show the tertiary sex ratio

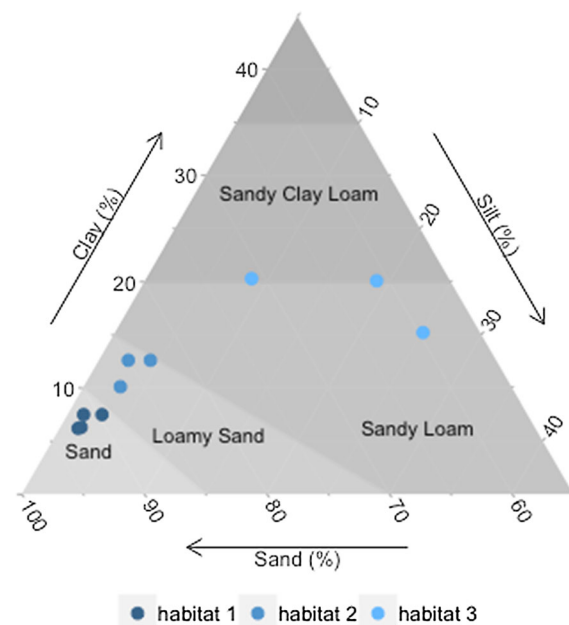
(male:female), suggesting significant deviation from the expected 1:1 ratio as determined by the *Chi-square* test. Asterisks above bars denote significant sexual bias (*P* < 0.5)

mainly flat to smooth-wavy relief, flat areas, or in the lower third of slopes, with potential movement or a

shallow water table. These three sites are formed, predominantly, by Latosols, Plinthosols, and Neosols

with a Loamy Sand texture (Fig. 4–Habitat 2). In the other three sites, the populations are distributed between Mirassol do Oeste and Cáceres, among the elevations of the rugged relief of the Província Serrana (IMP and VC), and in the valleys and sloped relief of Chapada dos Guimarães (SB). The main soils are Plinthosols and Neosols, with Sandy Clay Loam (SCL) transitioning to Sandy Loam (SL) textures (Fig. 4–Habitat 3). To summarize, 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 phytophysionomies is 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.

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 (Figs. 4, 5). Generally, the cycad was found in sandy soils of low-fertility, intermediate levels of incident light, compared to the average values obtained in the sampling of the sites, and marked climatic seasonality

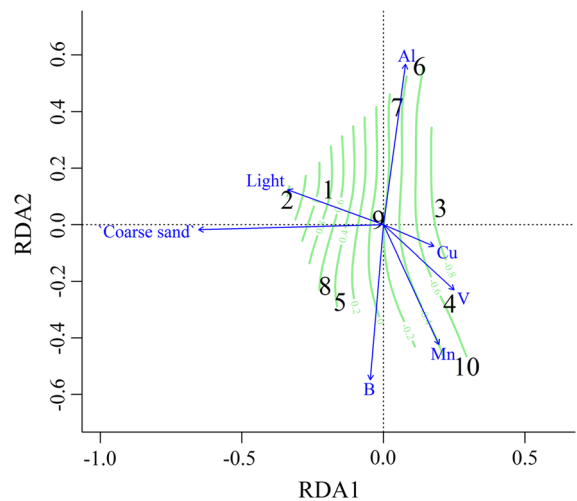


**Fig. 4** Soil texture triangle of the upper 0–30 cm soil layer in 10 types of habitats of *Z. boliviana* in localities from Mato Grosso state

in the Cerrado. The individuals form clumps mainly in the Cerrado (Figs. 2, 3, 4, 5) and the percentage of light reaching the habitat may exceed 70% during the dry period (Table 1). Cambisols are more permeable and, in this study, were associated with the development of plants with an extensive system of branched or clonal xylopodia (Table 1; Fig. 2g–h). In contrast to this, cycad populations associated with Red-Yellow Latosol have, predominantly, single (non-branched) and shallower xylopodia (Table 1; Fig. 2f).

#### Sex ratio correlation with environmental factors

The correlation of sex ratio with physical and chemical parameters of soil and incident light in 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



**Fig. 5** Biplot of the relationship between physicochemical parameters and tertiary 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 10 populations studied (1 = VB; 2 = TM; 3 = VC; 4 = IMP; 5 = JB; 6 = NP; 7 = SB; 8 = BV; 9 = BVB; 10 = DES)

**Table 3** A summary of the data files of redundancy analysis (RDA)

Species scores	RDA1	RDA2
Total area	– 0.293201	0.008652
Individual number	0.419165	– 0.027993
Male	0.223157	– 0.003366
Female	0.133318	0.118792
Strobili average	0.009515	0.005697
Sex ratio	0.023767	– 0.036592

exhibited significance in the composition of the model, showing a gradient in the studied populations ( $F_{1,2} = 8.900$ ;  $p = 0.037$ ; Fig. 5). The sandy texture was present in all populations, but decreasing, with the populations (number = population in RDA, Fig. 5, Table 3) NP'6, BV'8, BVB'9, and DES'10 presenting the highest percentage of sand in their soils, followed by the TM'2, JB'5, and VB'1 populations with intermediate values, and IMP'4, SB'7, and VC'3 with lower values.

## Discussion

Our investigations on the tertiary sex ratio of *Z. boliviana* populations in the Cerrado (Brazilian savanna) and local environmental factors revealed the following findings: (1) most populations present a male bias (70%); (2) *Z. boliviana* occupies different habitats within its area of distribution, with good adaptability to sandy and well-drained soils; and (3) we did not find any evidence that physical or chemical soil variables or light levels affect the sex ratio of *Z. boliviana*. However, we found an environmental gradient related to the soil properties. From these results, we discussed presumed causes, as well as similar studies.

### Sex ratio in *Zamia boliviana*

The male bias in the tertiary sex ratio of *Z. boliviana* appears to be related to a differential energy expenditure to produce ovulate strobili compared to pollen strobili, as reported for other species of the families Cycadaceae and Zamiaceae and as argued by Tang (1990) and Calonje et al. (2011). At the biological

level, the male bias in Zamiaceae may possibly be caused by the following factors or a combination thereof, according to Calonje et al. (2011) and the *Z. boliviana* phenology study (Soares 2020): (1) male individuals produce reproductive structures at a younger age than females; (2) male individuals can produce strobili more regularly than females; (3) male individuals of a population have a longer variation in time of the emergence and development of strobili within the same reproductive season than females; and (4) microstrobili have a shorter lifespan (~ two months) than megastrobili (~ ten months or more), preventing, oftentimes, the identification of sex in phenotypic evaluations. All these factors increase the chances of male sex identification in tertiary assessments.

Presumably the production of pollen strobili requires less energy expenditure than that of ovulate strobili and they are therefore produced more often during consecutive episodes of reproduction (Tang 1990; Grobbelaar 2002). The deviations toward the male sex occur in particular due to asynchronous reproductive phenological activity and short-term sampling (Tang 1990; Grobbelaar 2002; Calonje et al. 2011; Soares 2020). Thus, we assumed that the differential energy expenditure to produce sexual structures is preponderant in tertiary sex ratio variation in *Z. boliviana*, explaining the deviation from a genotypic sex ratio of 1:1, as expected in dioecious populations (Ornduff 1987; Barrett et al. 2010). A few other studies have also demonstrated greater female reproductive effort in dioecious species (see Allen and Antos 1988, 1993; Antos and Allen 1990, 1994; Delph 2019). We believe that there is no biological evidence that differential mortality in female plants of *Z. boliviana* causes male deviations in the sex ratio, although this needs to be further investigated. We attribute the morphology and ecological adaptations of this perennial cycad from the Cerrado, such as the presence of a xylopodium, to a large nutrient reserve and brevideciduous events, as the main reason to refute, at least partially, the mortality in female plants. Oliveira (1996) suggested that of the ca. 30% of dioecious species of Cerrado, 12% are in open areas, with a predominantly brevideciduous habit. Additionally, according to the findings of Octavio-Aguilar et al. (2017), mortality is not a crucial factor involved in the differential performance of the sexes in the genus *Zamia*.



Sex ratios of cycad populations have been investigated for different genera, such as *Ceratozamia* Brongniart, *Dioon* Lindley, *Encephalartos* Lehmann, *Lepidozamia*, *Macrozamia* Miquel, *Microcycas* (Miq.) A. DC., and *Zamia* L. (reviewed by 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 tertiary sex ratio (Clark and Clark 1987). In these cases, the bias toward males is usually higher (Calonje et al. 2011). However, most cumulative studies indicate no significant differences in genotypic or primary sex ratio, either for living collections (Ornduff 1987; Calonje et al. 2011) or in situ populations (Grobbeelaar 2002; Octavio-Aguilar et al. 2017), tending toward equality in the sex ratio.

Still with regard to cycads, studies showed that, whereas the gender ratio appears to change as cycad populations decrease in size, large and vigorous populations of *Encephalartos* spp. have a sex ratio of approximately 1:1 (as reviewed by Cousins and Witkowski 2017). Although the potential causes of deviations in the tertiary sex ratio include a higher mortality of older females relative to males as suggested by Grobbeelaar et al. (1989), and strong male-biased ratios in small cycad populations, attributed to selective harvesting of females by cycad collectors, such as in South Africa (as reviewed by Cousins and Witkowski 2017), these factors do not seem applicable to *Z. boliviana*. However, we found that up until ~ 50 years ago, the DES and NP site areas were used for crops, with frequent weeding and cutting of vegetation at ground level. These activities probably stimulated individuals to branch and form a clonal xylopod tendency intensified by Cambisol characteristics. These conditions, together with the fire that occurred in those populations, may have combined to increase the frequency and reproductive asynchronicity of the sexes, facts that also concur with the sampling method, and consequently may have contributed to the male bias in these populations.

Finally, the tertiary sex ratio results of this study may have been influenced by a set of deterministic biological and ecological factors, but also by stochastic events, such as precious cropping and fires (Table 1), affecting the cycad populations. Although in some cycads strobili may be regulated, or at least influenced, by summer fires (Jones 2002), no study has been conducted to specifically assess the effects of fire

on plants of *Z. boliviana*. However, *Z. boliviana* seems well adapted to some habitat conditions because the energy reserves in the underground organs allow it to develop new shoots when the aerial parts are lost to fire, brevideciduous events (Fig. 2g-h), or other related causes. These effects may interact with other aspects deterministic of life history, such as longevity, or stochastic to cause amplification of the sex ratio bias in recurrent episodes of reproduction (Field et al. 2012). This is supported since the biased sex ratio may impact the dioecious species' viability by reducing the effective reproductive population size, influencing female fecundity and/or decreasing 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).

#### Sex ratio correlation with environmental factors

With regard to cycad environmental conditions, our results have shown that *Z. boliviana* can occur in a considerable diversity of soil types and their physical and chemical compositions. The Cerrado's soils among our studied sites are characterized, in general, as alkaline (Reatto et al. 2008), with low natural fertility but good structure, and are predominantly sandy and well drained (SEPLAN 2000). *Zamia boliviana* also seems well adapted to intermediate levels of incident light, inherent to the climatic seasonality of the Cerrado. The pedological zone where this cycad occurs also includes Oxisols, mostly dystrophic and acidic but with good water permeability; Cambisols, chemically dystrophic with low chemical fertility and a predominantly sandy texture; Plinthosols, generally compact but hydromorphic with great variability in their chemical properties; and Neosols, 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 (Reatto et al. 2008).

The presence of *Z. boliviana* in different environmental contexts suggests occupation and growth in a wide variety of habitats throughout its distribution, revealing fitness for, and ecological plasticity to adapt to, different micro-conditions, with no interference in sex ratio. For instance, the absence of an association between incident light and the tertiary sex ratio

probably reflects the adaptability of *Z. boliviana* to their habitat of occurrence. This was also found in dioic *Mercurialis perennis* EU., where no correlation was found between light and the sex ratio (Vandepitte et al. 2009). 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.

The coarse sand was the only soil attribute that separated *Z. boliviana* populations and seems to reflect the soil typology of the cycad habitats. In particular, the populations BV, BVB, NP, and DES occur in habitats composed predominantly of sandy soils, typical of Cambisols, which probably influences the fitness of *Z. boliviana*, a matter for future research. Given the assumed differences in the energy expenditure of the sexes and the occurrence of these populations in characteristically sandy habitats, sandy soils may drive differences in the allocation of resources between the sexes. Male plants in these sites would meet their energy requirements for vegetative and reproductive functions and maintain a more frequent reproduction without interruption. In contrast, female plants have additional requirements, which can lead to irregular reproduction, accentuating the male bias in those populations.

Therefore, physiological differences between males and females can influence the sex ratio within a population when environmental conditions are heterogeneous (as reviewed in Krieg et al. 2017). The classic study of Ornduff (1987) with populations of *Zamia pumila* L. was the first to record the idea that soil quality affects the primary sex ratio. This researcher found that, 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 on an ecological scale, stochastic forces and the founder effect (e.g. small populations consisting predominantly of either males or females) can also act on natural habitats and cause a biased sex ratio. Variation in population density, the spatial distribution of sexes, resource availability, the structure of the founder population, pollinator visits, anthropic actions, spontaneous colonization, and genetic and demographic characteristics are examples of such forces we want to highlight. These factors were also pointed out as relevant in other species (Cunha

et al. 2014; Zhang et al. 2015) and cycad studies (Pérez-Farrera et al. 2017; Mankga and Yessoufou 2017; Octavio-Aguilar et al. 2017).

It should be noted that the small number of populations sampled in our study ( $N = 10$ ) is common for the rare, restricted *Z. boliviana* and other cycads. According to Yu and Lu (2011) and Vandepitte et al. (2009), the male-biased sex ratio may be quite pronounced in small populations and corroborate our results, since *Z. boliviana* populations are mostly small and fragmented. Small endemic populations, such as in *Z. boliviana*, 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. Additionally, this fact limits opportunities to detect robust patterns, assess the health of populations, and to distinguish which forces act most strongly to cause sex ratio deviations in long-living 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 improve our knowledge, as emphasized by Juvany and Munné-Bosch (2015).

## Conclusions

The male-biased sex ratio dominates in most in situ populations of *Z. boliviana*. However, environmental variables did not explain the biased sex ratios in this cycad. The biased sex ratios are likely explained by the differential energy expenditure of sexes in reproduction. Our study is the first systematic assessment of the tertiary sex ratio for *Zamia* and other dioecious species from central South America. It showed the current tertiary sex ratio pattern of a *Zamia* species, in a scenario of continuing decline in the extent and/or quality of habitats of cycad populations (see Calonje et al. 2021). Our finding may help us improve the understanding of the mechanisms causing biased sex ratios in dioecious species (e.g. impact of costs of reproductions). Based on the findings and also the limitations of this study, we highlight three general considerations to guide others' research in dioecious species from tropical ecosystems: (i) a precise determination of the stages at which the sexual 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; Field 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; Field et al. 2012). The different genomic techniques currently available may help identify several ecological processes within sex ratio determination, including cycad populations (on primary, secondary, and tertiary levels), 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); and (iii) 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).

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#### Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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