

Reproductive biology of the South American cycad *Zamia boliviana*, involving brood-site pollination

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Abstract

Brood-site pollination mutualisms represent extreme levels of reciprocal specialization between plants and insects. Several studies have revealed intimate associations between cycads and their insect pollinators across different continents. However, the reproductive biology of South American cycads is still poorly understood, with limited empirical data available. Here, we investigated the plant–pollinator interactions of *Zamia boliviana* (Cycadales, Zamiaceae), which is distributed on savanna patches in Brazil and Bolivia. Our aim was to examine reproductive biology (strobili development and their visitors) to identify potential pollinators from the perspective of dioecy and expectations of obligatory mutualism. We monitored reproductive cohorts and registered the strobili's visitors. We classified the visitors' activities and described the behavior of the most active and recurring ones in both reproductive structures to ascertain the potential pollinators and their relationship with the *Z. boliviana*. In situ experiments were used to demonstrate efficacy in seed production. Although other insects visit the strobili, only one Coleoptera (Erotylidae: *Pharaxonotha*) was present seasonally. *Pharaxonotha cerradensis* was the single species capable of transporting pollen and developing a life cycle associated with polleniferous strobili, while concomitantly visiting ovuliferous strobili. Experimental tests associated with observational evidence in the cycad indicated brood-site pollination mutualism with the Erotylidae beetle, a potential pollinator of *Z. boliviana*. Our investigation confirmed a new case of obligatory mutualism between cycads and *P. cerradensis* in the New World. Future research on *Zamia* from South America should address pollination systems of this genus to better understand the ecology and evolution of plant–pollinator interactions.

KEYWORDS

beetle life cycle, biotic vectors, dioecious plants, ecological interaction, plant–insect interactions

1 | INTRODUCTION

Reciprocally specialized plant–pollinator interactions are among the most intriguing ecological interactions (Solga, Harmon, & Ganguli, 2014; Suinyuy, Donaldson, & Johnson, 2015). The degree of ecological dependence of

plants on pollen vectors to produce seeds depends on their reproductive systems (Johnson, Harris, & Procheş, 2009; Richards, 1997). The dependence may range from completely obligatory, as in vector species that use particular plant reproductive structures as brood sites or sources of food, to facultative, as in vectors that have generalist

diets, including resources from flowers (Faegri & Van der Pijl, 1979; Johnson et al., 2009; Richards, 1997). Dioecious plants, such as cycads, or genetically self-incompatible species, are entirely dependent on cross-pollination to produce seeds (Norstog & Fawcett, 1989; Richards, 1997). In this context, brood-site pollination mutualism represents an extreme level of reciprocal specialization between plants and insect pollinators (Suinyuy et al., 2015).

In members of Zamiaceae, there is a striking sexual dimorphism in the reproductive structures of the two sexes (Grobelaar, 2002; Jones, 2002; Segalla, Telles, Pinheiro, & Morellato, 2019). The reliance on specialized and obligatory biotic vectors for reproduction increases the risks of reproductive failure 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, in some cases described as a cycad brood-site pollination mutualism (Hall, Walter, Bergstrom, & Machin, 2004; Norstog & Fawcett, 1989; Norstog, Stevenson, & Niklas, 1986; Segalla et al., 2019; Suinyuy, Donaldson, & Johnson, 2009; Tang, 1987; Terry, 2001; Terry et al., 2005; Toon, Terry, Tang, Walter, & Cook, 2020; Valencia-Montoya, Tuberquia, Guzmán, & Cardona-Duque, 2017; Vovides, 1991).

Despite their rarity and fascinating biological history, cycads are among the most threatened groups of plants worldwide (IUCN, International Union for Conservation of Nature and Natural Resources, 2019; Stevenson, Stanberg, & Calonje, 2018). The codependency on specialized mutualism may render both insect and cycad especially sensitive to the properties associated with cycad population declines (Lopez-Gallego, 2015; Segalla et al., 2019; Terry et al., 2005, 2012). The ancient origins of cycads provide an opportunity to study the evolution and diversity of plant 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 such brood site interactions (Roemer, Terry, & Walter, 2008). Pollination investigations of cycads, especially those in understudied regions, may provide new insights into the evolutionary history of those cycads, (e.g., New World cycads), helping to identify vulnerabilities that may support conservation efforts (Roemer et al., 2008; Roemer, Booth, Terry, & Walter, 2017; Tang et al., 2018; Tang, Skelley, & Pérez-Farrera, 2018; Terry et al., 2005; Terry, Roemer, Booth, Moore, & Walter, 2016).

Native South America cycads are placed in the prominent New World genus *Zamia* Linnaeus (see Calonje et al., 2019) and include *Zamia boliviana* (Brongn.) A. DC. endemic to Brazil and Bolivia (Segalla & Calonje, 2019).

The biological knowledge about *Zamia* species in South America is still notably scarce, and in situ conservation efforts will need to target not only the cycads but also the permanence and health of their pollinators and herbivores (Schneider, Wink, Sporer, & Lounibos, 2002; Segalla et al., 2019; Solga et al., 2014; Terry et al., 2005; Valencia-Montoya et al., 2017). Few systematic tests, including exclusion studies to evaluate the effectiveness of wind and insects as pollen vectors, have been conducted in South American cycads. The presence of insects at receptive strobili (e.g., Skelley & Segalla, 2019) raise questions as to their effectiveness as pollinators.

In this work, we studied the reproductive biology of *Z. boliviana*, monitored the polleniferous and ovuliferous strobili phenophases, and observed the activities of their visitors. We aimed to (a) examine the plant-pollinator reproductive systems of cycads; (b) identify common visitors of the strobili and the type of interaction (pollination, herbivory) with the cycad; and (c) investigate the efficiency of pollination vectors (insects and wind) in the reproductive biology of *Z. boliviana*, based on vector exclusion studies. We hypothesized that *Z. boliviana* and its likely pollinator *Pharaxothona cerradensis* Skelley and Segalla (Skelley & Segalla, 2019) represent a case of brood site mutualism.

2 | METHODS

2.1 | Study species and study site

Zamia boliviana populations occur in the central portion of South America (Figure 1a) in the Cerrado across the Brazilian state of Mato Grosso (MT) and Bolivia, characterized by high total solar radiation throughout the year (Deblauwe et al., 2016; SEPLAN Servicio Nacional de Meteorología e Hidrología, 2000). Equatorial and tropical hot climates prevail, with little seasonal and annual thermal variation (Deblauwe et al., 2016; SEPLAN Secretaria de Estado de Planejamento, 2000). The average minimum daily temperature is 20.3°C, and the average maximum daily temperature is 32.7°C (SENAMHI Servicio Nacional de Meteorología e Hidrología, 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 Servicio Nacional de Meteorología e Hidrología, 2019). *Zamia boliviana* is a small perennial plant (up to 0.80 m tall) with a subterranean stem (xylopodium) and from one to three leaves in each crown (Figure 1b). The reproductive structures are formed on an underground stem, emerging aboveground throughout development, from June to November. Male plants develop one to six pollen strobili per stem or branch

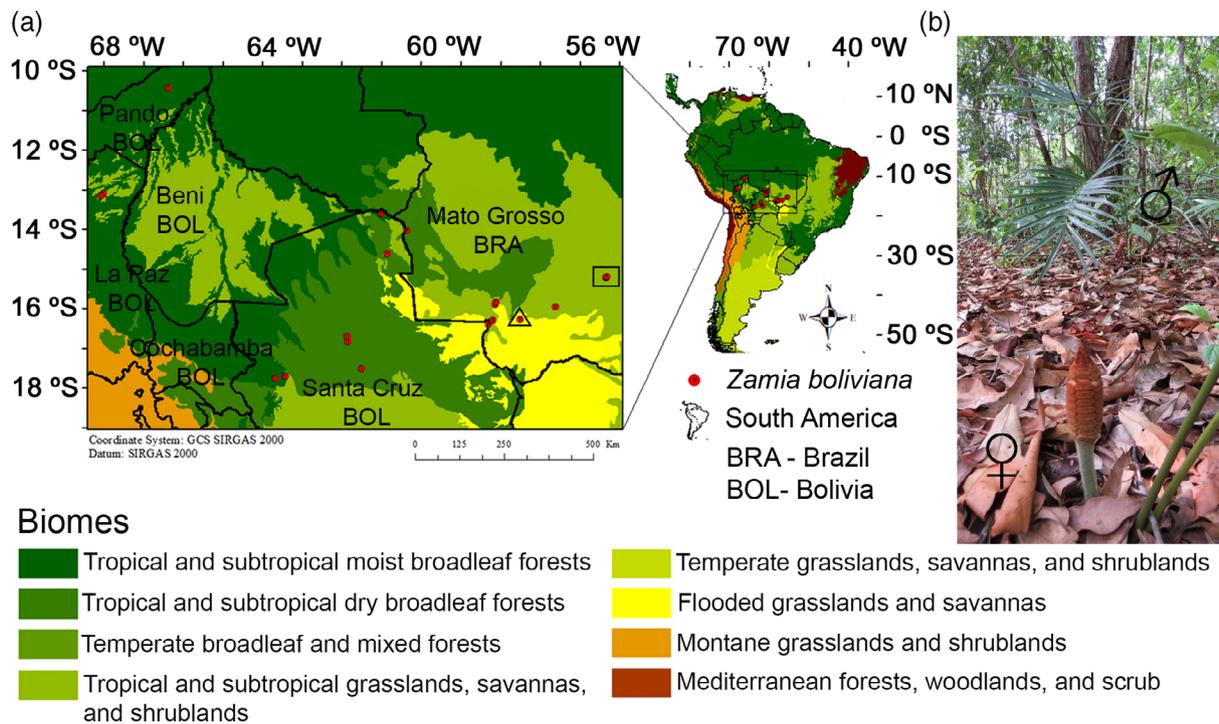


FIGURE 1 Geographic distribution and place of occurrence, according to the type of biome, of *Zamia boliviana* in South America, state of Mato Grosso (Brazil), and region of the Brazil–Bolivia border (a). Habit of *Z. boliviana* at place of occurrence (b). Locations of populations studied are marked with a triangle (Cáceres) and with a square (Chapada dos Guimarães). Credit: Rosane Segalla, personal collection

of xylopodium, whereas female plants produce only one ovuliferous strobilus. Phenological records indicate that this cycad has a life cycle intrinsically related to rainfall seasonality of its habitats (Soares, 2020). *Z. boliviana* strobili are visited by *P. cerradensis* (Skelley & Segalla, 2019).

2.2 | Strobili visitors: Diversity, frequency and behavior

The occasional (non-systematic) registration of visitors, and their diversity, frequency and behavior in polleniferous and ovuliferous strobili was performed in situ from 2016 to 2019 in reproductive cohorts of Brazilian cycad populations (Figure 1a). In 2017, systematic records were made in a population of Cáceres (Figure 1a, triangle) and in 2018 and 2019, in populations of Chapada dos Guimarães, MT (Figure 1a, square). Focal observations were carried out in the morning (6:00–12:00 a.m.), afternoon (2:00–6:00 p.m.) and evening (8:00–11:00 p.m.). Insect samples found in or on the strobili were collected in 70% ethanol and deposited in the biological collections of the Laboratório de Scarabaeologia at Universidade Federal de Mato Grosso, Instituto de Biociências, Cuiabá, Mato Grosso, Brazil (UFMI), and the Museu de Entomologia Pe. Jesus Santiago Moure, Universidade Federal do Paraná, Curitiba, Paraná, Brazil (DZUP), for identification and 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).

2.3 | Efficiency of vectors and cycad–pollinators relationship

2.3.1 | Pollination vector tests

The efficacy of vectors (wind and insects) in the pollination of *Z. boliviana* was tested and compared in exclusion experiments (modified from Norstog et al., 1986; Tang, 1987; Terry, 2001). (a) To determine whether pollen was transported by insects and evaluate their effectiveness in pollination, 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 ($n = 10$). (b) To test whether the wind acted as a subsidiary in the pollination of *Z. boliviana*, we covered the strobili with a polyester mesh cloth (mesh openings ~ 0.15 mm) and sealed 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 ($n = 10$). (c) To test

for self-pollination or agamospermy (negative control), we excluded both vectors (wind and insects) from the ovulate strobili. We used a similar approach as the wind and insect exclusion treatment and sealed the peduncle with a cord ($n = 10$). (d) As a control treatment (positive control), the ovuliferous strobili were left unprotected ($n = 21$). The plants were located by actively searching for populations in Chapada dos Guimarães (Figure 1a). All treatments were applied to the ovuliferous strobili prior to the pollen receptivity phase. Beetles (Coleoptera, Erotylidae) collected from polleniferous 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) 300–350 days after emergence. The pollinated and non-pollinated ovules were distinguished by their morphological aspects after the ovuliferous strobili's development. Pollinated ovules increased in size and developed a hard sclerotesta and red sarcotesta; ovules not pollinated remained small, opaque in color, and with undeveloped sclerotesta and sarcotesta (Figure 4c). 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 (Bousquet, Boucheron, & Lugosi, 2004) and estimation of the confidence interval.

3 | RESULTS

3.1 | Insect visitors, behavior, and efficiency of vectors in pollination

3.1.1 | Visitors and their behavior in the strobili

Visitors were classified into two groups: regular (insects that were present in every pollination season; pollinators and herbivores) and sporadic (insects opportunistically visiting; pollen collectors, herbivores and predators). 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 in the strobili's phenophases. Figure 2a–i presents the occurrences of visitors in the strobili and summarizes their main behavior during the visits.

Pharaxonotha cerradensis (Coleoptera: Erotylidae) was consistently observed in the successive reproductive cohorts (2016 to 2019) of *Z. boliviana*. *Pharaxonotha*

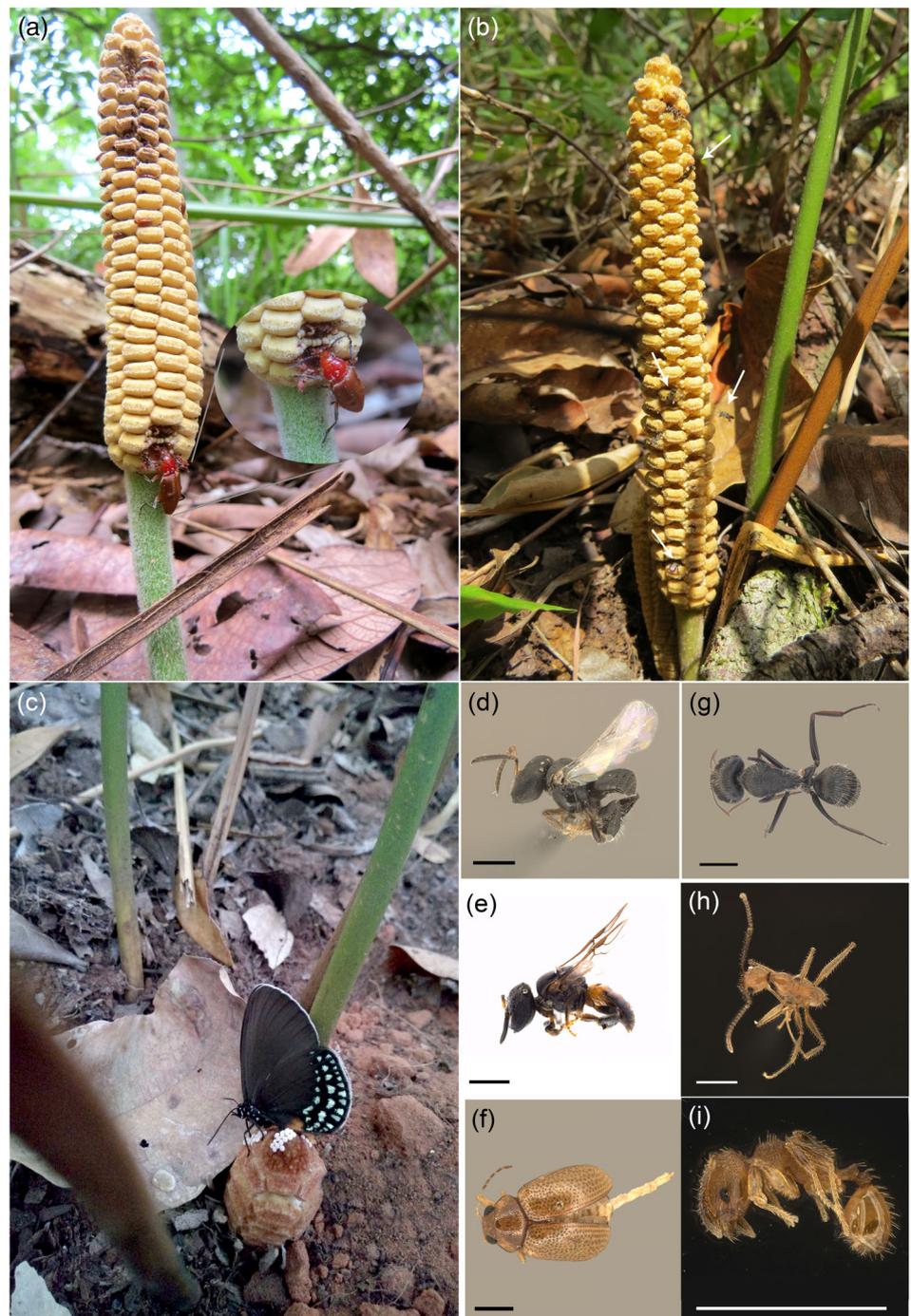
cerradensis individuals were also found in the soil, near male and female reproductive plants, and in the polleniferous and ovuliferous strobili (Figure 3), as described below.

In the polleniferous strobili, the beetle was attracted when the strobili were expanding and microsporophylls were separating from each other (Figure 3a [1.1]). This phase occurs in ~50 days of the strobil's lifespan, which is followed in 1 or 2 days by the onset of pollen dehiscence, with an average duration of 3 to 4 days of pollen dehiscence. The number of insects in pollen structure increased gradually, concomitantly with the distension of microsporophylls and opening of microsporangia. Thus, the polleniferous strobili's physiological state, its density when fully mature and the plant's pollen availability determined the number of *P. cerradensis* individuals in the strobili.

The estimated number of *P. cerradensis* beetles per microstrobilus was 60 individuals in the pollen release phase. In the pre-pollen dehiscence phase, these beetles begin patrolling the soil structure for polleniferous strobili. As the microsporophylls distended and the microsporangia opened, *P. cerradensis* repeatedly visited and foraged the external region and the spaces between the microsporophylls, usually from the base to the apex of the pollinic structure. When beetles found an open microsporangium, they accessed the internal parenchymatic content (pollen), resulting in a body structure immersed in pollen grains (Figure 3[3]). Generally, the patrolling and foraging of the outer surface alternated with the accommodation of individuals in the microsporophylls' spaces. The colonization occurred until the microsporangium was completely opened and declined with the decrease in pollen, when the individuals gradually moved to other polleniferous strobili undergoing a similar maturation process, as shown in Figure 3a.

Pharaxonotha cerradensis often demonstrated poor light tolerance and commonly foraged on the opposite side of the light source or on the parts of the strobili in contact with the leaf litter. Although it visited strobili during the day and night, it seemed more active at night, preferably at dusk, on cloudy days and in plants growing in full shade. *Pharaxonotha cerradensis* moves rapidly on the ground and seems to prefer land to air but can take flight frequently. When *P. cerradensis* was foraging or housed in the strobilus, it commonly exhibited elusive behavior when sensing touch and vibration of the strobili, moving immediately, falling or flying down from the peduncle to the ground. The beetle was lodged in the bracts next to the cycad's xylopodium. The microsporophyll dissection demonstrated that the beetle reproduced within the polleniferous strobili (Figure 3 (5)). Over the process of colonization, the microstrobilus served as a place of copulation, incubation of eggs and

FIGURE 2 Visitors of *Zamia 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* sp₁. (i) *Pheidole* sp₂. Scale bars = 1 mm. Credit: Rosane Segalla, personal collection



larval development, shelter and food for adults and their offspring (Figure 3). *Pharaxonotha cerradensis* was the only visitor with occurrence in the ovuliferous strobili, a high pollen load over its stay, and foraging activity in the polleniferous strobili (Figure 3 [2;3]).

The beetles moved into and out of the ovuliferous strobili during the day and night. Although the beetles moved into and out of the ovuliferous strobili during all times of day, they most frequently moved at dawn and dusk, avoiding periods of full sun. The morphology of the ovuliferous strobili (Figure 4a) made 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 spaces of the mid-section (Figure 3 [3.2]). The number of individuals was estimated from samples of bagged and

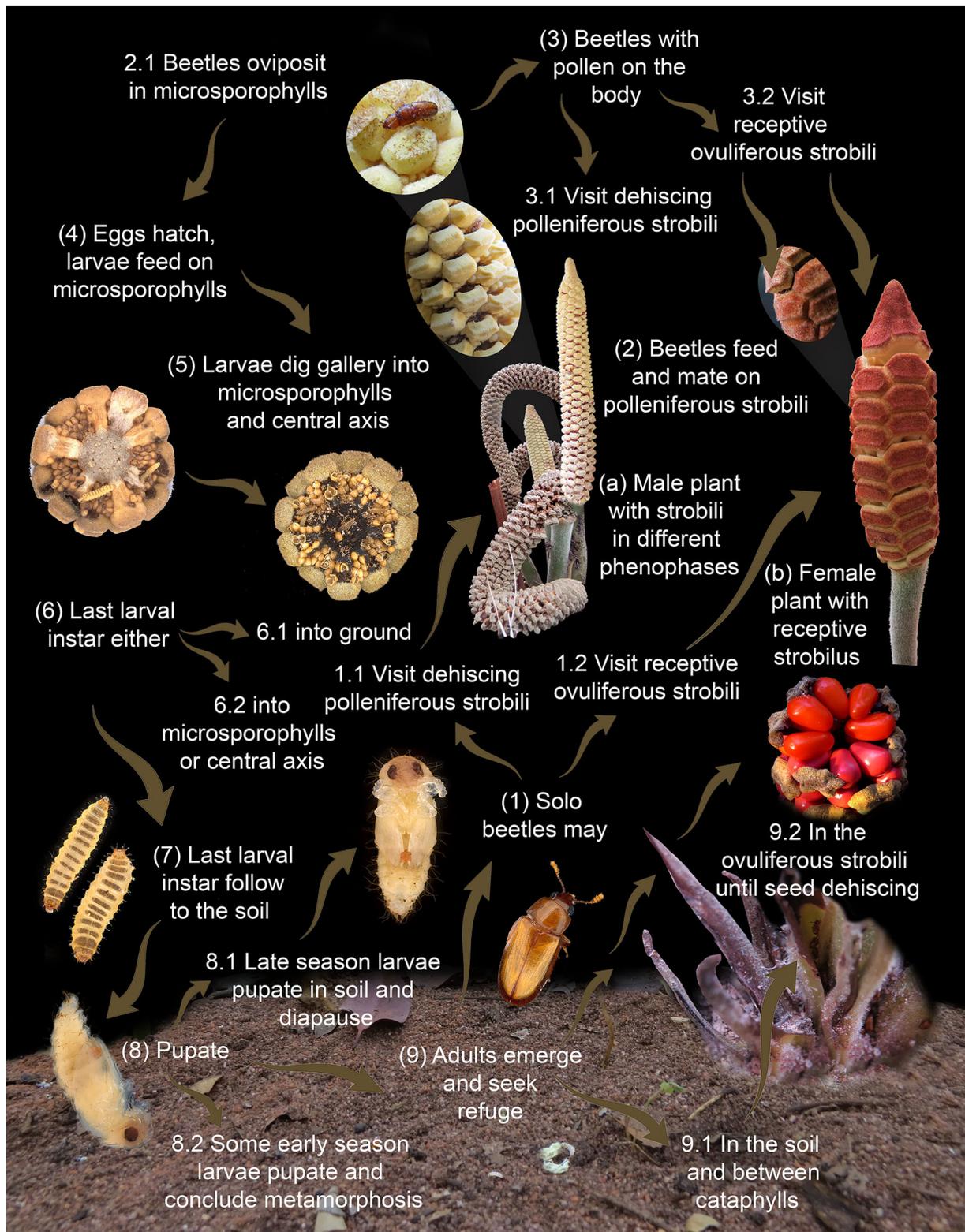
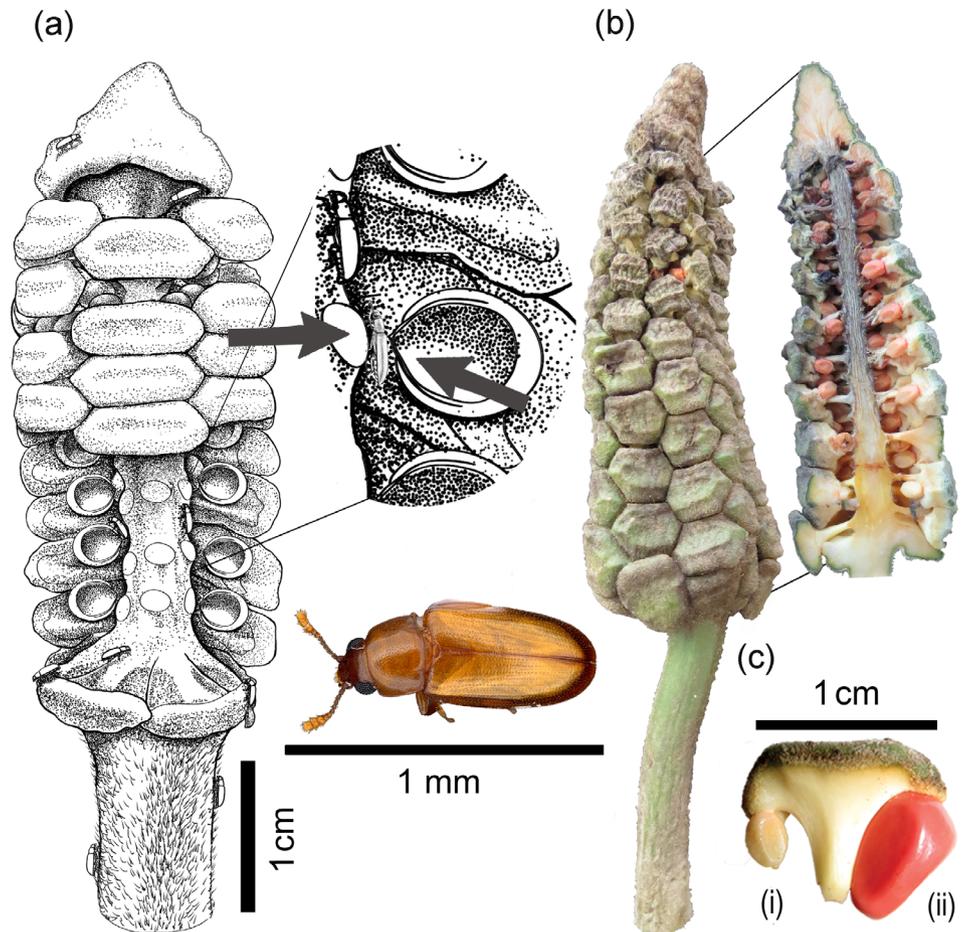


FIGURE 3 Reproductive biology of *Zamia boliviana* with brood-site pollination by *Pharaaxonotha cerradensis* (Coleoptera: Erotylidae). Male plant with strobili in different phenophases (a). Female plant with receptive strobili (b). Behavior of beetles in polleniferous strobili (1.1; 2; 2.1; 3.1) and 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 on the strobili and soil (4; 5; 6; 7; 8; 9). Some late season larvae pupate in soil and diapause and await the emergence of new strobili in the next reproductive season (8.1). Other early season larvae pupate and conclude metamorphosis (8.2) (according to Norstog & Fawcett, 1989). 9. Emergence of new adults. The entire life cycle of non-diapausing beetles is completed in 8–10 days. Credit: Rosane Segalla, personal collection

FIGURE 4 External and internal morphological structure of ovuliferous strobili of *Zamia boliviana*. Strobilus open with receptive ovules and with *Pharaxonotha cerradensis* (a). The arrows indicate the gap between the central axis of the megastrobili and the walls of the megasporophylls. Strobilus with non-pollinated ovules (b). Megasporophyll detail with non-pollinated ovules (c): pollinated ovules (i) or seed (ii). Credit: Rosane Segalla, personal collection; Pedro Lorenzo, art letter 'a'



dissected ovuliferous strobili. We found an average of six *P. cerradensis* individuals per receptive ovuliferous strobilus. Observations of the displacement behavior of *P. cerradensis* in the host plant indicated that the beetle moved through the strobilus axis that supports the megasporangia, possibly brushing the ovules and delivering pollen to the micropyle. The ovules were attached to the megasporophyll and were oriented so that the micropylar tip faces inward toward the strobilus axis. The spaces between the ovules and central axis (3 mm) corresponded to the body size of *P. cerradensis* (Figure 4a).

We were not able to assess pollination droplets or any kind of reward on the ovuliferous strobili. We also did not find predation of ovules by *P. cerradensis*, although they appeared to be guarding the ovuliferous strobili. Individual beetles were in spaces between megasporophylls while facing the outside of the organ, constantly moving their antennae.

We also observed the Lepidoptera *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 and ovuliferous strobili is recurrent. The visits started during the strobili's

emergence phase, preferably to plants growing under 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 (Figure 2c). *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 (Figure 2a). In low numbers ($n = 3$) and active in the morning and late afternoon, the beetles consumed microsporophyll tissues.

Seasonally or opportunistically, visitors of Hymenoptera such as bees of the family Apidae *Plebeia* aff. *minima* (Gribodo, 1893) (Figure 2e) and *Trigonisca* Moure, 1950 (Figure 2d) were observed sporadically visiting only polleniferous strobili in the pollen release phase, with a low number of visits, both among and within reproductive cohorts. Their activity was restricted to foraging and collecting pollen. Ten individuals of a Coleoptera (Chrysomelidae, Eumolpinae) (Figure 2f) species were observed exclusively foraging in polleniferous strobili in the pollen release phase. We also registered three ant species (Figure 2g–i) with predominantly patrolling and foraging behavior. *Pheidole* sp₁ and *Pheidole* sp₂ Westwood, 1839 were observed obtaining food in the pollen release

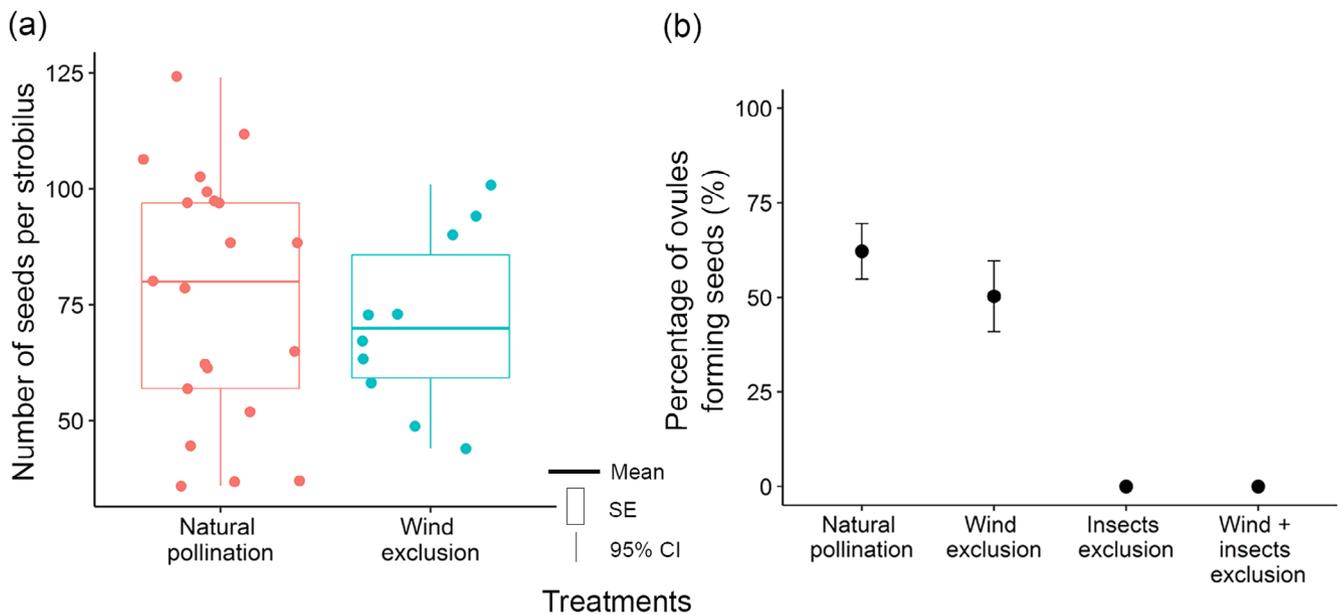


FIGURE 5 Analysis of the pollination experiments of *Zamia boliviana*. Natural pollination and wind exclusion (a and b) include all the treatments. Control treatment (ovuliferous strobili kept under natural conditions, without excluding pollination agents) (a). The error bars indicate 95% confidence intervals. The exclusion of insects and wind and exclusion of insects only were not included in the model and their error bars are not displayed because their replicates equaled zero. Percentage of ovules forming seeds (b). 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 higher than the results for exclusion of insects and of wind and insects. The results for exclusion of insects and of wind and insects did not differ from each other

phase, exclusively in polleniferous strobili. *Camponotus crassus* Mayr, 1862 (Figure 2g) visited both structures at different lifespan phases, including the pollen release and receptivity phases, but was not observed entering the ovuliferous strobili. These species showed no remarkable body pollen load.

3.1.2 | Pollination vector exclusion tests

The reproductive success of *Z. boliviana* measured in the pollination experiments revealed that wind exclusion did not affect the number of seeds developed ($t = 0.6306$; $p = .5358$) (Monte Carlo Permutation). Conversely, seed yield was significantly affected ($p = .000001$; Figure 5a,b), with complete failure of the seeds to develop when beetles or when these and winds were excluded from the pollination process (Figure 4). The control showed a seed production average of 77.23% in the set of 21 plants.

4 | DISCUSSION

Our study revealed a specialized plant–pollinator interaction between *Z. boliviana* and *P. cerradensis*, in an intimate interaction that we considered a case of brood-site pollination mutualism. *Pharaxonotha cerradensis* was

present during almost all reproductive phases of male and female plants, acting as the main pollinator. *Pharaxonotha cerradensis* also used *Zamia* reproductive organs to reproduce, characterizing the brood-site pollination mutualism. This study adds to the current body of knowledge accumulated over the last three decades suggesting that insects are the primary pollination vectors of cycads, and that the role of wind in pollinating New World cycads is negligible (Segalla et al., 2019; Tang, Xu, et al., 2018). Thus, our findings provide evidence that the conservation management of cycads must include management of their pollinators, especially for highly threatened species such as this *Zamia* of the Cerrado, a South American ecosystem with a high frequency of habitat loss.

4.1 | Insect visitors, behavior and efficiency of vectors in pollination

The follow-up of four reproductive cohorts of *Z. boliviana* and the observational and experimental studies corroborated our hypothesis that the pollination of this cycad is mediated by a biotic vector and is a case of brood-site pollination, as described for other *Zamia* species (Franz & Skelley, 2008; Norstog et al., 1986; Norstog & Fawcett, 1989; Tang, 1987; Valencia-Montoya et al., 2017). We found that a single species of Erotylidae beetle, *Pharaxonotha*

cerradensis, co-occurred with populations of *Zamia boliviana*, performing its life cycle in association with the pollen strobili, during synchronous and seasonal reproductive events (Skelley & Segalla, 2019), and also visiting the ovulate strobili (Figure 3). Similar to other interactions reported for cycads, we identified that *P. cerradensis* were obligately associated with their host species *Z. boliviana*; they fed, mated, laid eggs and completed larval development on their cycad host (Figure 5). Indeed, studies have demonstrated that beetles (Coleoptera) in the family Erotylidae (Cucujoidea) are important pollinators of cycads (reviewed in Skelley et al., 2017). Our findings revealed that *P. cerradensis* was the only visitor that demonstrated the requirements of a potential pollinator of *Z. boliviana*. The behavior, when in direct interaction with the plant, was consistent with the obligatory and specialized process of brood-site pollination mutualism (Johnson et al., 2009).

Our results showed that wind plays no subsidiary role in pollinating *Z. boliviana*, which is most likely pollinated only by a beetle. The exclusion experiments demonstrated that pollination was unfeasible in the absence of *P. cerradensis*, although the experiments were not designed to test the effectiveness of *P. cerradensis* specifically in the pollination of *Z. boliviana*, but only to test whether excluding insects and wind affected pollination and whether allowing insects but excluding wind affected pollination. Additionally, the morphological-architectural structure of *Z. boliviana* polleniferous and ovuliferous strobili, arranged near the ground, at an average height of 12 cm during the pollen release and receptivity phase, resulted in most of the pollen mass being shed by gravity near the polleniferous strobili (~5 cm in circumference). This plant habit and the horizontal oriented openings at ~0.5 mm at the base and apex of the ovuliferous strobili, possibly were a natural barrier to the arrival of pollen at the micropyles by air. Other features preventing wind-blown pollen from reaching the micropyle were 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. Additionally, the receptivity phase of the ovuliferous strobili was not always synchronized with the pollen release phase by the polleniferous strobili. Indeed, observations showed that the receptive phase may be offset from the pollen dehiscence phase in this population of cycads.

Our observations and experiments with *Z. boliviana* showed that biotic pollination in this cycad substantially outweighed wind pollination and corroborated about 30 other biology and ecology studies showing the predominance of biotic pollination for the family (reviewed in Segalla et al., 2019). Indeed, previous studies with

Zamiaceae species found limited or no pollination in tests from which insects were excluded (Donaldson, 1997; Donaldson, Nanni, & Bösenberg, 1995; Hall et al., 2004; Norstog et al., 1986; Terry, 2001; Terry et al., 2005; Valencia-Montoya et al., 2017; Vovides, 1991) and, therefore, weak or no presence of wind pollination in the cycads studied. The *Pharaxonotha* Reitter (Coleoptera: Erotylidae) is a predominantly pantropical genus and is found in the strobili of all four genera of cycads (*Ceratozamia*, *Dioon*, *Microcycas* and *Zamia*), all endemic in the New World (Chaves & Genaro, 2005; Franz & Skelley, 2008; Tang, Xu, et al., 2018b; Terry et al., 2012; Vovides, 1991), and is the first cycad pollinator recorded in Brazil. Although the majority of cycads in the New World host more than one species of beetle associated with their strobili and some of them host as many as three species (Tang, Xu, et al., 2018b), only *P. cerradensis* was recorded in *Z. boliviana*.

The palynivore habit and other behaviors of *P. cerradensis* corroborate an obligate association with their particular *Z. boliviana* host species as a potential pollinator of this cycad. A number of traits confirmed the brood-site plant-pollinator interaction between *Z. boliviana* and *P. cerradensis*, such as: (a) absence of consistent damage to ovuliferous strobili; (b) insect reproduction did not compromise the pollination process; (c) high frequencies and densities of *P. cerradensis* in the polleniferous and ovuliferous strobili compared to other visitors; and (d) the seasonal appearance of *P. cerradensis* concomitant with the emergence of strobili both among and within reproductive cohorts of the cycad. Additionally, morphological characteristics of *P. cerradensis*, mainly the presence of hair on the dorsal region and on the antennae (Skelley & Segalla, 2019), combined with the characteristics of the ovuliferous strobili, favored the adherence of pollen grains to the beetle and deposition on the ovule's micropyle upon contact with the insect as it walked along the central axis of the megasporangia.

The natural history of specialized brood pollinators associated with generalist plants is reported for other tropical species (e.g., Arecaceae species; Barfod, Hagen, & Borchsenius, 2011; De Medeiros, Núñez-Avellaneda, Hernandez, & Farrell, 2019). A number of pollination mechanisms in cycads and palms have provided direct or indirect evidence for a framework of interactions, which combines morphology attributes involving beetle-pollinated species (Barfod et al., 2011). The most common beetle visitors of cycads strobilis, palm flowers and Annonaceae species are the weevils from the Curculionidae family (Barfod et al., 2011; Chen, Liu, & Saunders, 2020). Exclusive pollination by beetles (or more rarely thrips) that breed on plant tissues is a common condition in palms (De Medeiros et al., 2019), as in Zamiaceae species (Segalla et al., 2019).

Nutritive tissues, pollen and oviposition sites are the major pollinator rewards (Barfod et al., 2011; Simpson & Neff, 1983) restricted to pollen strobili in cycads. Whether pollinators receive any reward from the ovuliferous strobili for their pollination services is still unknown (Terry, 2001). We assumed that *P. cerradensis* was probably mistakenly attracted to the ovuliferous strobili of *Z. boliviana*, when volatiles were released from the plant (Suinyuy et al., 2015; Terry et al., 2004), but may temporarily remain in the structure, induced by comfort and thermal shelter. We also assumed that the sentinel-like behavior was inherent to *P. cerradensis*'s interaction with the host plant *Z. boliviana*. Most likely, the female rewards are insignificant compared with polleniferous strobili that provide warmth, as well as a nourishing breeding and feeding environment (Terry, 2001). However, the costs of delivering pollen to the ovuliferous strobili may be small if the insects are only temporarily duped by similar cues such as polleniferous strobili (Terry, 2001).

Studies on the ecology and evolution of brood-site pollination mutualisms and associations may provide new insights into the evolutionary history (e.g., conditions in which these interactions are favored, according to De Medeiros et al., 2019; Chen et al., 2020). In the current global scenario of species loss, vulnerability and their intricate pollination systems, such as New World cycads, such studies are fundamental to direct applicability of conservation (de Medeiros et al., 2019; Nunes, Maruyama, Azevedo-Silva, & Sazima, 2018; Roemer et al., 2008; Tang, Xu, et al., 2018).

4.2 | Seasonally regular visitors: Cycad–herbivore relationships

Although the butterfly *Eumaeus minyas* visited strobili seasonally, its participation did not result in the pollination of this cycad. The main interaction of the adult *E. minyas* with the cycad was oviposition in the reproductive and vegetative structures of *Z. boliviana*, because it is an obligatory herbivore in the larval stage (Prado, Ledezma, Cubilla-Rios, Bede, & Windsor, 2011; Segalla & Morellato, 2019). *Janbechynea* aff. *paradoxa* had low frequency in the cycad populations monitored and was found in only one population, relatively isolated from the others. Prado, Mckenna, and Windsor (2012) and Prado, Sierra, Windsor, and Bede (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, Luna-Ferrer, González-Gándara, Cruz-Morales, and Domínguez-Barradas (2016). However, these episodes would be less likely in in situ populations due to the distribution of cycads in biodiverse habitats, as is the case for *Z. boliviana*. Future studies about these relationships can provide new insights into their origins and behaviors (Prado et al., 2011).

The ants observed on *Z. boliviana* strobili feeding on pollen were considered opportunistic visitors. *Camponotus crassus* visited strobili of both sexes, but was not considered a pollinator due to inconstancy and poor fidelity to the strobili. In general, ants have limitations in pollination due to their lack of hairs or smooth integuments and the frequency with which they clean their bodies, limiting the adhesion of pollen (Conceição, Delabie, & Costa Neto, 2004). In addition, the presence of the metapleural gland in most species, producing lipophilic substances, may inactivate the pollen (Beattie, 1985). Knowledge about the reliability of plant-visiting ants is scarce (Del-Claro, Rodríguez-Morales, Calixto, Martins, & Torezan-Silingardi, 2019), and the true pollination potential of these insects extends to tropical cycads, and awaits further research.

5 | CONCLUSIONS

Knowledge of natural history and of the reproductive biology of cycads and their pollinators is fundamental, especially in tropical regions. Indeed, insect pollination is a critical facet of cycad biology and conservation (e.g., Roemer et al., 2008; Tang, Skelley, & Pérez-Farrera, 2018; Tang, Xu, et al., 2018; Terry et al., 2016). In this study, we showed that species of Coleoptera, Hymenoptera and Lepidoptera visit *Z. boliviana* strobili, but two of them have an obligatory exclusive relationship. Our investigations revealed the existence of essential and specialized brood-site pollination mutualism between the beetle *P. cerradensis* and *Z. boliviana*, an obligatory insect–plant relationship for the viability of both life cycles. The ovuliferous strobili's morphology, exclusion of wind as a pollen vector, absence of agamospermy and high seed formation upon pollination reinforce our findings that *P. cerradensis* is the effective pollinator for *Z. boliviana*. The Lepidoptera *Eumaeus minyas* is an obligatory herbivore of *Z. boliviana* during larval stages. All stages of *E. minyas* contain cycasin, a compound sequestered from its host plant, *Zamia loddigesii*, and used for defense among predators; the same might be true for *Z. boliviana* (Castillo-Guevara & Rico-Gray, 2002). Our research enriches the knowledge of South American *Zamia* pollination biology and ecology, providing crucial information for testing hypotheses about the taxonomy and biogeography of cycads (e.g., Tang, Xu, et al., 2018).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rosane Segalla and Leonor Patrícia Cerdeira Morellato developed the concept and designed the study; Rosane Segalla conducted all field work, analyzed and interpreted data, and wrote the first draft of the manuscript. Rosane Segalla, Fábio Pinheiro and Leonor Patrícia Cerdeira Morellato contributed to further versions of the manuscript. All authors read and agreed with the final content of the manuscript.

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