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**Dichogamous sexual system in *Cissus spinosa* (Vitaceae): floral  
biology, synchronicity, and visitors**

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synchronicity, and visitors**

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Dissertation presented as a requisite to obtain the Master's degree in Ecology, from the Ecology and Conservation Graduate Program at the Universidade Federal de Mato Grosso do Sul.

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To Charles “Chuck” Martinez aka Gramps, for your contagious passion for the natural world, especially botany, I dedicate this work to you and express the deepest gratitude for your interest and enthusiasm in this research project, you were truly an inspiration

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# Index

	Page
<b>Abstract</b>	01
<b>Resumo</b>	02
<b>Introduction</b>	04
<b>Methods</b>	07
<i>Study site and species</i>	07
<i>Floral biology, dichogamous system, and synchronicity</i>	09
<i>Mating system</i>	11
<i>Floral visitors</i>	12
<i>Data analysis</i>	13
<b>Results</b>	14
<i>Floral biology and mating system</i>	14
<i>Dichogamy and synchronicity</i>	20
<i>Floral visitors</i>	22
<b>Discussion</b>	25
<i>Dichogamy and synchronicity</i>	25
<i>Mating system</i>	26
<i>Potential pollinators and floral visitors</i>	27
<i>Conclusions</i>	29
<b>References</b>	29

## Abstract

Separation of male and female sex organs in angiosperms is essential to their reproductive success, either temporally or spatially, as it promotes outcrossing, reduces self-interference, and produces more fit progeny. Dichogamy is one such strategy with several sub categories related to specific timing or maturation and presentation of stamens and pistils, making it a complex phenomenon to diagnose, and is thought to have evolved for the avoidance of self-pollination. In particular, synchronization of presentation of male and female phase flowers within flowers, inflorescences, and plants is an essential aspect in avoiding selfing for dichogamous plant species. In addition, to ensure reproductive success, many dichogamous species need pollen vectors, such as insects, that visit both male and female flowers to ensure pollen flow and seed production. Herein, I investigated the reproductive and gender system of the liana species *Cissus spinosa* (Vitaceae) in the Brazilian Pantanal, which was preliminarily observed with a seemingly synchronized protandrous dichogamy, and asked: what is the specific dichogamous gender system and mating system for this species and who are potential pollinators and floral visitors? Using specimens of the scandent liana *Cissus spinosa* in the field, I made observations of floral biology, collections of flower development (i.e. reproductive phase), compatibility system and floral visitation between 2014-2016. Through analysis of data on floral morphology and gender presentation through time I determined that *Cissus spinosa* exhibits multi-cycle synchronous dichogamy, with protandrous flowers which had a high level of synchronization both within and between inflorescences of individual plants. *Cissus spinosa* was self-compatible, forming fruits in all treatments of the mating system test. Potential pollinators were found to be Apidae, including *Apis mellifera* and *Trigona spinipes*, along with Crabronidae and Vespidae species. Other visitors included beetles (Chrysomelidae), butterflies (Nymphalidae), and flies (Sarcophagidae & Syrphidae). Overall, the specific dichogamous system reported herein could be a strategy to avoid selfing in this self-compatible species, as inflorescences are synchronously male or female and plants within the same area offer both floral phases consistently throughout the day. This research represents the first report for this specific type of dichogamy in the large genus *Cissus* and could point to trends among this genus or family, especially those in tropical regions.

## Resumo

A separação de órgãos sexuais masculinos e femininos em angiospermas é essencial para o seu sucesso reprodutivo, temporalmente ou espacialmente, pois reduz o autocruzamento e a auto-interferência, e produz proles mais saudáveis. A dicogamia é uma dessas estratégias com várias subcategorias relacionadas ao momento específico em que ocorre a maturação e apresentação de estames e pistilos, tornando-a um fenômeno de diagnóstico complexo, que se acredita ter evoluído para evitar a autopolinização. Em particular, a sincronização do surgimento de estruturas reprodutivas masculinas e femininas, dentro das flores, em inflorescências, e em toda planta é um aspecto essencial para evitar a autofecundação de espécies de plantas dicogâmicas. Além disso, para garantir o sucesso reprodutivo, as espécies dicogâmicas necessitam de vetores de pólen, como insetos, que visitam as flores masculinas e femininas para garantir o fluxo de pólen e a produção de sementes. Neste trabalho, investiguei o sistema sexual e reprodutivo da espécie de liana *Cissus spinosa* (Vitaceae) no Pantanal brasileiro, que foi preliminarmente observada como uma espécie com protândria aparentemente sincronizada, posteriormente questionamos: qual é o sistema sexual de dicogâmico específico e o sistema de acasalamento desta espécie e quais são os potenciais polinizadores e visitantes florais? Utilizando espécimes da liana escandente *Cissus spinosa* no campo, fiz observações da biologia floral, coletas de todos os estágios do desenvolvimento floral (isto é, fase reprodutiva), sistema de acasalamento e visitação floral entre os anos de 2014-2016. Através da análise de dados sobre morfologia floral e maturação de estames e pistilo ao longo do tempo, verifiquei que *Cissus spinosa* apresentou uma dicogamia síncrona de vários ciclos, com flores protândricas que apresentavam um alto nível de sincronização dentro e entre inflorescências de plantas individuais. *Cissus spinosa* é auto-compatível, formando frutos em todos os tratamentos do teste do sistema de acasalamento. Os polinizadores potenciais foram abelhas Apidae, tais como *Apis mellifera* e *Trigona spinipes*, juntamente com espécies de Crabronidae e Vespidae. Outros visitantes incluíram besouros (Chrysomelidae), borboletas (Nymphalidae), e moscas (Sarcophagidae & Syrphidae). Em geral, o tipo de dicogamia aqui relatado pode ser uma estratégia que ocorre para evitar a autofecundação nesta espécie autocompatível, uma vez que as inflorescências são sincronicamente masculinas ou femininas e as plantas dentro da mesma área

oferecem ambas as fases florais consistentemente ao longo do dia. Esta pesquisa representa o primeiro relato para este tipo específico de dicogamia no grande gênero de *Cissus* e pode apontar tendências entre este gênero ou família, especialmente aquelas em regiões tropicais.

## Introduction

About 72% of angiosperms are hermaphrodites with perfect flowers, which present both male and female functions (Richards 1997). Compared to unisexual flowers, perfect flowers are evolutionarily more vulnerable to self-pollination (selfing), and thus also to inbreeding depression and pollen discounting that, in turn, lead to less fit offspring and maternal parents (Barrett 1998, Bawa 1979). To optimize reproductive success, many angiosperms with perfect flowers exhibit diverse mechanisms to avoid pollen-stigma interference and to minimize selfing (Lloyd & Webb 1986). Such mechanisms involve spatial and/or temporal separation of male and female structures within flowers or inflorescences (Bennett 1870, Muller 1883, Lloyd & Webb 1986, Barrett et al. 2000, Barrett 2003). One of these mechanisms is dichogamy, the temporal separation of the functionally male (staminate) and female (pistillate) phases within a flower, inflorescence, or plant (Lloyd & Webb 1986, Bertin & Newman 1993, Çetinbaş & Unal 2014). Dichogamy is a complex mechanism, as several intricate variations exist among species in terms of time and type of change between staminate and pistillate phases.

The two principal types of dichogamy are protandry, when pollen availability precedes stigma receptivity, and protogyny, when stigmas become receptive before pollen is available. Both types provide the unique benefits of avoiding selfing and promoting outcrossing (Table 1, Faegri & Van der Pijl 1979, Bawa and Beach 1981, Webb 1981). Another common dichogamous mechanism thought to improve the avoidance of selfing is synchronicity, which involves the functionally male and female stages alternating synchronously within and between individuals. Different levels of synchronicity and subclasses regarding the number of cycles of floral phases exist which are important in classifying the particular kind of dichogamy which a species presents (Table 1). Synchronicity further reduces self-pollination and maybe an important adaptation for multi-flowered inflorescences, especially how this

functions within or between inflorescences and between plants (Bhardwaj & Eckert 2001).

Therefore, the intricate ways in which dichogamy presents itself, especially in terms of synchronicity, are important for understanding how a plant species avoids selfing, as well as providing clues about its evolutionary history and current ecological interactions.

Table 1. Categories of dichogamy adapted from Çetinbaş & Unal (2014)

Types of Dichogamy				
Order of presentation	Floral elements	Degree of stamen and pistil separation	Degree of plant synchronization	Time interval between stamen and pistil emergence
1.Protandry 2.Protogyny	1.Intrafloral 2.Interfloral	1.Complete 2.Incomplete	1.Asynchronous 2.Hemi-synchronous 3.Synchronous a) Multiple cycles b) Duodichogamy c) Single cycle d) Heterodichogamy	Different time intervals

Several works have focused on the importance of dichogamy in relation to self-incompatibility systems and support that this sexual system can reduce self-fertilization in species with partial or weak self-incompatibility (Lloyd and Webb 1986; Willemstein 1987). Different types of dichogamy affect the evolution of pollinator behavior and many protandrous species tend to depend on pollen vectors to insure the success of this mechanism in providing crosspollination, as well as to increase pollen dispersal to other plants (Barrett 1998; Ollerton et al. 2011, Çetinbaş & Unal 2014). Interactions between plants and pollinators may be complex and highly dynamic, with consequences for reproduction and evolution for both groups (Gómez et al. 2007; Burkle and Alarcón 2011). Animal-pollinated plants exchange resources for the service of pollination provided by the visitors (Waser and Price 1983; Bronstein 1994). The efficacy of floral visitation in pollinating depends on which floral visitors are actual pollinators and which are robbers of flower rewards, such as pollen or nectar, subsequently offering no pollination service (Alves-dos-Santos et al. 2016, Bronstein 2001). To ensure their reproductive success, dichogamous species need pollen vectors that visit flowers in both phases (male and female) to ensure pollen flow and seed production. Therefore, studying how the floral biology, morphology, and sexual system of plant species, prevent inbreeding, and promote pollen flow is essential for understanding plant reproductive patterns and evolutionary processes.

Studies on plant reproduction are especially scarce in Vitaceae, a family with 14 genera and about 900 species (Gerrath et al 2015). *Vitis* is the most studied genus due to the economically important grape species (Gerrath & Posluszny 1989). Dioecy has been reported for some wild *Vitis* species and self-compatible hermaphrodite flowers are commonly found in cultivated varieties (Negi & Olmo 1966, Freeman et al. 1980, Ramos et al. 2014, Zito et al. 2016). Protandrous flowers are reported in the genera *Cayratia* and *Ampelopsis* (Gerrath & Posluszny 1989; Kakutani et al. 1989), but potential dichogamous gender systems are

unknown for most genera. Likewise, few records are available on floral visitors and pollinators among Vitaceae species. *Apis mellifera* and Halictidae bees were reported as pollinators of *Vitis* and *Cayratia* species, beyond other insect visitors as Cerambycidae beetles (Kakutani et al. 1989, Zito et al. 2016). For *Cissus*, which contains over 300 species, reports on floral traits, mating systems, and pollinators are even more sporadic. Protandrous flowers has been reported for *Cissus alata* Jacq. and *Cissus erosa* L.C.Rich. in Venezuela and *Cissus antarctica* Vent. from Australia (Gerrath & Posluszy 1994, Ramirez et al. 2005); and bird-pollination reported for *Cissus simsiana* in the Amazon (Quirino & Machado 2014). Another species, *Cissus ulmifolia*, in the Brazilian Amazon has been reported as a keystone plant resource, due to flowering and fruiting throughout the year and dominant growth patterns (Diaz-Martin 2014).

*Cissus spinosa*, a scandent vine common in the Brazilian Pantanal, was preliminary observed presenting protandrous flowers with a seemingly high level of synchronicity within inflorescences. Nevertheless, how dichogamy is expressed in time and space, the compatibility system (i.e. whether plants are self-compatible versus self-incompatible), and potential pollinators for this species was poorly understood. In this study, I asked what is the specific type of dichogamy and compatibility system of *C. spinosa*, and who are its potential pollinators and floral visitors in regards to this dichogamous behavior? I predicted that this species was self-compatible and presented synchronous dichogamy within inflorescences.

## **Methods**

### *Study site and species*

Field work was carried out during the months of August 2015 and January, June, August 2016, near the Base de Estudos do Pantanal (19°35'S, 57°01'W), Universidade Federal de Mato Grosso do Sul, in the Miranda region of the Pantanal, Mato Grosso do Sul state, Brazil

(Figure 1). The climate is Aw of Köppen, with distinct wet and dry seasons caused by multi-annual flood pulses. Annual rainfall varies between 800 and 1400 mm, with 80% occurring between November and March (Junk et al. 2006). The study site was located next to the Miranda River with segments of disturbed riparian forests and “paratudal”, an arboreal savanna formation with predominance of *Tabebuia aurea* (Bignoniaceae) (Pott et al. 2011) (Figure 1). Individual plants used in this study were selected within a region of “paratudal”, specifically semi-aquatic areas, close to fences or road/trail borders, from two distinct sites, Site1 along the Estrada Parque roadside and Site 2 along a trail edge (Figure 1).

*Cissus spinosa* is distributed throughout Venezuela, Suriname, French Guiana, Brazil, Bolivia, and Paraguay. It has been found from one to 500 m a.s.l. near water bodies or flooded areas (Pott and Pott 2000). Inflorescences are red and horizontal plagiotropic; flowering and fruiting year round (Lombardi 1994; Pott and Pott 2000). Individuals of this species can extend for many meters on top of other vegetation and can be found along riverbanks, road edges, borders, and fences, flowering and offering floral resources to insect visitors throughout the year and seasonal flooding pulses of the Pantanal wetland (Figure 2). Voucher material was deposited in the Campo Grande Herbarium (CGMS) of the Universidade Federal de Mato Grosso do Sul.

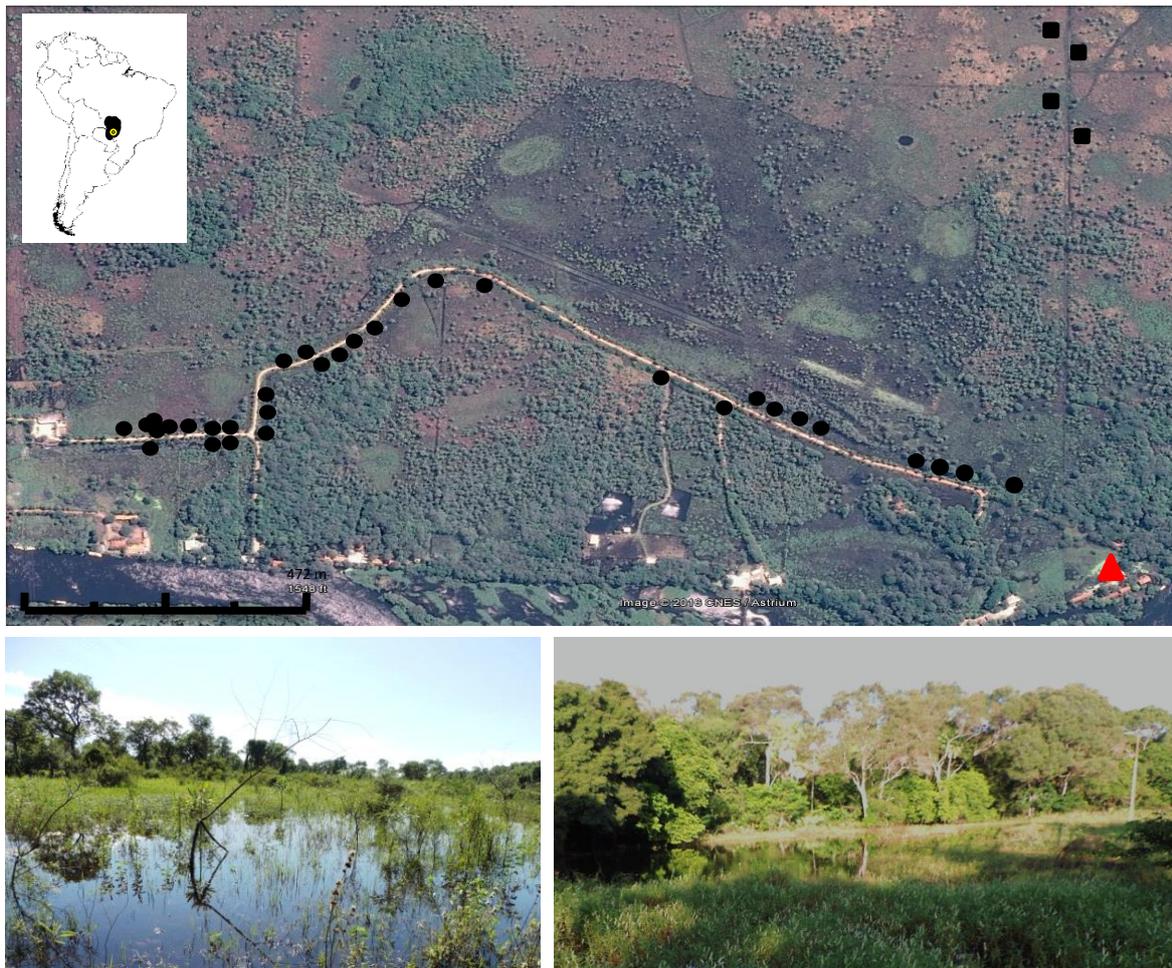


Figure 1. Study site in the Miranda region of the Pantanal in central South America. Above, location of sampled *Cissus spinosa* (Vitaceae) individuals of Site 1 (circles) and Site 2 (squares); and the Base de Estudos do Pantanal (red triangle). Bottom left, “paratudal” formation; bottom right, road borders of riparian forest.

#### *Floral biology, dichogamous system, and synchronicity*

Data collection on floral biology and morphology of *Cissus spinosa* was carried out in the field using live specimens and in the laboratory with material preserved in FAA (70%) or alcohol (70%). To record general floral rhythm, I marked 10 pre-anthesis buds in five plants and accompanied changes throughout anthesis (following methods in Cascante-Marín et al. 2005). I recorded movements of bud and flower structures, pollen viability, stigma

receptivity, and nectar sugar concentration. I estimated the proportion of viable pollen grains of 10 pre-anthesis buds using acetic carmine in pollen slides under microscope (Dafni 1992). I determined stigma receptivity by the formation of air bubbles after adding hydrogen peroxide 3% ( $H_2O_2$ ) to the stigmatic surface of 40 flowers (20 in male and 20 in female phase) from five plants (Dafni 1992), and through observation of stigma exudates with a magnifying glass (“Lupenbrille”) and stereomicroscope (Sigrist & Sazima 2004). Nectar sugar concentration was measured with a digital pocket refractometer (0+53 Brix, Pocket Pal-1 Atago) from recently opened flowers (20 flowers in each phase from five plants). I verified the location of nectar production and storage under stereomicroscope from 10 fresh flowers of different individual plants. I measured style length and flower length and width of 15 male and 15 female phase flowers from six individual plants with a caliper (0.01 mm precision).

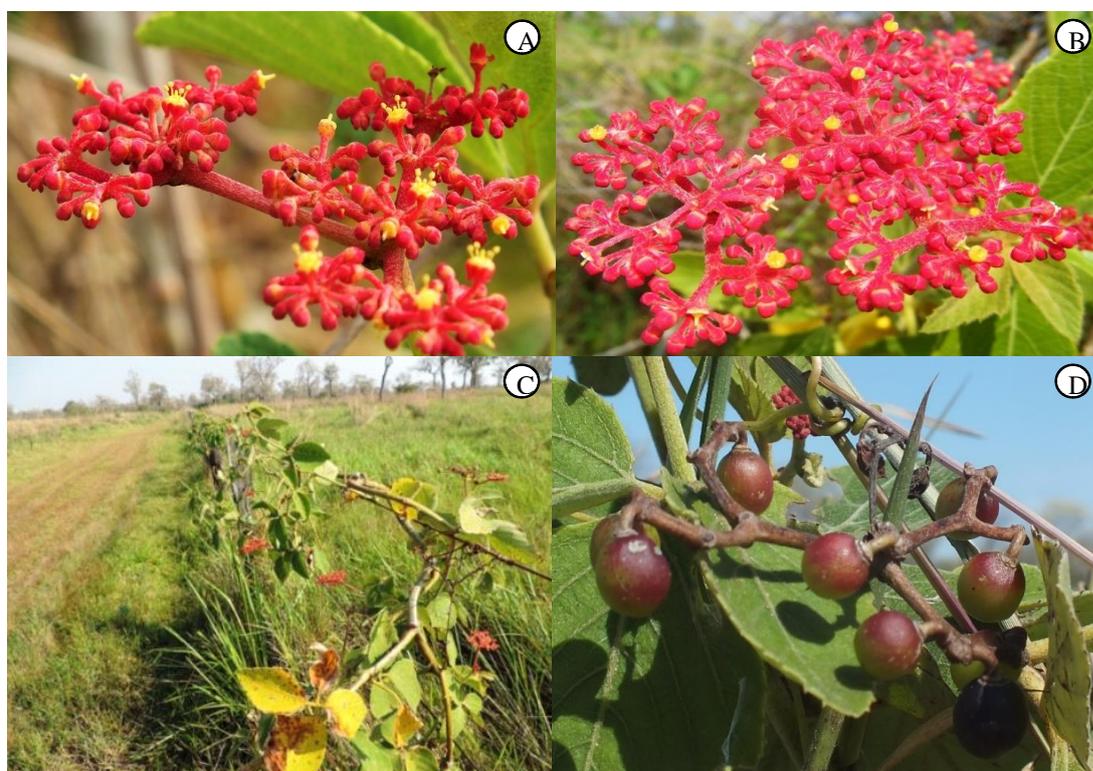


Figure 2. Inflorescences of *Cissus spinosa* (Vitaceae) with all male (A) or female (B) flowers; plants growing on fences in flooded area (C); and infructescence (D).

To study inflorescence and plant gender synchronicity within populations and within individuals, I visually characterized the male and female flower phases. Flowers were considered functionally male when stamens were erect with pollen available and functionally female when styles were extended and stigmas were receptive (wet surfaces that responded positively to hydrogen peroxide test). At the plant level, synchronicity was recorded from 33 individuals at Site 1 (Figure 1) at least 5 m apart from one another to ensure that data represented different individual plants. I marked 3-5 inflorescences per plant and counted the number of male and female flowers throughout the anthesis period (0800-1700 h) during five consecutive days. To determine sexual synchronicity within inflorescences, I marked unopened inflorescences from Site 2 (Figure 1) and recorded floral development in the field through photos and counts of open flowers every hour between 0700 h and 1800 h for seven consecutive days. I followed Lloyd & Webb (1986) and Çetinbaş & Unal (2014) to determine the specific type of dichogamy.

#### *Compatibility system*

I carried out tests of self-compatibility versus self-incompatibility in the field through manipulative experiments. I used 20 to 25 bagged flowers (4 individuals) for each of the three treatments, manual self-pollination spontaneous self-pollination, and cross-pollination (xenogamy) (Kearns & Inouye 1993). For all three treatments I used mesh netting to cover inflorescences with unopened buds, when buds began to open I removed the mesh and either removed stamens/anthers from newly opened flowers and placed that pollen directly on the flowers unextended style (manual self-pollination), left floral buds enclosed with in the mesh (spontaneous self-pollination), or used pollen collected from other plants (within 2km) which I placed on the unextended styles of newly opened flowers which had stamens/anthers removed (cross-pollination). Additionally, I haphazardly selected open flowers accessible to

floral visitors and marked them to verify the pollination under natural conditions (natural or “open” pollination). Fifteen days after the treatments, I counted the number of manipulated or marked flowers that started fruit formation (one ovule per fruit with one seed). Furthermore, to estimate fruit set in natural conditions I counted the number of floral elements (buds) and the number of fruits produced in 32 inflorescences from four different plants. I also calculated the auto fertility index (AFI), the self-compatibility index (SCI), and the reproductive efficiency (RE) (Lloyd & Schoen 1992, Zapata & Arroyo 1978).

### *Floral visitors*

Direct observations were used to sample visits by insects to flowers of 78 individuals. One visit was considered when the insect landed on floral structures for two seconds or more. From site 2 visitors were recorded within intermittent periods of 15 min between 0800 and 1700 h (when visitors were most active), totaling 30 observation hours completed in January and August 2016. In each visit, I recorded the sexual phase of the visited flowers, the visitor species, anther/stigma contact, and visiting behavior during the visits. For each species, I calculated the average visitation frequency (# of visits of species/total number of visits observed) and ratio of visitation between male and female flower phases for each species. In different periods, visiting insects were photographed and collected with butterfly nets for identification of species by specialists, as well as were examined for pollen adherence under stereomicroscope. Potential pollinators were considered those with legitimate anther and stigma contact, pollen adhered to body parts that also touched floral reproductive parts, and high visitation frequency (as well as frequency between both male and female morphs). All specimens were incorporated into the Zoological Collection at the Universidade Federal do Mato Grosso do Sul (ZUFMS).

### *Data analysis*

To evaluate differences in floral dimensions between male and female phases (cues for pollination) I first tested normality of floral dimensions data using the Kolmogorov-Smirnov test (Zar 2010). Non-parametric data (nectar concentration) was tested using Mann-Whitney test and parametric data (style length, floral width, floral length) were tested using Student t-tests. In order to confirm if *Cissus spinosa* was self-compatible, I evaluated fruit formation from pollination treatments through a Pearson chi-squared test, using the number of fruits formed per treatment divided by total number of flowers per treatment (R Core Team 2016). To calculate if gender expression between inflorescences on the same plant was synchronized I log transformed the total number of open male and female flowers per plant at each time step to determine the relationship between number of male and female flowers. Afterwards, with log transformed data I was able to perform a Pearson test to determine the relationship between male and female flowers within plants. Furthermore, results of Pearson test were compared to Monte Carlo simulations (random distribution) to determine if relationship between male and female flowers observed was a random phenomenon or if inflorescences within plants presented significant correlation of the presentation of male and female flowers.

To evaluate synchronicity of gender expression within inflorescences and the population, I also transformed gender expression data (total number of open male and female flowers per inflorescence) using the Shannon entropy ( $\sum_{i=1}^n p_i * \log_2(p_i)$ ), which expresses a value of disorder within the data, with 1 being complete disorder and 0 being no disorder. For flower gender expression, numbers close to 1 meant that both male and female flowers were present in the level tested (inflorescence or population) and numbers close to 0 meant that only one phase was present within a time step. For both synchronicity tests, inflorescences with zero open flowers were discarded as they did not represent a sex. All analysis was performed in R version 3.24 (R Core Team 2016).

## Results

### *Floral biology and compatibility system*

*Cissus spinosa* showed a remarkable floral display, with red and composed summit inflorescences (Figure 2). Flowering individuals presented 1 or more inflorescences, each one containing an average of ~97 buds. Within each inflorescence 2-12 flowers opened daily, with all flowers opening within 15-20 days. Flowers were about 5 mm long and pedicels nearly 3 mm long. Each flower showed four reddish sepals that folded back when flowers opened. The interior of the flower, the stamens and pistil were all yellow (Figure 2), with a nectariferous disc at the base of the ovary that produced nectar throughout anthesis and accumulated at the exposed base of the ovary. New flowers opened throughout the day starting between 6:00am-8:00am with anthesis of individual flowers lasting for an average of six to eight hours. The periods of pollen availability and stigma receptivity were completely separated within the flowers.

The pre-anthesis buds (phase 1 in Figure 3) were positioned downward in the inflorescence, then started to rise and reached phase 2 after about 10 min and phase 3 after 20 min, when buds were erect and started opening (Figure 3). In the phase 4, erect stamens were functional with pollen available but stigmas were not receptive; this male phase lasted between one to two hours and then the stamens senesced. Following, the flowers entered in a brief (< 30 min) neuter state (phase 5) in which stigma was not receptive nor pollen was available. During this period the style grew and then became receptive (phase 6), when flowers were functionally female. Style length during the female phase ( $1.89 \pm 0.59$  mm) was almost twice ( $p < 0.0001$ ;  $f = 6.89$ ;  $DF = 27$ ) the length in the male phase ( $1.07 \pm 0.22$  mm). After 2-3h in the female phase, the flowers begin to turn down and stigmas became brown (phase 7) (Figure 3). Immature fruits began forming within 7-15 days after anthesis; dark purple fruits contained one seed and were ripe after 15-25 days (Figure 2D). When in male

phase flowers measured  $4.7 \pm 0.3$  mm wide and  $3.9 \pm 0.26$  mm long, and during the female phase, flowers measured  $1.8 \pm 0.3$  mm wide and  $4.4 \pm 0.30$  mm long (Figure 4B). Nectar sugar concentration was higher in the female phase ( $p < 0.0001$ ; Mann-Whitney, Figure 4D). Female phase flowers had greater width ( $4.4 \pm 0.3$ ) than male phase ( $3.9 \pm 0.26$ ), while male phase flowers had greater average length ( $3.7 \pm 0.35$ ) than female phase ( $0.9 \pm 0.15$ ) (Figure 4).

*Cissus spinosa* was found to be self-compatible. All treatments of controlled pollinations produced fruits, with no significant differences of the proportion of fruits among self-pollination, cross-pollination and natural pollination while spontaneous pollination showed significant difference and the highest rate of fruit formation (Table 2). Natural fruit set was low in relation to number of buds produced per inflorescence. The overall fruit set, recorded through the total number of flowers that formed into fruits per inflorescence, was 6.1% on average (32 inflorescences, 260 fruits/4240 flowers). Pearson chi squared revealed that there was no significant difference between fruit formation for between Selfing, Cross, and Natural Pollination, while there was significant difference between Cross and Spontaneous, with spontaneous pollination forming the most fruits ( $\chi^2 = 14.426$ ,  $df = 3$ ,  $p\text{-value} = 0.0024$ ).

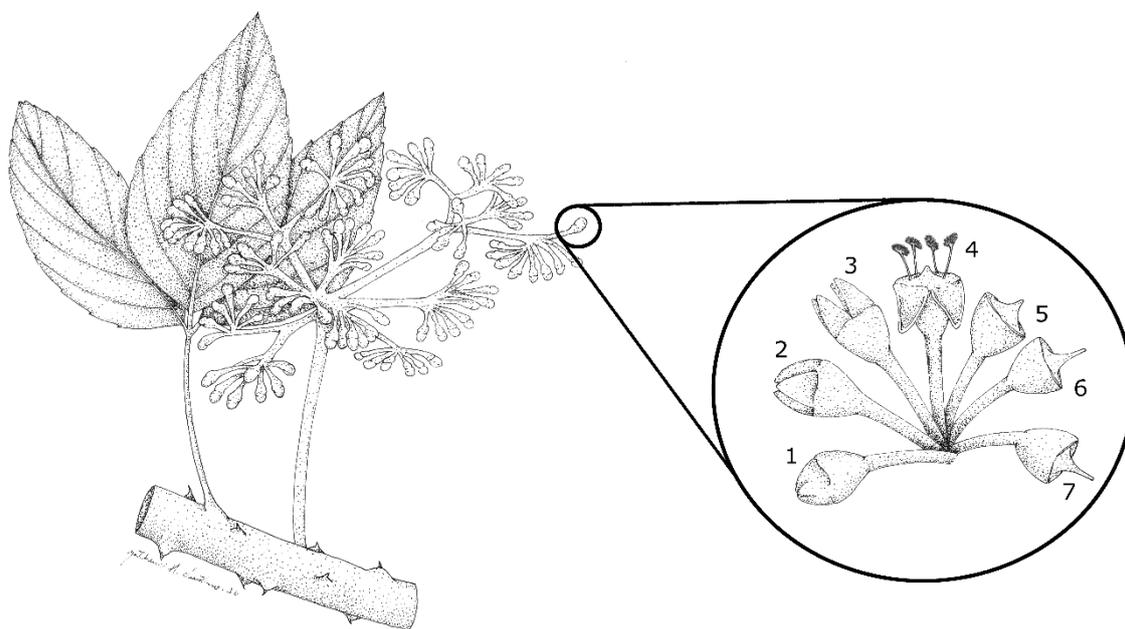


Figure 3. Inflorescence of *Cissus spinosa* (Vitaceae) with magnified schematic progression of floral development: (1) pre-anthesis bud, (2) bud begins to rise, (3) sepals begin to open, (4) male phase with stamens and anthers, (5) neuter phase, (6) female phase with receptive stigma, (7) end of anthesis (flower turns down for fruit formation or abortion). Phases 3 to 6 occur at 90 degrees within inflorescence.

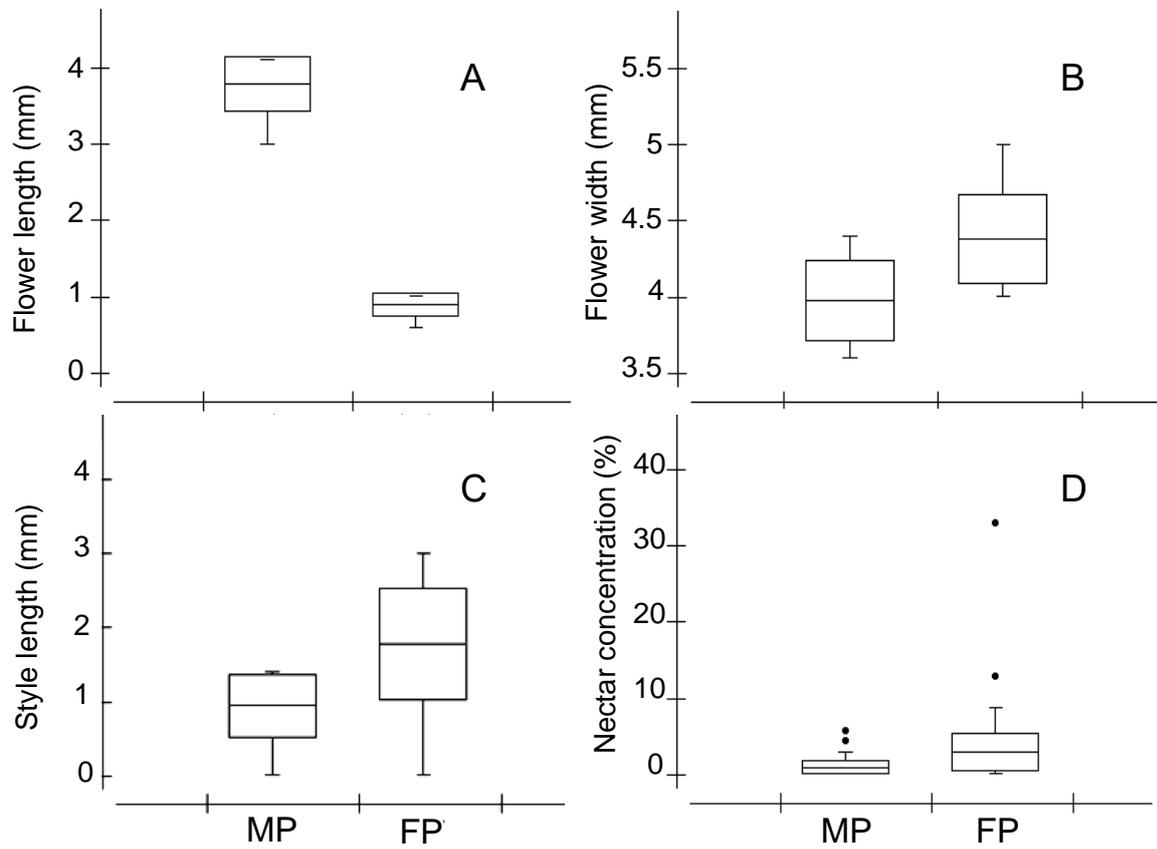


Figure 4. Flower length (A) and width, (B) style length, (C) and nectar sugar concentration (D) of flowers of *Cissus spinosa* (Vitaceae) during the male phase (MP) and female phase (FP)

Table 2. Results of pollination experiment for *Cissus spinosa* (Vitaceae) in the Pantanal wetland, Brazil. Autofertility and self-compatibility indices follow Lloyd & Schoen (1992) and reproductive efficiency follows Zapata & Arroyo (1978).

\*Significant difference in fruit formation, Pearson chi-squared ( $\chi^2= 14.426$ , DF= 3, p-value=0.0024).

Treatments	% (fruits/flowers)
Manual self-pollination	29 (6/21)
Spontaneous self-pollination*	55 (11/20)
Cross-pollination (xenogamy)	8 (2/25)
Natural pollination	15 (3/20)
Auto fertility index (AFI)	6.88
Self-compatibility index (SCI)	3.63
Reproductive efficiency (RE)	1.88

### *Dichogamy and synchronicity*

Throughout each day inflorescences presented flowers which changed phase, opening at multiple times of the day (Figure 5). Individual flowers of *C. spinosa* were markedly protandrous, with male flowers always present before female flowers. Within and between flowers on inflorescences dichogamy was intrafloral and complete, with no overlap between stamen and pistil presentation. Inflorescences within a plant showed a strongly synchronous pattern with a negative correlation between male and female flowers within inflorescences ( $r=-0.062$ ,  $p<0.01$ ), in other words when male flowers were present across inflorescences produced by a plant, female flowers were not and vice-versa. Pattern of synchrony for the population compared to separate inflorescences, as demonstrated through Shannon entropy (Figure 5) shows an asynchronous pattern of floral presentation for the population (Entropy  $\geq 0.5$ , Figure 5), while separate inflorescences show a more synchronous pattern of floral presentation (Entropy  $\leq 0.4$ , Figure 5). Overall, *C. spinosa* expressed a multi-cycle synchronous dichogamy with protandrous flowers that opened throughout the day, for every day of the year (Table 1).

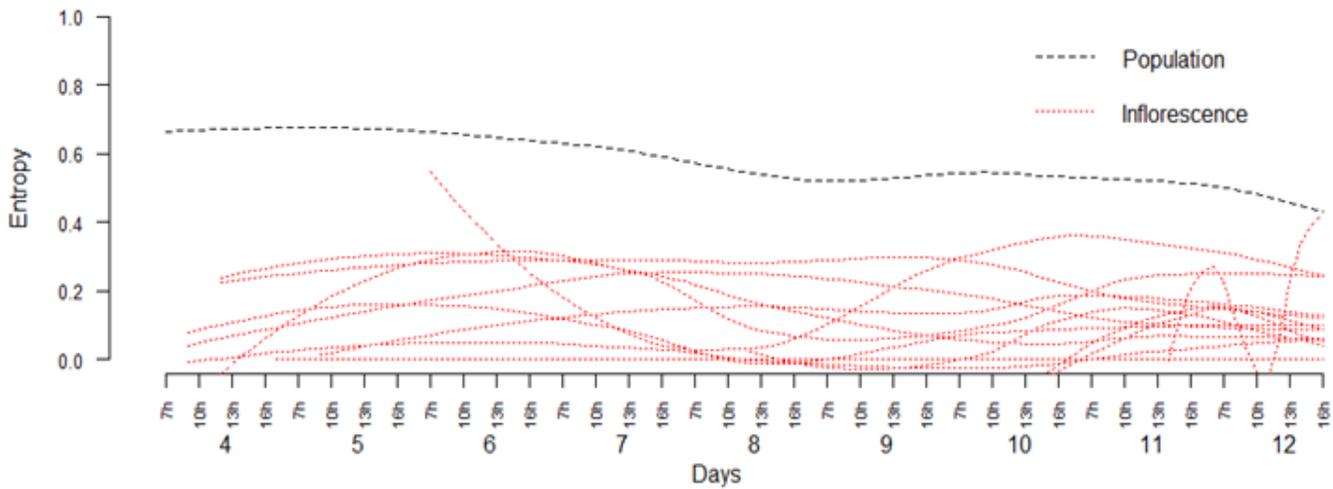


Figure 5. Shannon entropy  $(\sum_{i=1}^n p_i * \log_2(p_i))$  (representation of synchronization between male and female phase flowers, with numbers closer to 0 being fully synchronous and closer to 1 asynchronous) between twenty-one different inflorescences of *C. spinosa* compared with the Shannon entropy of total population throughout 12 consecutive days in August 2016 at the Base de Estudos in the Pantanal of Mato Grosso do Sul, Brazil.

### *Floral visitors*

In total I recorded 22 species visiting *C. spinosa* flowers (Table 3). Twelve of them visited flowers of both male and female phases, including the bees *Apis mellifera*, *Bombus* sp., *Trigona spinipes* and *Xyllocopa* sp. (Apidae), the wasps *Brachygastra* sp., *Polybia* sp., *Polystes* sp. (Vespidae), the butterfly *Dryadula phaetusa*, and undetermined species of Chrysomelidae, Culcidae, Syrphidae and Crabronidae-Philanthini (Table 3). Of these species, *Apis mellifera* was almost six times more frequent than the second most frequent visitor, *Brachygastra* sp., followed by *Polystes* sp., then other native bees and wasps, including *Trigona spinipes*, and lastly *Dryadula phaetusa*.

The bees, *Apis mellifera*, *Bombus* sp., *Trigona spinipes*, and *Xyllocopa* sp. are most likely main pollinators of *Cissus spinosa*. These species consistently contacted reproductive structures with the abaxial thorax while collecting pollen or nectar, pollen was found adhered abundantly on insect body (frontal abdomen, thorax, legs, and mouthparts), were the most frequent insect group, and were seen frequenting both male and female phase in equal proportion (Table 3, Figure 6A&B). Crabronidae, were found with abundant pollen throughout the bodies (abdomen, thorax, legs, mouthparts), contacted both reproductive structures flowers, and were fairly frequent visitors, thus could also be considered potential pollinators. Vespidae, as *Brachygastra* sp., *Polybia* sp., *Polystes* sp 1., and *Polystes* sp. 2 may be considered potential pollinators, as they represent the second most frequent group, found visiting both male and female phases, but had fewer pollen grains adhered to body (abdomen, thorax, leg attachments, mouthparts) and made minimal contact with reproductive structures during visits, due to elevated body morphology (Table 3, Figure 6 D& E). Furthermore, flies and butterflies, as Chrysomelidae, Sarcophagidae, Syrphidae, and Nymphalidae may be considered occasional

pollinators, as their frequency was relatively low, often avoided contact with reproductive parts, but were found visiting both phases and with sparse pollen adhered to bodies (Chrysomelidae: antennae, Syrphidae: head, abdomen, legs, Sarcophagidae: head) (Table 3, Figure 6C & F).

Table 3. Flower visitors of *Cissus spinosa* (Vitaceae) in the Pantanal wetland, Brazil, and number of visits, resource collected (N: nectar, P: pollen), visitation frequency (mean  $\pm$  SD) and female: male (F: M) ratio of visited flowers.

Order Family	Species	Visits (n)	Resource	Pollen adherence	Frequency % (n/total # of visits)	F:M phase ratio
Coleoptera						
Chrysomelidae	Undetermined	11	N	Sparse	2.08 $\pm$ 0.74	7:3
Diptera						
Culicidae	Undetermined	3	N		0.57 $\pm$ 1.08	6.6:3.4
Sarcophagidae	Undetermined	5	N	Sparse	0.94 $\pm$ 0.99	1:0
Syrphidae	Undetermined	4	P/N	Sparse	0.76 $\pm$ 1.04	3:7
Hymenoptera						
Apidae	<i>Apis mellifera</i>	329	P/N	Abundant	62.08 $\pm$ 13.03	1:1
	<i>Bombus</i> sp.	5	N		0.94 $\pm$ 0.99	2:3
	<i>Trigona spinipes</i>	34	P/N	Abundant	6.42 $\pm$ 0.26	5.3:4.7
	<i>Xylocopa</i> sp.	4	N		0.76 $\pm$ 1.04	4.5:5.5
Crabronidae	Philanthini	11	P/N	Abundant	2.08 $\pm$ 0.74	6.3:3.7
Sphecidae	Undetermined	1			0.19 $\pm$ 1.17	-
Formicidae	<i>Camponotus</i> sp.	1	N		0.19 $\pm$ 1.17	1:0
Vespidae	Undetermined	13			2.45 $\pm$ 0.65	2.7:7.3
	<i>Brachygastra</i> sp.	56		Sparse	10.57 $\pm$ 1.21	3:2
	<i>Polybia sericea</i>	1			0.19 $\pm$ 1.17	0:1
	<i>Polybia</i> sp.	2		Sparse	0.38 $\pm$ 1.12	1:1
	<i>Polystes</i> sp.1	29	N	Sparse	5.47 $\pm$ 0.04	1:1
	<i>Polystes</i> sp.2	3	N	Sparse	0.57 $\pm$ 1.08	0:1
Lepidoptera						
Lycaenidae	Undetermined					
Nymphalidae	Undetermined	6	N		1.13 $\pm$ 0.95	1:0
	<i>Dryadula phaetusa</i>	12	N		2.26 $\pm$ 0.69	1:3
	Satyrinae					

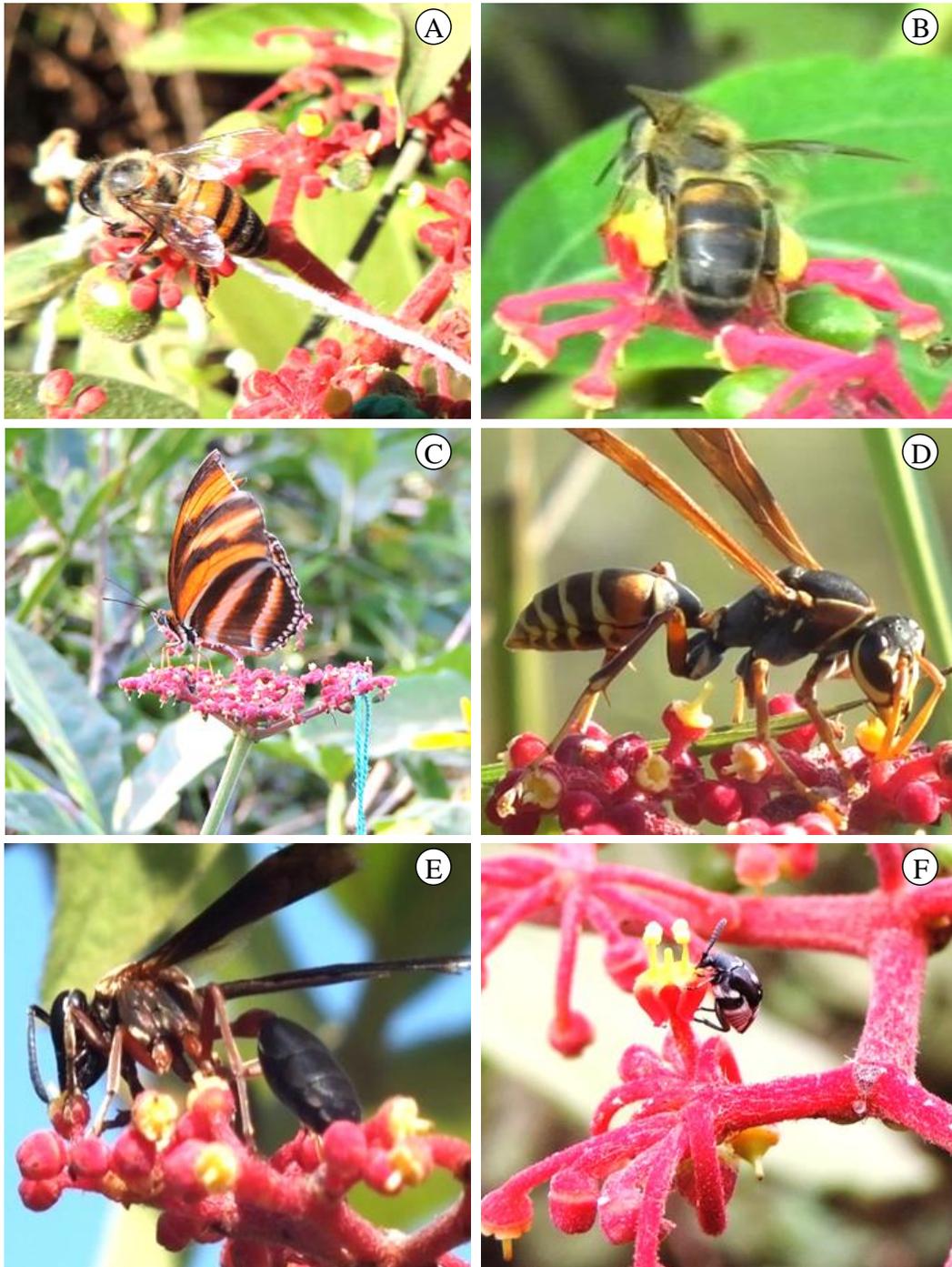


Figure 6. Floral visitors of *Cissus spinosa* (Vitaceae) in the Pantanal wetland, Brazil. (A-B) *Apis mellifera* bee contacting reproductive structures; (C) Nymphalidae butterfly visiting for nectar; (D) *Polystes* sp.1 and (E) *Polybia sericia* wasps collecting nectar; (F) Chrysomelidae beetle stealing nectar from a flower in male phase.

## Discussion

Overall this work supports a multi-cycle synchronous dichogamy within inflorescences, with protandrous flowers, as the gender system for *Cissus spinosa*, with functionally male and female flowers alternating synchronously throughout the day. *Cissus spinosa* is self-compatible, able to form fruits with its own pollen. Natural fruit set was relatively low when compared to number of buds produced per inflorescence. *Cissus spinosa* also needs pollen vectors to bring pollen to receptive stigmas and presented a fairly generalized pollination system, with open floral morphology and easily accessible pollen, as well as nectar available in both male and female phases. Main potential pollinators are bees, as *Apis mellifera*, *Trigona spinipes*, along with Crabronidae and Vespidae, such as *Brachygastra* sp. and *Polystes* sp. 1, while other visitors such as butterflies, flies, and beetles may be considered occasional or rare pollinators.

### *Dichogamy and synchronicity*

The specific kind of dichogamous gender strategy seen herein has gone widely undiagnosed in studies that focus on floral phenology and reproduction among angiosperms, which has been attributed to the lack of attention that Darwin allocated to this kind of plant gender system (Lloyd & Webb 1986, Cetinbas & Unal 2014). All individual flowers of *Cissus spinosa* were found to have intrafloral protandry, with pollen and stamens consistently available before stigma receptivity within each flower. This protandry was rapid and daily (lasting six to eight hours), with reproductive structures completely separated in time and space, causing no overlap between receptive stigmas and viable pollen within a flower, which may have been affected by insect visitation rates or climatic and environmental conditions (Devlin & Stephenson 1984, Borges 1998, Bell & Cresswell 1998, Molina 2009). These findings for *Cissus spinosa* corroborate

with previous trends seen for protandrous flowers, being commonly seen in one-day flowering plants, as well as in species from tropical regions where stamen development may be accelerated (Bawa & Beach 1981, Cetinbas & Unal 2014). I also confirmed that within inflorescences flowers generally presented male and female phases asynchronously, which could be promoting the avoidance of pollen-stigma contact between flowers within the inflorescence (evading autogamous selfing). Likewise, inflorescences within the same individual plant showed a generally synchronous gender at each time step, which may be aiding in the avoidance of geitonogamous selfing.

Though floral synchrony was found at the flower, inflorescence, and plant level, I recorded asynchronous gender expression between neighboring plants within the study sites. I also noted a consistent multi-cycle dichogamy for this species, which may allow for more chances of pollen adherence to reproductive style, as *Cissus spinosa* has many small flowers within in the same inflorescences (Table 1, Figure 2). Reviews on this gender strategy confirm that multi-cycle is the most common type of synchronized protandry, as described in *Aralia hispida* (Thomson & Barrett 1981). This rate at which this multi-cycle dichogamy occurs is remarkable, further supporting the importance of asynchrony in gender phases within inflorescences and within individuals.

### *Mating system*

*Cissus spinosa* was found to be self-compatible, producing fruits in all pollination treatments. Herein the highest number of fruits were formed in the spontaneous pollination treatment, which may be attributed to squatter species (spiders, beetles, and ants) that could have remained in the bagged inflorescences, or to rain or wind, which could have caused contact between mesh and

reproductive structures, subsequently transferring self-pollen to receptive stigmas (Lloyd & Schoen 1992). Furthermore, this treatment may not have been effective in determining if the species spontaneously self-pollinates or forms fruit, but may still be used to support self-compatibility within this species.

The combination of asynchrony in gender at the plant level probably helps to avoid self-pollination within plants, while asynchrony between neighboring plants helps guarantee cross-pollination, and outcrossed progeny (Ims 1990). Overall, this particular temporal strategy would be optimal in avoiding high levels of selfing in inflorescences that present many proximal tiny flowers within an inflorescence, as spatial separation within these inflorescences may be costly and lead to increased interference or selfing rates. Furthermore, the markedly protandrous flowers and synchronized behavior described herein make selfing, within and between flowers, difficult both spatially and temporally, as anthers dehisce and flower enters short style phase is which stigma is not receptive. This particular floral adaptation could have been maintained due to self-compatibility within the species. The evolution of self-incompatibly (SI) strategies are complex and the reversal of SI is more common than the gain within a species (Igic et al 2008), therefore causing self-compatible species to depend greatly on floral morphology and pollen vectors to avoid selfing, as the likelihood of developing genetic SI would be low (Barrett 1988, Igic et al 2008). Therefore, floral adaptations and rapid cycle of male and female phases reported for *Cissus spinosa* may be an example of this type of morphological adaptation to avoid selfing.

#### *Pollinators and floral visitors*

Overall the inflorescence of *C. spinosa* contains many small bright red buds (~75-200), which when open are bright yellow with nectar available in both male and female phases. Such factors

give the plant greater likelihood of visualization by insects, such as bees, butterflies, and flies (Rech et al. 2015). As described by Alves-dos-Santos et al (2016), determining successful pollinators is quite complex and potential pollinators may be considered those that have legitimate visits, are faithful to flowers, and have pollen located on insect bodies. Following this criteria, main pollinators can be considered Apidae, including *Apis mellifera*, *Trigona spinipes*, *Bombus* and *Xylocopa*. Though bee pollination is yet unreported for *Cissus*, a related liana species, *Cayratia japonica* Vitaceae, which also presents protandrous dichogamy within individual flowers, was found being pollinated by the native bee *Apis ceana* (Kakutani et al. 1989). Other than bees, Crabronidae and Vespidae could also be considered potential pollinators, as they were frequently seen visiting *Cissus*, while most other species were considered occasional or rare pollinators. Even though a pollinator may be a rare visitor in relation to other species, they could be an important vector for cross pollination, as they may have longer flight patterns, which has been seen with butterfly species (Herrera 1987). Morphological qualities, as open flowers and easily accessible floral resources, may also help explain why I recorded diverse floral visitor species (Table 3; Figure 6; Ollerton et al. 2007). Selection favors floral traits that influence the transfer of pollen, increasing quantity and quality of the seeds produced and the amount of pollen exported to congeners (Barrett 1998). For pollinators, selection favors traits that maximize the rate of energy gain through foraging behavior (Pyke 1984), which could lead to a transfer of non-optimal pollen to plants. Therefore, while plants and pollinators are involved in true mutualism, with reciprocal benefits, conflict of interests still exist (Bronstein 2001, Dufay & Anstett 2003, De Jong & Klinkhamer 2005). For *Cissus spinosa* this conflict was evident with the wide range of visitors that used nectar and pollen resources, ranging from ants (pure nectar robbers) to bees (who visit frequently, carry large pollen loads, and collect both pollen and nectar

resources). Conversely, from the plants point of view, reproductive dichogamy tends to affect the evolution of pollination behavior through selection of location of reproductive parts, with protandrous flowers typically pollinated by birds and bees (Çetinbaş & Unal). Thus, this may explain why I witnessed the protandrous flowers of *C. spinosa*, which offered pollen, being primarily pollinated by bee species, both native and invasive, as protandrous floral position was adapted to their body morphology.

### *Conclusions*

Though dichogamy is commonly seen among angiosperms, it has gone fairly unreported within the Vitaceae family, herein I report for the first time multi-cycle synchronous protandrous dichogamy for *Cissus spinosa* in the Brazilian Pantanal. I suggest that this particular type of dichogamy could be a trend within genus *Cissus*, as I personally observed similar floral development in *Cissus verticilata*, with protandrous flowers being reported in other species of *Cissus* (Gerrath & Posluszy 1994, Ramirez et al. 2005). Further exploration of floral biology is necessary for the genus, and arguably the family, in order to determine specific phylogenetic relationships, as trends in dichogamy tend to be family and or genus wide patterns (Gerrath et al 2015, Çetinbaş & Unal 2014). Regarding floral visitors, bee species are the main pollinators of *Cissus spinosa* in the Brazilian Pantanal, especially *Apis mellifera*, a non- native bee species.

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