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Reproductive biology and selection of floral attractiveness traits by legitimate and illegitimate visitors in the narrow endemic *Collaea cipoensis* (Fabaceae)

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"Just living is not enough," said the butterfly,

"one must have sunshine, freedom, and a little flower."

Hans Christian Andersen
The Complete Fairy Tales

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SUMÁRIO

Abstract

Plant interactions with flower visitors are essential to understand the reproductive biology, evolution and distribution of flowering plants. Attractiveness to pollinators plays a decisive role in the reproductive ecology of such species. Although, attractiveness involves that flowers undergo interactions with mutualistic pollinators, and simultaneously with antagonist visitors (i.e. nectar-robber, nectar-thieves, floral enemies). Pollinators are considered one of the main drivers of evolution on floral traits related to attractiveness, and nectar-robbers can affect plant reproductive success causing direct or indirect effects. The present study describes the floral morphology and reproductive aspects of *Collaea cipoensis*, addressing the selective effects of floral legitimate and illegitimate visitors on floral attractiveness. Was hypothesized that visitors counteract the selective positive effects of pollinators on flower size and number through female fitness components. A total of 43 species (with 4164 events of visitation) were recorded. Hummingbirds *Eupetomena macroura* and *Colibri serrirostris* were identified as the potential pollinators of the especies. The results demonstrate that plants with large number of open flowers are more attractive to both mutualistic (i.e potential pollinators) and antagonistic (i.e nectar-robbers, nectar-thieves) visitors, and also that nectar-robbers affect negatively the female reproductive capacity of the species by interfering with its production of fruits and seeds, and its seed number. The findings of this study, reinforce the idea that endemic and limited distributed species of the rupestrian grasslands have a very specific niche and are highly adapted to this complex scenario, in which strong selective pressures diminish the species likelihood to succeed in other areas.

Key words: antagonism, floral attractiveness, floral visitors, illegitimate visitors, legitimate visitors, phenotypic selection, reproductive success.

Resumo

O conhecimento das interações entre plantas e seus visitantes florais é essencial no entendimento da biologia reprodutiva, evolução e distribuição das plantas com flores. A atratividade floral desempenha um papel decisivo na ecologia reprodutiva dessas espécies, embora, a atratividade atue, simultaneamente, sobre polinizadores mutualistas e antagonistas (i.e. ladrões de néctar, pilhadores, inimigos florais). Os polinizadores mutualistas (legítimos) são considerados um dos principais motores da evolução nos traços florais relacionados à atratividade, enquanto os antagonistas (ilegítimos) podem afetar o sucesso reprodutivo das plantas causando efeitos diretos ou indiretos. No presente estudo são descritos os aspectos da morfologia floral e reprodutiva de *Collaea cipoensis*, quantificando os efeitos seletivos de visitantes legítimos e ilegítimos e sua relação com a atratividade floral. A hipótese testada é que os visitantes ilegítimos neutralizam os efeitos positivos que selecionam os polinizadores na atratividade floral, avaliada por meio de componentes do fitness feminino. Foram registadas 43 espécies de visitantes, em 4.164 visitas. Os beija-flores *Eupetomena macroura* e *Colibri serrirostris* foram identificados como potenciais polinizadores de *C. cipoensis*. Os resultados demonstram que indivíduos com maior número de flores abertas são mais atraentes para ambos visitantes. Os pilhadores de néctar afetam negativamente a capacidade reprodutiva feminina dessa espécie, interferindo na sua produção de frutos e no número de sementes produzidas. Esses resultados reforçam a ideia de que as espécies endêmicas dos campos rupestres têm um nicho específico e são altamente adaptadas a este cenário complexo, em que fortes pressões seletivas diminuem a probabilidade de serem bem sucedidas em outras áreas.

Palavras Chave: antagonismo, atratividade floral, seleção fenotípica, sucesso reprodutivo, visitantes florais, visitantes ilegítimos, visitantes legítimos.

Chapter 1

Reproductive biology and floral visitors of *Collaea cipoensis* (Fabaceae), an endemic shrub of the rupestrian grasslands

Irene Gélvez-Zúñiga, Ana Carolina O. Neves, Alberto L. Teixido, G. Wilson Fernandes

Introduction

Interactions of plants with flower visitors are essential to fully understand plant's reproductive biology, evolution and distribution. Selective pressures have allowed flowers to maximize their attraction to pollinator agents (Fenster et al., 2004; Rosas-Guerrero et al., 2014; Stebbins, 1970). However, attractiveness involves that flowers not only experience interactions with mutualistic pollinators, but simultaneously also with antagonist visitors, such as florivores, nectar-robbers, nectar-thieves and fungal pathogens (Bronstein, 2001; Inouye, 1980; McCall and Irwin, 2006; Shykoff et al., 1996). The balance between costs and benefits of concurrent floral attraction to potential pollinators and floral enemies can switch from positive (Navarro, 2000) to neutral (Maloof, 2001) to, mostly, negative (Irwin, 2009; Irwin and Maloof, 2002; Navarro and Medel, 2009) according to the ecological context and inherent biotic and abiotic conditions faced by the plants (Bronstein, 2001).

Endemic species with restricted distributions are frequent in Brazilian rupestrian grasslands and such a limitation is strongly associated with wide altitudinal range, habitat heterogeneity, isolation among vegetation islands, low nutritional soil quality and low water retention (Fernandes, 2016; Giulietti et al., 1997; Silveira et al., 2016). Hereby, endemic species of the rupestrian grasslands are under strong selective pressure (Fernandes, 2016). Although of paramount importance for their conservation and management, the understanding of the ecology and evolution of geographically restricted species is still poor in this singular savannah ecosystems (but see Barbosa et al., 2015; Moreira et al., 2008). More specifically, studies on flower antagonist visitors and their roles as nectar-robbers, nectar-thieves or florivores remain scarce (Guerra et al., 2016).

The South American *Collaea* genus has seven species occurring in Peru, Bolivia, Paraguay, Brazil and Argentina (Burkart, 1987; Lewis et al., 2005). Brazil, with four species reported, is considered its center of occurrence (JBRJ, 2014). *Collaea cipoensis* Fortunato is an endemic shrub limited geographically to rupestrian grasslands, distributed between 1100 and 1400 m a.s.l. (Fortunato, 1995). The species is restricted to very few small patches with few individuals, watercourse-dependent, and associated with acidic and nutrient-poor soils (Negreiros et al., 2008). The species has a red tubular corolla and is hummingbird-pollinated. Although many bee species visit its flowers, almost 50% of them are attacked by nectar-robbers (Gélvez-Zúñiga et al., in press). In spite of *C. cipoensis* restricted distribution, it has been successfully used in projects of restoration of degraded areas (Le Stradic et al., 2014), with sizeable survival rates and seed production (Gomes et al., 2015).

This study aims to describe, for the first time, floral biology and reproductive aspects of *C. cipoensis*, with special emphasis on the importance and functional role of floral visitors. Specifically, the goals of this study were: (1) to evaluate the floral morphology, floral biology and reproductive phenology of *C. cipoensis*; (2) to test the species dependence on pollinators to achieve reproduction by determining its mating systems; and (3) to determine the floral visitor assemblage, their behavior, and their potential role as legitimate (i.e. pollinators) or illegitimate visitors (i.e. nectar-robbers, nectar-thieves or florivores).

Material and methods

Species and study site

Collaea cipoensis is an endemic shrub (1-4 m in height) solely found in Serra do Cipó, Minas Gerais, Brazil, that flowers and fructifies throughout the year. Flowers are zygomorphic, hermaphrodite and odorless, with a tubular red corolla (Fortunato, 1995). The region where it is found has a humid subtropical climate (Type "Cwb" in the Köppen classification) (Alvares et al., 2013), with a hot, rainy season, from November to February, a post-rain period in April and May, a dry cold season from June to August, and a post-dry period in September and October (Rocha et al., 2016). Rupestrian grasslands are characterized by nutrient-impoverished soils, seasonal water deficit, high solar exposure, strong winds and properties influenced by acid, chemically-poor materials (Fernandes, 2016; Oliveira et al., 2014; Silveira et al., 2016). With about 6,000 plant species (Fernandes, 2016), this region harbors the highest levels of plant

endemism of Brazil (Giulietti et al., 1997; Rapini et al., 2008) and one of the most diverse ecosystems in the world (Fernandes, 2016; Silveira et al., 2016).

Flower development and reproductive biology

Between September 2015 and May 2016 two different natural patches of *C. cipoensis* were selected to carry out the observations and experiments. Patches differ in location and altitude, and were chosen to include the highest possible variation in flower development, floral traits and floral visitors within the species. The first patch (hereafter Vellozia) is located at the Reserva Vellozia (19°16'46"S 43°35'13"W) at 1100 m a.s.l., whereas the second one (hereafter Travessão) is located in the Parque Nacional da Serra do Cipó (19°18'52"S 43°32'34"W) at 1300 m a.s.l. At each patch, 31 and 36 individuals were monitored, respectively.

Flower development of 10 individuals in each patch was monitored every two weeks, using five randomly selected floral buds per plant to evaluate their growth, anthesis, floral longevity and stages of female and male organ maturity. Number of floral buds, flowers and fruits were recorded for all marked plants in each census, and marked flowers were accompanied until fruit maturity. Stigma receptivity was examined in three different stages: (1) floral buds, (2) recently open flowers and (3) 24h post-anthesis flowers. When receptive, stigmas release enzymes reacting with a 3% hydrogen peroxide solution producing bubbles (according to Zeisler, 1938 and detailed in Kearns and Inouye, 1993). As papilionaceous flowers have a distinctive corolla morphology, four traits were measured to estimate floral size: banner width, banner length, wing length and keel length (Gong and Huang, 2009).

Mating systems were studied in 15 randomly selected individuals in each patch. On each individual, five different treatments were conducted using 5 randomly chosen flowers (i.e. one flower per treatment and plant): pollination-excluded flowers to test spontaneous self-pollination (selfing), hand-pollinated flowers with pollen from same flower (autogamy), hand-pollinated flowers with pollen from different flowers from the same plant (geitonogamy), hand-pollinated flowers with pollen from different individuals (xenogamy), and unmanipulated naturally pollinated flowers (control). All hand-pollination treatments were conducted with a paintbrush by carefully placing pollen on stigmas. For xenogamy treatment, pollen of two flowers of 10 plants away a 10-m radius of the recipient flower was collected and placed on stigmas. The

selfing treatment was conducted by bagging flowers within tulle bags (Kearns and Inouye, 1993).

Floral visitors

To determine the floral visitor assemblage, focal observations were performed in 15 out of 31 and 36, respectively, randomly chosen individuals from each patch. Flowering individuals were observed during 15-min periods between 06:00 h and 18:00 h, except between 12:00 h and 14:00 h. Observations were conducted on warm sunny days, with no extreme wind conditions. All plants were observed for ten periods in two different days, totalizing 20 observation periods per plant, and 600 periods during a total of 150 hours. As flowers remain open at night, random night walks were also performed in 5 individuals from each patch once during 15-min periods, between 20:00 h and 22:00 h. Overall, diurnal and nocturnal censuses comprised 152.5 observation hours.

For each observation period, the total number of open flowers on the focal plant was recorded. For each visit, the number of visited flowers, type of visit (legitimate or illegitimate), desired floral reward (nectar, pollen or tissue) and interactions with other visitors as territorial behavior to account for possible interferences between visitor species were also recorded. A legitimate visit was defined as any physical contact between a visitor's body and a flower's anther or stigma, whereas an illegitimate visit was considered as any other event with no physical contact with anthers or stigma. Desired floral rewards were identified as the preferred structures of the visitor while foraging flowers. Different taxonomic groups were grouped according to their functionality (i.e. potential pollinators, nectar-robbers, nectar-thieves, or florivores) (Fenster et al., 2004). Hummingbirds were identified by direct visual observations and photographs taken during visits. Insects were collected and preserved for later identification at species level.

Results

Flower development and reproductive biology

Collaea cipoensis plants develop axillary inflorescences with 2–6 flowers. Initially, the flowering shoots are erect but as the inflorescence develops they become pendulous because of increasing weight of flowers. Floral longevity spans 5–7 days (mean ± SD = 5.9 ± 0.7 days; N = 20). Banner is elliptic and narrowed at the base, 20.45±2.8/26.88±3.3 mm in width/length. Wings

and keels are oblong, 29.24±3.3 and 28.87±3.1 mm in length, respectively (N = 339 for all the measurements).

The anthesis occurs when the banner is reflected and stamens and stigma extend beyond the keel to become exposed to visitors, occurring between 06:00 and 17:00 h. The androecium is composed of 10 monadelphous stamens with dorsifixed ellipsoid anthers. Anthers release pollen grains just before anthesis, so when banner is extended, anthers are exerted from petals, facilitating pollen dispersal. Pollen grains are visible during the first three days of anthesis and, after this, anthers become completely dry.

The gynoecium forms a curve that follows the keel shape, it is filiform, and exerted from keel at anthesis. Stigma reaches the level of the anthers before anthesis and remains in this position until floral senescence. Ovary is pubescent, containing 9–23 ovules (mean ± SD = 16.70±2.38 ovules/flower; N = 688). Flowers secrete nectar at the apical part of floral buds, from corolla in pre-anthesis flowers and from the base of corolla during anthesis. Stigmas are receptive to pollen even before anthesis, since the three floral stages (i.e. floral buds, recently open flowers and 24h post-anthesis flowers) reacted positively (by bubbling) to hydrogen peroxide enzyme, indicating functional activity of stigma (Fig. 1).
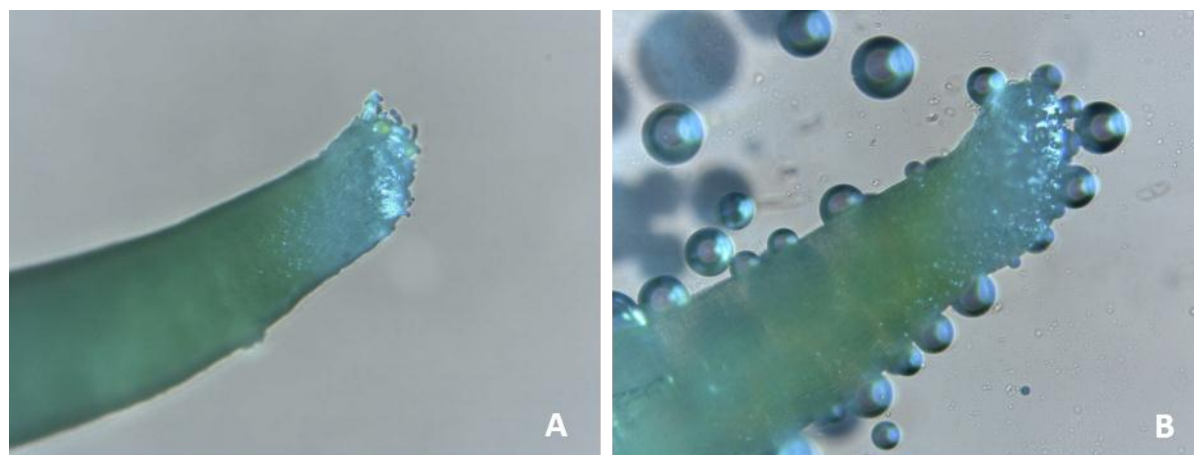

Figure 1. Stigmatic receptivity test by means of hydrogen peroxide 3% solution of flowers of *C. cipoensis* (Fabaceae). A – Intact stigmatic surface; B - Receptive stigmatic surface (bubbling).

Collaea cipoensis is self-incompatible since flowers from selfing, autogamy, and geitonogamy treatments did not develop any fruit. Flowers under these treatments did not show

any sign of fertilization and wilted over time. For the xenogamy treatment, 20% of flowers (6 out of 30) set mature fruits. Relative to the control treatment, 13.33% of flowers (4 out of 30) developed mature fruits. Fertilized flowers took 4 days to show an enlarged style and, subsequently, flowers start wilting. Fruits, forming velutinous brown pods, reached maturity after two months and then dehisced along the lateral margins. Each side curled releasing seeds, which are firstly dispersed by autochory (ballistics dispersal) and secondarily by hydrochory. Seeds are brown, ovoid and range 0–18 per fruit (mean $\pm$ SD = 6.03 $\pm$ 4.82 seeds per fruit, N = 90). Seed production in control flowers was fairly limited as seed set averaged 36%.

Floral visitors

A total of 4,164 visits (133 legitimate and 4,031 illegitimate) were recorded, distributed in 10 taxonomic groups and 43species (Table 1). Visitor functional groups were represented by 87% of nectar-robbers, 9% of nectar-thieves, 3% of potential pollinators and 1% of florivores. Hummingbirds and bees were the most abundant taxonomic groups visiting *C. cipoensis* flowers, and they both made legitimate and illegitimate visits (Fig. 2). Nectar-robbers represented 90% of the illegitimate visits, while nectar-thieves represented 9% and florivores 1%. Bees were the main nectar-robbers of *C. cipoensis* flowers with nearly 68% out of the total of illegitimate visits (Fig. 2).
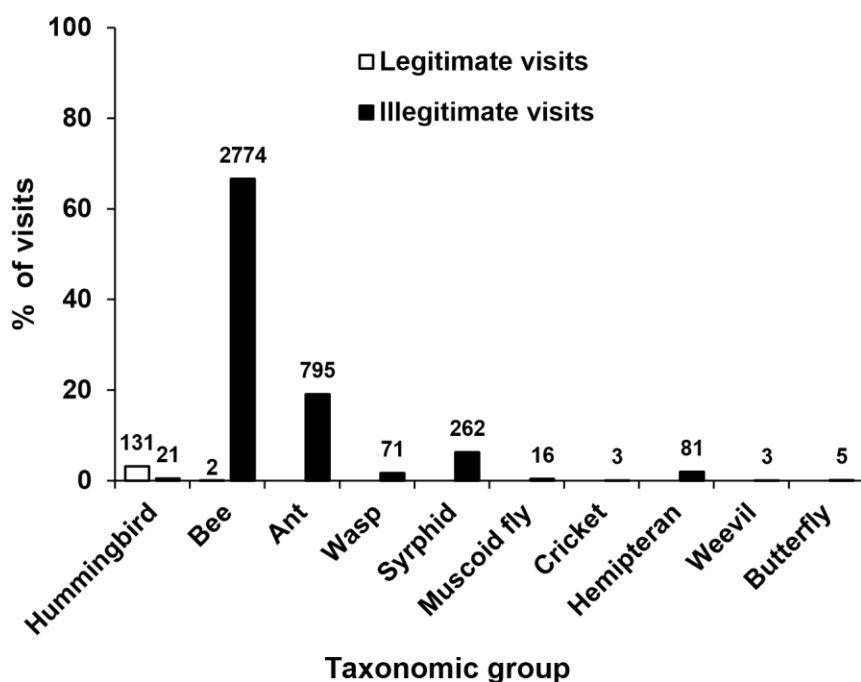
Figure 2. Percentage of flower visitor group to flowers of *Collaea cipoensis* (Fabaceae). Numbers on each bar indicate total number of visits.

Visits were distinctly distributed by functional groups along the day. Potential pollinators visited flowers more often in the early morning (06:00–09:00 h) and afternoon (14:00–15:00 h). Nectar-robbers as bees, syrphids, wasps, and muscoid flies were more frequent between 09:00–15:00 h, whereas ants visited more often from 09:00 to 12:00 h, and florivore crickets were more frequent after 17:00 h. Nectar-thieves as weevils and aphids were found throughout the day on the flowers due to a more "sessile" life cycle. Behavior of the most common species per functional group visiting *C. cipoensis* flowers are detailed below.

Hummingbird *Eupetomena macroura* (Gmelin, 1788) –Swallow-tailed hummingbird– is the main potential pollinator of *C. cipoensis*. It makes strictly legitimate visits, yet brief and fast (between 2-5 sec), choosing no more than 2 or 3 open mature flowers per plant (Fig. 3A). The species preferred recently open flowers or no longer than 24 post-anthesis flowers. Nectar is collected from the base of banner and keel, contacting the reproductive parts with its crown. This species did not show a territorial behavior or struggled with other species.

Colibri serrirostris (Vieillot, 1816) –White-vented violetear– is the most frequent hummingbird visiting the study species and it acts as a potential pollinator and a nectar-thief. When making legitimate visits, it choses open mature flowers and contacts the reproductive parts with its crown (Fig. 3B). This opportunistic species, act as a nectar-thief by collecting nectar from the space between sepals and petals (Fig. 3C). This species shows a territorial behavior and chases bees, wasps and ants. It usually visits more than a flower per plant (range: 1–9), varying between legitimate and illegitimate visits.

Hummingbird *Chlorostilbon lucidus* (Shaw, 1812) –Glittering-bellied emerald– behaves as a nectar-robber by cutting calyces of close and open flowers and collecting nectar from the free margins between sepals and petals, without contacting the reproductive parts. It also makes triangular holes in the calyx, which are later used by several secondary nectar-robber species (Fig. 3D). This bird selects pre-anthesis or 24-hour post-anthesis flowers.

Figure 3. Hummingbird species visiting *Collaea cipoensis* flowers and their visit behavior. A- Legitimatte visit by *Eupetomena macroura* (Gmelin, 1788); B- Legitimate visit by *Colibri serrirostris* (Vieillot, 1816)*;* C- Nectar-thieving visit by *Colibri serrirostris* (Vieillot, 1816); D- Nectar-robbing by *Chlorostilbon lucidus* (Shaw, 1812).

Honeybee *Apis mellifera* (Linnaeus, 1758) is also an opportunistic visitor. It is mainly a nectar-robber, but its behavior includes foraging for pollen and, occasionally, floral tissue. It also acts as a potential pollinator. Its activity peak differs from other bees, being more frequent before

09:00 h (Fig. 4A). The carpenter bee *Xylocopa muscaria* (Fabricius, 1775) was only recorded twice, making legitimate visits, acting as a potential pollinator.

Trigona spinipes (Fabricius, 1793) is the most common nectar-robber of *C. cipoensis*. It makes rounded holes in the calyx base and also eats petals and anthers (Fig. 4B). This species spends much time choosing the host individual and shows a territorial behavior, attacking other visitors such as carpenter bees and wasps. The species damages floral buds, pre-anthesis, open and even senescent flowers, and it is more frequent in plants with higher floral displays.

Wasp species use floral resources of *C. cipoensis* as nectar-robbers, occasionally foraging for pollen, but they are found preferably on floral buds. Both *Polistes* sp. and *Mischocyttarus* sp. wasps are secondary nectar-robbers (Figs. 4C - D), which collect nectar from the calyx base from an existing hole (made by *T. spinipes* or *C. lucidus*), as well as pollen consumers by foraging directly on corolla or anthers.

Other four taxonomic groups exhibit secondary nectar-robber behavior. Syrphid *Toxomerus musicus* (Fabricius, 1805) collected nectar from an existing hole in the calyx (Fig. 3E), whereas muscoid flies were represented by two species (Table 1). Butterflies were always recorded making secondary robbery directly from the calyx holes, as shown in Fig. 3F. Further aspects of less frequent species are detailed in Table 1.

Ants behave as nectar-robbers or nectar-thieves. They usually forage on all flower surface and, when flowers show a hole in calyx, they act as secondary nectar-robbers (see details in Table 1). Only small-sized ants, as *Linepithema anathema* (Wild, 2007) and *Brachymyrmex* pr. *Cordemoyi* (Forel, 1895), spend most of time inside the petals base and are most frequently found on intact flowers (nectar-thieving). The two most common ant species are *Camponotus melanoticus* (Emery, 1894) (Fig. 4G) and *Cephalotes pusillus* (Klug, 1824) (Fig. 4H).

Figure 4. Visiting insects on *Collaea cipoensis* (Fabaceae) flowers. A- *Apis mellifera* (Linnaeus, 1758); B- *Trigona spinipes* (Fabricius, 1793); C- *Polistes* sp.; D- *Mischocyttarus* sp.; E- *Toxomerus musicus* (Fabricius, 1805); F- *Paryphthimoides* sp.; G- *Camponotus melanoticus* (Emery, 1894); H- *Cephalotes pusillus* (Klug, 1824).

Table 1. Insect visitors of *Collaea cipoensis* (Fabaceae) and their behavior on flowers. Group (PP: potential pollinator, NR: nectar robber, SNR: secondary nectar robber, NT: nectar thieve), activity peaks (D: diurnal, N: nocturnal, C: crepuscular) and desired floral reward (N: nectar, P: pollen, T: tissue).

Group	Taxa	Species	Activity	Floral reward
PP	Bee	*Xylocopa muscaria* (Fabricius, 1775)	D	N
PP - NR	Bee	*Apis mellifera* (Linnaeus, 1758)	D	N - P - T
NR	Bee	*Trigona spinipes* (Fabricius, 1793)	D	N
NR	Bee	*Exomalopsis* sp.	D	N - P
NR	Ant	*Camponotus rufipes* (Fabricius, 1775)	D - N	N - T
NR	Ant	*Camponotus melanoticus* (Emery, 1894)	D - N	N - T
NR	Ant	*Dorymyrmex* cf. *goetschi* (Goetsch, 1933)	D	N - P
NR	Ant	*Camponotus novogranadensis* (Mayr, 1870)	D - N	N - T
NR	Ant	*Camponotus crassus* (Mayr, 1862)	D - N	N - T
NR	Wasp	Vespidae sp.	D	N - P
NR	Wasp	*Mischocyttarus* sp.	D	N - P
NR	Wasp	*Polistes* sp.	D	N - P
NR	Beetle	*Pantomorus* sp.	D	T
NR	Beetle	*Apion* sp.	D	T
NR	Beetle	*Chalcodermus* sp.	D	T
SNR	Ant	*Cephalotes pusillus* (Klug, 1824)	D	N - T
SNR	Syrphid	*Toxomerus musicus* (Fabricius, 1805)	D	N
SNR	Muscoid Fly	Sarcophagidae sp.	D	N - P
SNR	Muscoid Fly	Agromyzidae sp.	D	N - P
SNR	Butterfly	*Pyrisitia leuce* (Boisduval, 1836)	D	N

SNR	Butterfly	*Paryphthimoides* sp.	D	N
SNR	Butterfly	*Leptotes cassius* (Cramer, 1775)	D	N
NT	Bee	*Megachile* sp.	D	N - T
NT	Bee	*Melipona marginata* (Lepeletier, 1836)	D	N - P
NT	Bee	*Augochloropsis* sp.	D	N
NT	Bee	*Ceratina* sp.	D	N - P
NT	Ant	*Pseudomyrmex* gr. *pallidus* (Smith, 1855)	D	N
NT	Ant	*Pseudomyrmex gracilis* (Fabricius, 1804)	D - N	N - P
NT	Ant	*Linepithema anathena* (Wild, 2007)	D - N	N
NT	Ant	*Brachymyrmex* pr. *cordemoyi* (Forel, 1895)	D - N	N
NT	Ant	*Crematogaster sp.*	D	N
NT	Hemipteran	*Crinocerus sanctus* (Fabricius, 1775)	D	N
NT	Hemipteran	Aphididae sp.	D-N	N
FL	Beetle	Cryptocephalinae sp1.	D-N	T
FL	Beetle	Alticinae sp1.	D-N	T
FL	Beetle	*Parapantomorus flexuosus* (Boheman, 1840)	D	T
FL	Cricket	*Homotoicha* sp.	C	P -T
FL	Cricket	*Baeacris* cf. *pseudopunctulata* (Ronderos, 1964)	D	P -T
FL	Cricket	*Dasyscelus* sp.	C	T - P
FL	Cricket	*Dichromatos* sp.	C	T - P

Discussion

This study reports novel data on flower morphology and development, reproductive biology, and combined observational and experimental evidence of floral visitors and mating systems of *Collaea cipoensis.* This is a self-incompatible species strictly xenogamous and potentially dependent on two hummingbird species (*Eupetonema macroura* and *Colibri serrirostris*) to achieve reproduction, despite the wide spectrum of taxa and behaviors of flower visitors. Most of the flower visitors were predominantly illegitimate by robbing nectar. The self-incompatible, cross-pollination mating system of *C. cipoensis* limits its reproductive output as flowers increased more than 50% of fruit production due to hand-pollination. Altogether, the results here suggest that reproduction in this species may be limited by floral enemies, and this can lead to reduction of the species fitness components.

Collaea cipoensis shows some pollen limitation as hand-pollinated flowers developed, on average, a higher fruit set than control flowers left to natural pollination. Besides pollen limitation and reproductive fails mediated by hummingbirds as potential pollinators, *C. cipoensis* showed a low reproductive output in terms of fruit production (*ca*. 20%) even in hand-pollinated flowers. This result suggests that other non-pollinator agents may be potentially reducing fruit set alternatively to limitation pollen conditions. The results reported here show that a high proportion and diversity of illegitimate visitors such as florivores and nectar-robbers collect floral rewards by causing mechanical damage in flowers without providing pollinator service (see also Gélvez-Zúñiga et al., in press).

It is broadly known that florivores and nectar-robbers may reduce fruit and seed production by degrading the attractive properties of flowers for pollinator service or by direct consumption of viable gametes (e.g. Carper et al., 2016; Irwin et al., 2015, 2001; Krupnick et al., 1999; Schemske and Horvitz, 1989). Otherwise, low resource availability, especially in terms of water, could alternatively (or simultaneously) impose constrains to reproduction output. Water is a crucial resource to maintain attractiveness and flowers physiologically active while remaining open (Galen, 1989; Patiño and Grace, 2002; Teixido and Valladares, 2014). The study area, as commonly happens in rupestrian grasslands, is influenced by high temperatures and a marked dry season (Madeira and Fernandes, 1999; Rocha et al., 2016). Acting together, heat and drought can disrupt the normal performance of flowers, affecting fruit production (Erickson and

Markhart, 2002; Konsens et al., 1991). However, these conclusions should be taken with caution as no study has yet tested this hypothesis in the rupestrian grasslands.

In the rupestrian grasslands, insect-pollinated species (mostly bee-pollinated) represent about 70% of plant species, whereas bird-pollinated plants are represented by 10-15% of animal-pollinated plant diversity (Carstensen et al., 2014; Jacobi and Carmo, 2011). Some endemic species are exclusively bee-pollinated (Franco and Gimenes, 2011; Jacobi et al., 2000; Matias et al., 1996), and others involve bees and hummingbirds as pollinators (Jacobi and Antonini, 2008; Sazima and Sazima, 1990). To avoid temporal bias on floral visitors community, here we conducted observations in both rainy and dry season, since a high variation in pollination assemblage has been reported between seasons along the year, with ornithophily prevailing during the dry season (Conceição et al., 2007). In *C. cipoensis*, bees are more frequent visitors but with a strong nectar-robbing behavior, whereas hummingbirds do not visit flowers so often, although exert an important role as potential pollinators. In our study area, hummingbirds are usually highly territorial and defend flower resources against other visitors (Guerra et al., 2014; Jacobi and Antonini, 2008; Sazima and Sazima, 1990; Vasconcelos and Lombardi, 2001). However, *E. macroura* did not show a territorial behavior when foraging *C. cipoensis* flowers. Hummingbirds may also be frequent visitors of non-ornitophilous plant species (Machado, 2009; Rodrigues, LC & Rodrigues, 2014; Vasconcelos and Lombardi, 2001), which represents an extra challenge for reproduction of narrow endemic hummingbird-pollinated species as *C. cipoensis* in rupestrian grasslands.

As predicted by the OCBIL (Old Climatically-Buffered Infertile Landscapes) theory (Hopper, 2009), evolution of reproductive strategies in these ecosystems, among which the rupestrian grasslands are found, should reduce inbreeding of endemic species by means of mechanisms of cross-pollination (Silveira et al., 2016), which agrees with the findings of reproductive systems and potential pollinator visitors in *C. cipoensis* reported here. Otherwise, the limited distribution range of *C. cipoensis* is congruent with the sympatric speciation mechanisms in narrow endemic species of rupestrian grasslands (the hummingbird-pollinated species *C. speciosa* (Buzato et al., 2000), wherein intrinsic habitat heterogeneity may set barriers to gene flow (Lousada et al., 2011).

In conclusion, the hummingbird *E. macroura* and *C. serrirostris* are vital to attain reproduction of the self-incompatible and narrowly-distributed *C. cipoensis.* However, production of flowers and their nectar contents are energetically expensive resources, especially when dealing with nutrient-poor soils, high temperatures and water shortage, and a high incidence of floral enemies in the rupestrian grassland environment. The facultative nectar-thieve and nectar-robber behavior of other hummingbird visitors, such as *C. serrirostris* and *C. lucidus,* together with the high florivory mediated by insects seem to limit reproductive output of *C. cipoensis*. Altogether, the dependence on cross-pollination, the stressful environmental conditions and the high floral visitation rates by illegitimate visitors increase the harsh scenario for this species. Perhaps, the rarity of *C. cipoensis* in the rupestrian grasslands landscape may be influenced by these selective pressures that diminish the species likelihood to succeed.

Acknowledgements

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Capítulo 2

Selection of floral attractiveness traits by legitimate and illegitimate visitors in the narrow endemic *Collaea cipoensis* (Fabaceae)

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Introduction

Attractiveness to pollinators plays a decisive role in the reproductive ecology of zoophilous plants. The importance of the number of displayed flowers for pollinator visitation rates and fruit production has been broadly reported (Brody & Mitchell, 1997; Thompson, 2001; Harder & Johnson, 2005; Delmas et al., 2014). Likewise, larger flowers have also been associated with higher pollinator attraction and a subsequent increase in cross-pollination and reproductive success (Galen, 1989; Kudoh & Whigham, 1998; Aigner, 2005; Teixido & Valladares, 2014). As a consequence, pollinators are considered one of the main drivers of evolution on floral traits directly related to attractiveness (reviewed in Fenster et al., 2004). Indeed, phenotypic selection formal studies in natural populations have confirmed significant positive selection on flower size and number under natural conditions in pollen-limited populations, when pollinators have the potential to increase reproductive output (e.g. Conner & Rush, 1997; Totland, 2001; Harder & Johnson, 2009; Sletvold et al., 2010; Zhao & Wang, 2015).

Despite its benefits, floral attractiveness can also be related with greater plant-animal illegitimate interactions. Illegitimate visitors can forage the same nutritional resources collected by pollinators, such as pollen and nectar, or can instead feed from floral tissues, without providing pollination service (Strauss & Irwin, 2004). For example, floral herbivores cause damage to open flowers by eating bracts, sepals, petals, androecium and/or gynoecium (McCall & Irwin, 2006). Otherwise, nectar-robbers cause damage to open flowers by producing holes in flowers to remove nectar (Inouye, 1980). Nectar robbing is not uncommon among flowering plants, especially in flowers having tubular corollas or nectar spurs (Irwin et al., 2010). The total effects of nectar-robbers and florivores, including direct effects (i.e. damage to floral reproductive organs) and indirect effects (i.e. changes in pollination derived from decreasing attractiveness or nectar availability) can strongly affect plant fitness by reducing reproductive

output (Schemske & Horvitz, 1988; Krupnick et al., 1999; Irwin et al., 2001, 2015; Carper et al., 2016).

There is evidence that increasing components of plant attractiveness to pollinators such as the number of flowers displayed and flower size intensify florivory (Galen, 1999; Teixido et al., 2011; Ruane et al., 2014) and nectar-robber incidence (Irwin & Brody, 1998; Wang et al., 2013; Lobo et al., 2016). From an evolutionary perspective, both florivores and nectar-robbers can exert negative selective pressures on the same floral attractiveness traits positively selected for pollinators (Irwin et al., 2001, 2010; Strauss & Whittall, 2006; Castro et al., 2008; Wang et al., 2013). Therefore, multiple species interactions jointly may potentially exert selective pressures on floral attractiveness traits and shape flower evolution (Strauss & Irwin, 2004; Strauss & Whittall, 2006). In this way, comparing the relative importance of different agents in selection on floral traits related to pollinator attractiveness to achieve reproduction is of paramount importance to fully understand the forces of floral evolution. However, combined effects of pollinator- and illegitimate visitor-mediated selection have been only scarcely studied (Irwin, 2006; Wang et al., 2013) and deserves more attention.

The present study addresses the selective effects of floral legitimate and illegitimate visitors on floral attractiveness traits through female fitness components of *Collaea cipoensis* (Fabaceae), an endemic shrub from Brazilian rupestrian grasslands. *Collaea cipoensis* is a self-incompatible species with red tubular corollas, almost exclusively pollinated by hummingbirds (Fortunato, 1995), but also visited by several florivore and nectar robber insects (Gélvez-Zúñiga et al., in press; see also Chapter 1). Pollen limitation is a mandatory condition so that pollinator-mediated selection operates in natural populations (Knight et al., 2005). Likewise, constrains in reproductive output beyond pollen limitation involve that other non-pollinator selective agents influence floral traits (Galen, 1999; Strauss & Whittall, 2006; Teixido et al., 2016). Chapter 1 demonstrates that *C. cipoensis* is a pollen-limited species, which also shows limitation in reproductive output beyond pollinator effects as many hand-pollinated flowers did not set fruits. Thus, a positive relationship between floral attractiveness (flower size and number of displayed flowers) and legitimate (i.e. pollinators) and illegitimate visitors (i.e. nectar-robber, or florivores) is hypothesized. And, it is expected the illegitimate visitors to counteract the selective positive effects of pollinators on flower size and number, entailing a conflicting selection on floral

attractiveness traits through female fitness components of *C. cipoensis* mediated by the combined effect of pollinators and floral enemies.

Material and methods

Species and study area

    Collaea cipoensis (Fabaceae) is a narrow endemic species only known from Serra do Cipó, Minas Gerais, Brazil (Fortunato, 1995). The species is a perennial shrub approximately 1-4 m in height flowering and fruiting continuously along the year. Flowers are disposed in axillary inflorescences containing 2-6 individual flowers, which are hermaphrodite, with 4-lobed and red tubular corollas 3-5 cm in size, odorless and pollinated by hummingbirds (Fortunato, 1995; see Chapter 1). Fruits are oblong and elastically dehiscent, comprising a maximum of 18 ellipsoid seeds (Fortunato, 1995; see Chapter 1). Natural patches are small and distributed along watercourses, in open areas of Rupestrian grasslands between 1100 and 1400 m, and associated with poor, acid soils (Negreiros et al., 2008).

    The study was conducted in two patches of *C. cipoensis* located at Serra do Cipó between September 2015 and May 2016. The first patch (hereafter Vellozia) is located at the Reserva Vellozia (19°16'46"S 43°35'13"W) and the second patch (hereafter Travessão) is located at the Parque Nacional da Serra do Cipó (19°18'52"S 43°32'34"W). The distance between both patches is 9 km. Differences between patches were tested, however due to the low genetic variation in other narrow endemic species in the area (see Ribeiro & Fernandes, 2000; Moreira et al., 2008), patches were assumed as being from a single *C. cipoensis* population. The vegetation in the region is predominated by Rupestrian grasslands on quartzite, sandstone and ironstone formations. Rupestrian grasslands are characterized by nutrient-impoverished soils, seasonal water deficit, high solar exposure, strong winds and properties influenced by acid, chemically-poor materials (Oliveira et al., 2015; Silveira et al., 2016)

Floral visitors

    To quantify floral visitation rates, 15 flowering individuals were observed over 15-min periods between 06:00 and 18:00 h, except from 12:00 to 14:00h, on each patch. Observations were made at each ten hours, in two different days, totalizing 20 observation periods per plant, and 600 periods with a total of 150 hours. Warm, sunny days were chosen to make the

observations. For each period, the number of open flowers and the number of flowers visited were recorded. Visitors were categorized by their behavior as nectar-robbers, nectar-thieves or potential pollinators. Nectar-robbers are visitors that damage floral structures by cutting flowers when foraging for nectar rewards, sometimes without providing pollination services (Inouye, 1980). Nectar-thieves otherwise forage for floral rewards (mostly nectar) without causing structural damages to the flowers, but equally without providing pollination (Inouye, 1980). Potential pollinators are organisms that provide legitimate visits (i.e. pollination service) (Fenster et a., 2004). Visits were considered legitimate when physical contact between a visitor's body and a flower's anther or stigma occurred. Different taxonomic groups were grouped according to their functionality (i.e. potential pollinators, nectar-robbers, nectar-thieves, or florivores) (Fenster et al., 2004). The groups were hummingbirds, bees (including solitary bees and honeybees), bumblebees, ants (Formicidae), wasps (Vespidae), hover flies (Syrphidae), muscoid flies (Sarcophagidae and Agromyzidae), crickets (Tettigoniidae and Acrididae), phytofagous Hemiptera (Coreidae and Aphididae), butterflies (diurnal Lepidoptera) and beetles (Curculionidae, Brentidae and Crisomelidae).

Floral attractiveness

To determine flower attractiveness, four traits related to flower size in Fabaceae were measured (Gong & Huang, 2009): banner width and length, keel length and wing length (cm to the nearest mm) from 5 to 15 open flowers per plant were measured in the field using a digital caliper. The number of open flowers per plant was also recorded daily. Then flower size and number of open flowers every day (hereafter floral display) on each plant were averaged. Mean floral size and display per plant were utilized for averaging these traits at each patch.

Female fitness components

To evaluate the effects caused by visitors on female fitness components, 31 flowering plants from Vellozia and 35 from Travessão were randomly selected. In each plant 2 to 30 flowers were marked according to whether they were visibly attacked or not, and followed until fruit ripping. Depending on total floral display and number of attacked flowers per individual, 1 to 15 attacked flowers were marked to have a representative sample of flowers over the total number of flowers displayed at each plant. Additionally, 1 to 15 intact flowers were randomly

marked per plant following the same criteria above. These flowers were intact when marked, but they could be subsequently attacked before setting fruits, although floral lifespan was not recorded. Therefore, it was not possible to know whether fruits set from marked intact flowers were developed from intact flowers *per se* or not. As fitness components from marked intact flowers ultimately comprised, on average, total reproductive output from both intact and attacked flowers, they were named "total flowers" to facilitate the interpretation of results. Attacked flowers showed conspicuous holes in the perianth as consequence of nectar-robbers. All marked flowers that remained on plant to subsequently set fruits were bagged in tulle bags before fruit maturity to avoid predispersal seed predation as long as possible.

All ripe fruits from previously marked flowers were picked before seed dispersal to estimate fruit set, seed number per plant (hereafter seed number) and seed set per fruit. The effect of illegitimate visits caused by nectar-robbers was also considered by estimating seed set of fruits from attacked flowers. According to Fenster et al. (2004), pollination efficiency can be estimated by fruit set as a proportion of pollinated flowers, while the quality of mating can be estimated by seed number and seed set. Fruit set per plant was calculated by dividing the number of mature fruits between all marked flowers per plant. To determine seed number, the total number of seeds from fruits per plant was recorded. Seed set was calculated as the number of seeds per fruit divided between the mean number of ovules per flower and plant. The number of ovules from ten randomly selected floral buds were counted to obtain a mean ovule number per plant.

Statistical analysis

To test differences between patches and frequency of visits (legitimate or illegitimate) of each visitors group to each plant PerMANOVA analyses were performed. PerMANOVA is a permutation-based version of the multivariate analysis of variance (Anderson, 2001) that uses the distances between samples to partition variance and permutations to produce the *p*-value for the hypothesis test. Data of each visitor group were square root transformed to ensure the normality and homogeneity of variance assumptions. Bray-Curtis similarity index was calculated before performing the analysis (Anderson, 2001). All PerMANOVA analyses were performed in Primer 6.0 (Clarke & Gorley, 2006).

To determine whether visitation rates increase with flower size and floral display, two General-linear model ANCOVAs were performed, one for legitimate visits and other for

illegitimate visits. Patch (fixed factor), plant (random factor within patch), banner width, banner length and floral display (covariates; fixed factors), and the interactions patch $\times$ banner width, patch $\times$ banner length and patch $\times$ floral display were considered as independent variables in each ANCOVA.

Correlations between floral size traits (banner width, banner length, keel length and wing length) and between flower size traits and floral display per patch were tested by means of the Pearson's correlation coefficient. According to correlation results, only banner width and length were used as floral size traits (see Appendix 1). To determine whether flower traits (i.e. banner width and length) and floral display significantly differ between patches, three General-linear model ANOVAs were fitted, considering patch (fixed factor) and plant (random factor nested within patch) as independent variables. To test significance in the difference of ovule number between patches, a new General-linear model ANOVA was fitted, including patch (fixed factor) and plant (random factor within patch) as independent variables. The assumptions of normality and homogeneity of variance were tested using Shapiro–Wilk's and Levene's tests, respectively.

To test the effects of flower size and floral display on the percentage of attacked flowers, an additional General-linear model ANCOVA was fitted, including patch (fixed factor), plant (random factor within patch), banner width, banner length and floral display (covariates; fixed factors), and the interactions patch $\times$ banner width, patch $\times$ banner length and patch $\times$ floral display were considered as independent variables. Data of attacked flowers were square root transformed to ensure the normality and homogeneity of variance assumptions.

To test for differences in the three female fitness components between patches, three General Linear Models (GLMs) were performed. For fruit set, a logistic GLM with binomial error distribution was used whereas for seed number and seed set General-linear model ANOVAs were performed. Seed set was square root transformed to ensure normality. In all cases, except seed number, patch (fixed factor) and plant (random factor within patch) were considered as independent variables. For seed number there was no any replicate per plant since this fitness component was determined as total number of seeds in each individual, so only patch (fixed factor) was included as independent variable. For seed set, type of marked flower (i.e. total or attacked, see "Female fitness components"; fixed factor) and the interaction patch $\times$ type

of marked flower were included as independent variables. GLMs were performed using the R software (R Development Core Team, 2016).

Phenotypic Selection

Selection on flower size was estimated through fruit set, seed number, seed set and seed set of fruits from attacked flowers in both patches following Lande & Arnold (1983). Multiple regression analyses were used to estimate linear selection (β) using fruit set, seed number, seed set and seed set from attacked flowers (individual fitness/patch mean fitness, *w*) as the response variables, and standardized floral size traits (with a mean of 0 and a variance of 1) as explanatory variables. Correlational selection between floral size traits (banner width × banner length) and floral size traits with floral display (banner width× floral display, banner length × floral display and banner width × banner length× floral display) were estimated. In addition, non-linear selection gradients (γ) were used to estimate stabilizing/disruptive selection, by obtaining quadratic deviations from the mean for both single and quadratic terms of characters (Lande & Arnold, 1983). Thus, banner width, banner length, floral display, and the interaction between of banner width × banner length, banner width× floral display, banner length × floral display and banner width × banner length× floral display, and the quadratic components of banner width^2, banner length^2, floral display^2 and (banner width × banner length)^2, (banner width× floral display)^2, (banner length × floral display)^2 and (banner width × banner length× floral display)^2 were used in the regression model. Quadratic regression coefficients were doubled to estimate stabilizing/disruptive selection gradients (Lande & Arnold, 1983; Stinchcombe et al., 2008). All regression models were performed using IBM SPSS Statistics version 22.0 (IBM Corp. Released 2013; IBM SPSS Statistics for Windows; Armonk, NY, USA).

Results

Floral visitors

Eleven taxonomic groups were identified in each patch groups, with a total of ten different groups in both areas (Fig. 1). Carpenter bees were absent in Vellozia and aphids in Travessão. Hummingbirds accounted for 100% out of 113 and 90% out of 20 legitimate visits in each patch, respectively. Carpenter bee *Xilocopa muscaria* accounted for the remaining 10% in Travessão. Bees and ants accounted for 82% out of 2258 illegitimate visits in Vellozia, followed

by syrphids (10 %) and aphids (5%) (Fig. 1A). In Travessão, bees were the dominant visitors, accounting for 65% out of 1773 illegitimate visits although ants were also abundant. Nectar-robber bee *Trigona spinipes* represented 99% of bee illegitimate visits. Overall, both groups accounted for 1662 out of 1773 (*ca*. 94%) illegitimate visits in this patch (Fig. 1B). Wasps and syrphids occurred at a low frequency (approx. 5% each; Fig. 1B). Assemblage and visitation rates of groups significantly differed between patches (Pseudo-$F_{1,34}$ = 4.43, *p* = 0.023) and between legitimate and illegitimate visitation (Pseudo-$F_{1,34}$ = 7.54, *p* = 0.009).
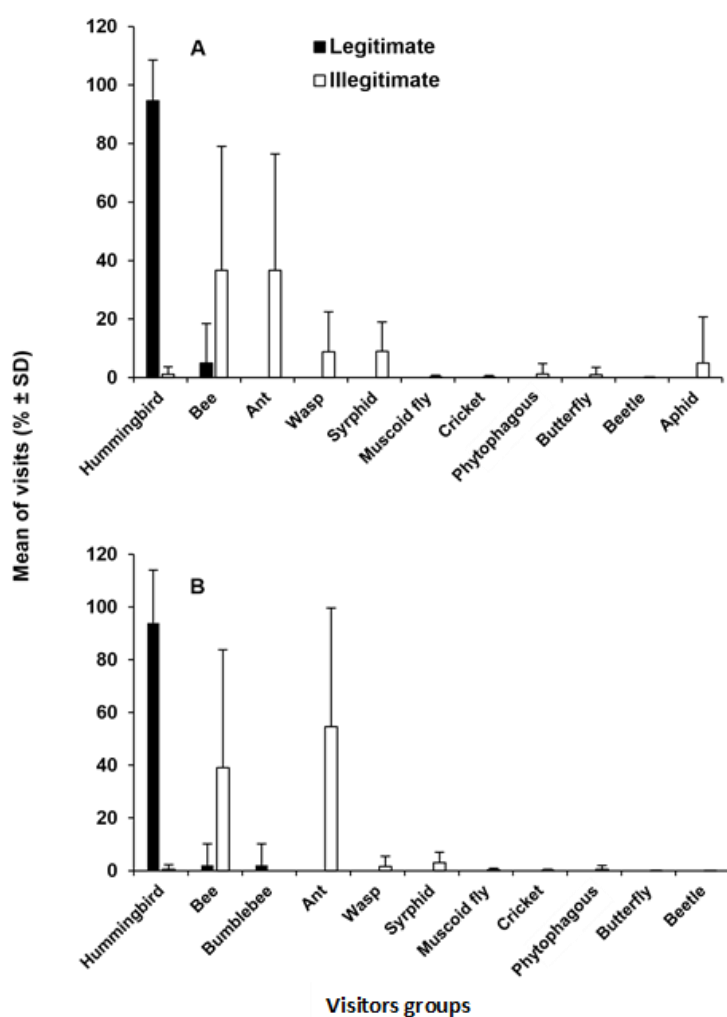

Figure 1. Frequency (% mean ± SE) of legitimate and illegitimate visits of each visitors group to *Collaea cipoensis* flowers in two patches; A – Vellozia, B – Travessão.

Table 1. Mean ± SD of floral attractiveness (flower size in terms of banner size –mm–* and floral display), proportion (%) of attacked flowers, flower visit rates (legitimate and illegitimate) and the three components of female fitness (seed set is shown for total and attacked flowers) of *Collaea cipoensis* (Fabaceae) in the study patches.
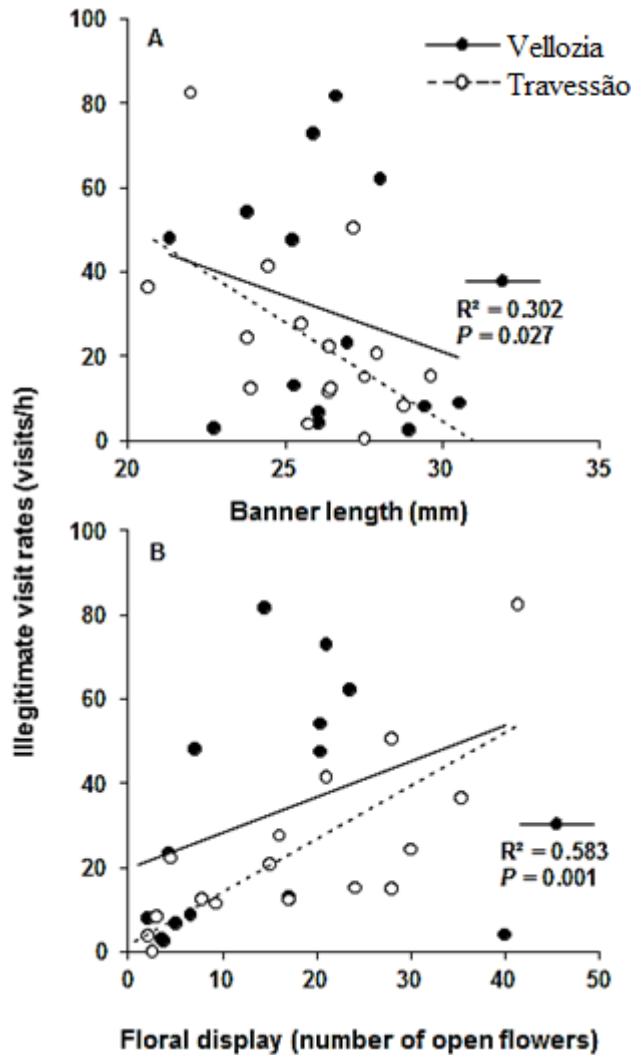
Patch	Floral attractiveness			Attacked flowers	Floral visits		Fitness components			
	Banner width	Banner length	Floral display		Legit.	Illegit.	Fruit set	Seed number	Seed set	
									Total	Attacked
Vellozia	21.0 ±2.3	26.4 ±3.3	9.1 ±9.3	0.4 ±0.3	1.7 ±1.3	29.3 ±28.7	0.5 ±0.3	26.2 ±34.7	0.4 ±0.2	0.3 ±0.2
Travessão	20.2 ±3.1	27.1 ±3.3	9.2 ±10.2	0.4 ±0.3	0.4 ±0.3	24.2 ±20.7	0.4 ±0.2	19.0 ±21.2	0.5 ±0.3	0.4 ±0.2

* See Appendix 1 for flower size measures Pearson correlation index.

Legitimate visitation rates were significantly four times higher in Vellozia than in Travessão ($F_{1,28} = 10.89$, $p = 0.002$; Table 1). These rates were positively correlated with banner width and floral display only in Vellozia (Patch × banner width and Patch × floral display significant; Fig. 2A, B), but were independent of banner length in both patches. Illegitimate visitation rates did not show differences between patches ($F_{1,28} = 0.29$, $p = 0.592$; Table 1) but were negatively correlated with banner length (Patch × banner length significant; Fig. 3A) and positively correlated with number of open flowers in Travessão (Patch × floral display significant; Fig. 3B). However, illegitimate visitation rates were independent of banner width in both patches.
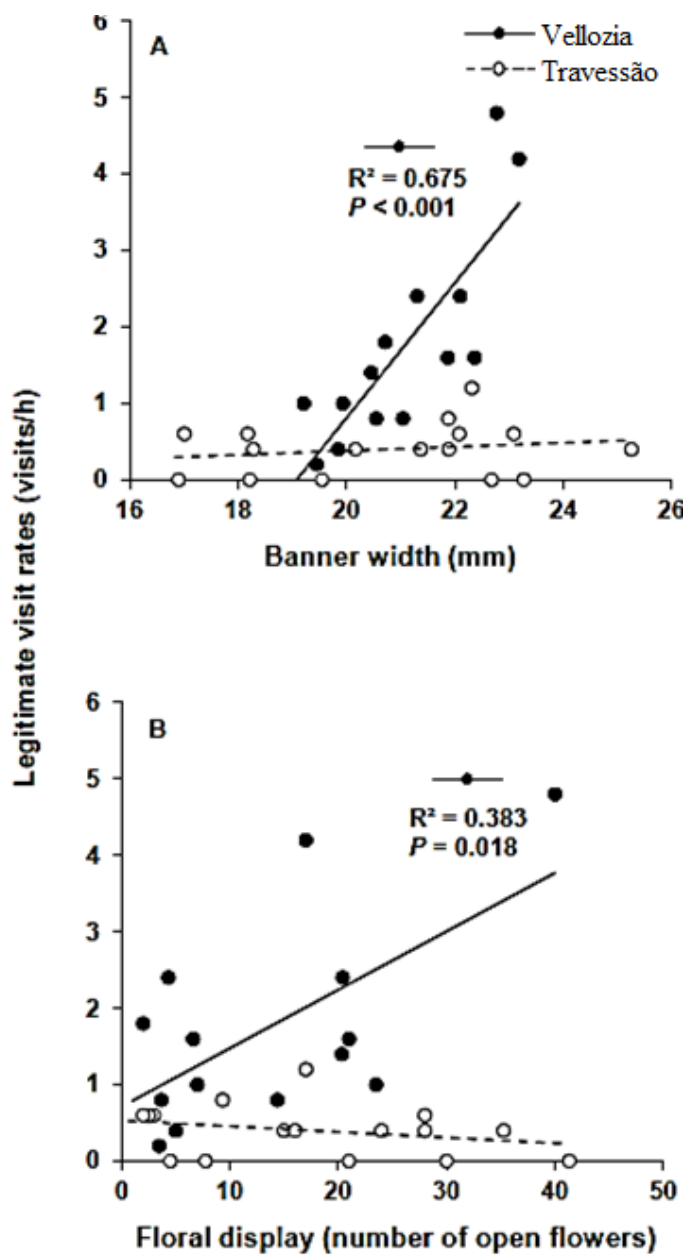
Figure 2. Interaction between patch and floral traits (banner length and floral display) in legitimate visitation rates on flowers of *Collaea cipoensis* (Fabaceae). Solid line: Vellozia, dotted line: Travessão.

Figure 3. Interaction between patch and floral traits (banner length and floral display) in illegitimate visitation rates on flowers of *Collaea cipoensis* (Fabaceae). Solid line: Vellozia, dotted line: Travessão.

Floral attractiveness and components of female fitness

Banner width (ranged from 10.4±3.5 to 29.9±3.3 mm) was similar between patches, but significantly differed among plants within patches (Tables 1 and 2). Banner length (ranged from 16.3±2.9 to 36.2±3.2 mm) was significantly higher in Travessão and significantly differed among plants within patches (Tables 1 and 2). Floral display ranged from 2 to 53 open flowers

(lowest and highest mean ± SD = 9.1±9.3 and 9.2±10.2, respectively) and only showed significant differences among plants within patches (Tables 1 and 2).

Table 2. General-linear model ANOVAs for differences in floral attractiveness traits of *Collaea cipoensis* (Fabaceae) between patches and plants within patches. Significant *p*-values are marked in bold.

Floral traits	Effect	*d.f.*	MS	F	*p*
Banner width	Patch	1	5660.5	2.38	0.192
	Plant (Patch)	61	10165.8	4.94	**<0.001**
	Error	337	6254.2		
Banner length	Patch	1	1514.9	5.40	**0.012**
	Plant (Patch)	61	6642.6	4.02	**<0.001**
	Error	337	3929.2		
Floral display	Patch	1	122.1	2.88	0.115
	Plant (Patch)	69	280.6	4.59	**<0.001**
	Error	202	38.1		

The number of attacked flowers by nectar-robbers ranged from 0 to 49 in Vellozia, and from 3 to 28 in Travessão and significantly differed between patches ($F_{1,50} = 7.84$, $p = 0.007$; Table 1). The number of flowers attacked significantly decreased with banner width in Travessão (Patch × banner width significant; Fig. 4A). At both patches, the number of attacked flowers was negatively correlated with banner length (Fig. 4B). Relative to floral display, the number of attacked flowers significantly increased with the number of open flowers in Travessão (Patch × floral display significant; Fig. 4C).
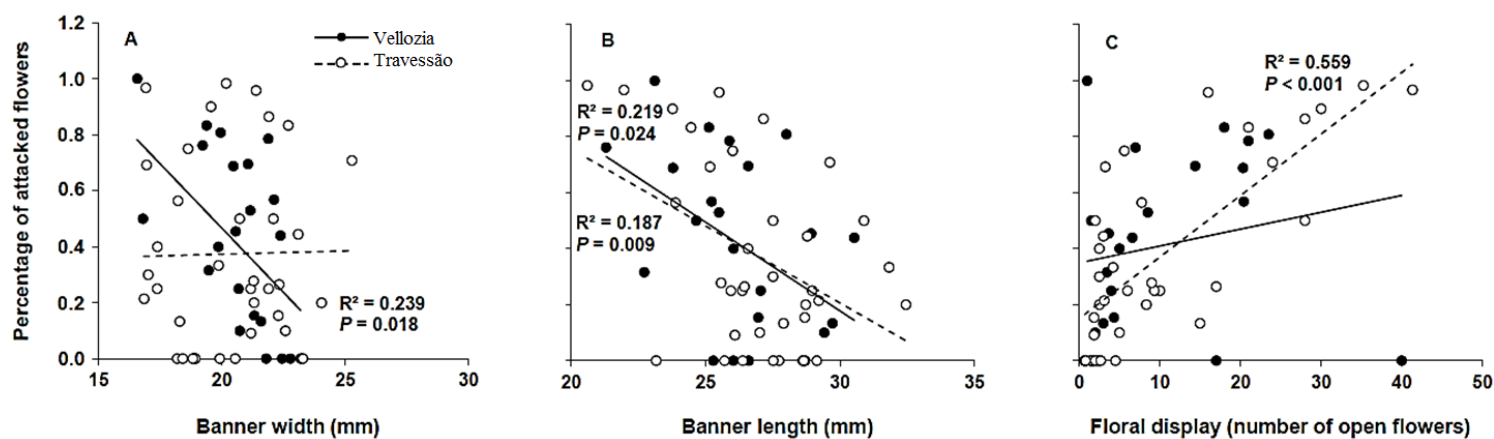
Figure 4. Interaction between patch and floral traits (banner width, banner length and floral display) in the percentage of flowers attacked by nectar-robbers in *Collaea cipoensis* (Fabaceae). Solid line: Vellozia, dotted line: Travessão.

Fruit set ranged from 0.05 to 1 and averaged 0.5±0.3 and 0.4±0.2 in Vellozia and Travessão, respectively (Table 1). However, these differences were not significant between patches, but exclusively among plants within patches (Table 3). Seed number ranged from 1 to 18 and mean seed number was similar between patches (Tables 1 and 3). Seed set component ranged from 0.17 to 0.99 for total flowers (i.e. intact more attacked) and from 0.06 to 1 for attacked flowers. Overall, seed set showed a marginally significant increase in Travessão and, especially, in attacked flowers (Table 3).

Table 3. Generalized linear models for differences in fruit set, seed number and seed set of *Collaea cipoensis* (Fabaceae) between patches and plant within patches (fruit set), patches (seed number) and patches, type of flower (i.e. total and attacked; see "Female fitness components" in "Material and methods"), plants within patches and patch × type of flower (seed set). Significant *p*-values are marked in bold. Type of flower: attacked or not attacked.

Fitness component	Effect	*d.f.*	MS	F	*p*
Fruit set	Patch	1	0.314	0.69	0.408

	Plant (Patch)	72	0.635	3.80	**< 0.001**
	Error	728	0.167		
Seed number	Patch	1	45,730	0.06	0.794
	Error	73	663,427		
Seed set	Patch	1	0.110	3.27	0.073
	Type of flower	1	0.204	3.92	0.051
	Patch × Type of flower	1	<0.001	<0.01	0.951
	Plant (Patch)	37	0.084	2.50	**< 0.001**
	Error	167	0.034		

Fruit set was analyzed by means of a logistic GLM with binomial error distribution; seed number and seed set were analyzed by means of General-linear model ANOVAs.

Phenotypic Selection

The results revealed negative directional selection on banner length but positive directional selection on floral display through seed number in Vellozia (Table 4). Additionally, disruptive selection on the interaction banner width × floral display through seed number in this patch was detected (significant positive quadratic term; Table 4). In Travessão, the results revealed positive directional selection on banner width × banner length through seed number, negative directional selection on banner width × floral display through both fruit set and seed number and negative directional selection on banner length × floral display and on banner width × banner length × floral display interactions through seed set from attacked flowers (Table 4). In quadratic terms, stabilizing selection was detected on banner width and floral display through fruit set, stabilizing selection on floral display through seed number and seed set, and on banner width × floral display through attacked seed set (significant negative quadratic terms; Table 4).

Table 4. Standardized selection gradients for floral attractiveness traits and their correlations in *Collaea cipoensis* (Fabaceae) on fruit set, seed number, seed set and seed set from attacked flowers on each Patch.

Vellozia

Floral trait	Fitness component							
	Fruit set		Seed number		Seed set		Seed set (attacked)	
	β'	γ'	β'	γ'	β'	γ'	β'	γ'
Banner width	0.16±0.17	-0.03±0.16	-0.16±0.35	0.18±0.42	0.21±0.24	0.22±0.20	-0.12±0.44	0.40±0.39
Banner length	-0.02±0.15	-0.11±0.13	**0.82±0.29**	-0.01±0.26	-0.33±0.20	0.21±0.16	0.39±0.33	-0.22±0.27
Floral display	-0.02±0.21	-0.00±0.52	**1.20±0.41**	-1.32±1.03	0.21±0.28	0.86±0.65	0.50±0.47	-1.00±1.12
Banner width× Banner length	-0.14±0.15	0.00±0.01	-0.34±0.40	-0.01±0.02	0.02±0.22	0.00±0.01	-0.13±0.44	-0.01±0.03
Banner width× Floral display	-0.37±0.59	-0.06±0.06	0.32±0.96	**-0.32±0.10**	0.35±0.93	0.02±0.08	0.46±0.99	-0.02±0.18
Banner length× Floral display	-0.29±0.21	-0.03±0.03	0.60±0.41	-0.08±0.06	0.24±0.30	0.00±0.00	0.38±0.51	0.03±0.08
Banner width× Banner length× Floral display	0.19±0.23	0.00±0.00	0.45±0.48	0.00±0.01	-0.27±0.33	0.00±0.00	-0.45±0.80	0.00±0.01

Linear (β') and quadratic (γ') coefficients ± SE are shown. Selection gradients with significant P values are marked in bold.

Travessão

Floral trait	Fitness component							
	Fruit set		Seed number		Seed set		Seed set (attacked)	
	β'	γ'	β'	γ'	β'	γ'	β'	γ'
Banner width	-0.17±0.11	**0.52±0.22**	0.09±0.21	0.42±0.44	-0.07±0.23	0.46±0.62	-0.05±0.31	0.48±0.37
Banner length	0.10±0.12	-0.08±0.14	0.16±0.23	-0.16±0.28	0.09±0.30	-0.36±0.38	-0.07±0.36	0.79±0.77
Floral display	-0.16±0.11	**0.38±0.20**	0.15±0.21	**-0.96±0.40**	019±0.27	**-1.04±0.56**	-0.08±0.44	1.01±0.90
Banner width× Banner length	0.10±0.10	0.00±0.01	**0.36±0.18**	-0.14±0.01	0.20±0.24	-0.01±0.01	-0.66±0.41	-0.03±0.01
Banner width× Floral display	-**0.18±0.09**	0.00±0.01	-**0.53±0.16**	0.01±0.30	0.38±0.23	0.03±0.40	-0.80±0.67	-**0.71±0.19**
Banner length× Floral display	-0.15±0.08	0.02±0.01	-0.25±0.16	0.03±0.02	0.26±0.21	0.02±0.03	**-1.30±0.54**	-0.36±0.28
Banner width× Banner length× Floral display	0.14±0.13	0.00±0.00	0.22±0.24	0.00±0.01	0.06±0.36	0.00±0.00	**-1.03±0.26**	0.02±0.05

Discussion

This study reports spatial variation in the patterns on floral visitation rates and visitors assemblage in patches of *C. cipoensis*. The results demonstrate that plants with large number of open flowers are more attractive to both mutualistic (i.e potential pollinators) and antagonistic (i.e nectar-robbers) visitors, corroborating the hypothesis of this study. Effects of nectar-robbers are considered to be context-dependent, shifting between the costs (Irwin & Maloof, 2002; Irwin, 2009; Navarro & Medel, 2009), the benefits (Navarro, 2009) or even none of these (Maloof, 2001). However, nectar-robbers are recorded most frequently as negative for plant species. Potential pollinators were more attracted by flowers with larger banner width, while nectar-robbers, nectar-thieves and flower herbivores were more attracted by flowers with smaller banner length. Nectar-robbers attacked smaller flowers, and individuals with greater number of open flowers. Interestingly, female fitness components shown no spatial differences (differences between patches), but fruit and seed set between plants within patches were different. The similarity between patches has been related to the idea that species with restricted distributions from rupestrian grasslands are in fact a single population, which resulted from the species adaptation to harsh environmental conditions, and may be prisoners of this this habitat, as reported for *Baccharis concinna* and *Coccoloba cereifera* (Ribeiro & Fernandes, 2000; Barbosa et al., 2015).

Collaea cipoensis depends on hummingbird pollination to set fruits and seeds, and its flowers fit into the ornitophilous pollination syndrome, showing red flowers with tubular corollas, odorless and with nectar production. Illegitimate visitors represented the 97% of the total flower visits, while hummingbirds represented the 96% of legitimate visitation. In other words, hummingbirds are extremely important visitors to the species reproduction, since it depends exclusively on cross-pollination to set fruits and seeds. However, the direct contribution of hummingbird to plant reproduction can be low, even when they are the most frequent visitors (Maruyama et al., 2012; Wattset al., 2012). As the results of this study suggest, *C. cipoensis* is specialist in terms of pollination syndromes (i.e. scentless, tubular corollas, nectar production and diurnal anthesis), but this relationship seems not to be reciprocal, since hummingbirds uses plant rewards as generalists. Also, hummingbirds may be very sensitive to plant resources (Irwin & Brody, 1999), which may explain the differences in visitation rates between patches and

usually avert individuals with high robbing levels and visit fewer flowers. Even though hummingbirds prefer ornitophilous species, they can use wider floral syndromes types depending on the ecological and environmental context (Dalsgaard et al., 2009; Abrahamczyk & Kessler, 2010). Indeed, hummingbirds appear to be less specialized and visiting more non-ornithophilous plants in dry, seasonal and open habitats (Arizmendi & Ornelas, 1990; Araujo & Sazima, 2003; Dalsgaard et al., 2009; Abrahamczyk & Kessler, 2010; Dalsgaard et al. 2011; Rodrigues & Araujo2011; Maruyama et al., 2012; Rodrigues & Rodrigues, 2014). Interestingly, *C. cipoensis* flowers do not seem to be attractive to *Heliactin bilopha* (Temminck, 1820) or the endemic *Augastes scutatus* (Temminck, 1824) hummingbirds, since both species were observed during the observation periods, but never visited the species.

The results presented novel data regarding the effect of nectar-robbers in *Collaea cipoensis* reproduction, and demonstrate that illegitimate visitors affect negatively the female reproductive capacity of the species by interfering with its fruit and seed production, and its seed number. Gélvez-Zúñiga et al. (2016) reported the bee *Trigona spinipes* as the primary nectar-robber of *C. cipoensis*, however, the present study found a wider scenario including hummingbird opportunistic behavior, and a variety of illegitimate visitors, and its negative effects on female fitness components, and also included floral attractiveness traits. According to Irwin et al. (2001), insect nectar-robbers exhibit strong negative effects on plant reproductive success especially in plants pollinated by birds. Additionally, Irwin & Brody (1999) report nectar-robbers as causing floral attractiveness reduction to pollinators, causing indirectly a decreasing pollinator visitation rates. At least for one of the floral attractiveness variables (floral display) the results here show an increase of both pollinators and nectar-robbers visitation rates in plants with greater floral displays. Additionally, difference of seed set of fruits from intact and attacked flowers, were marginally significant (See Table 3). However this marginal value may be more related to sample size than to the lack of interference of nectar-robbers in seed set. On the other hand, nectar-robbers can reduce significantly the pollen transfer and seed production of endemic and threatened plants species, as found by Castro et al. (2008), where the pollinator behavior was also modified by the presence of attacked flowers by decreasing the legitimate visitation rates, and therefore, raising important considerations for its management and conservation. Still, the role of flower visitors as nectar robbers, and its conflictive effects to rupestrian grasslands plant species remains poorly known (Guerra et al., 2016).

The selection patterns detected here help to understand some of the evolutionary pressures acting on flower size and display, and also its dynamic in *C. cipoensis* patches. A conflicting selection on floral attractiveness traits through female fitness components of *C. cipoensis* mediated by the combined effect of pollinators and floral enemies was expected. Negative directional selection on banner length and positive directional selection on floral display through seed number was detected for Vellozia. This means that smaller banner lengths have been selected by visitors (legitimate and illegitimate) through the seed number, while greater floral displays are favored through the same fitness component. Additionally, disruptive selection on the interaction banner width × floral display through seed number was detected, meaning that intermediated phenotypes are under selective disadvantage. So, for greater banner width and floral display the fitness increased, as well as for smaller banner width and less floral displays. This selection for increased flower number has also been reported in other studies in natural populations (Caruso et al., 2003; Sletvold, Grindeland & Ågren, 2010).

In Travessão, a positive directional selection on banner width × banner length through seed number, indicates that flowers with greater banner widths and lengths are favored via floral visitors. And negative directional selection on banner width × floral display through both, fruit set and seed number, indicating that fruit set and seed number increases with smaller flower widths and floral displays. In the seed set of attacked flowers a negative directional selection on banner length × floral display, and on banner width × banner length × floral display interactions was detected. This means that mediated by nectar-robbers, smaller banner length and displays widths result in an increased seed set. The stabilizing selection detected on banner width × floral display through attacked seed set shows that nectar-robber are mediating the selection on these floral traits through the seed set of attacked flowers. Higher flower number possible involve increased ovule discounting in hermaphrodite plants with different forms of self-incompatibility, thus decreasing fruit and seed set (Duffy & Johnson, 2011).

In general, the results shown here confirm the hypothesis that *C. cipoensis* is under a conflictive selection between legitimate visitor effectiveness and the cost of dealing with nectar-robbers effects. And also, that nectar-robbers reduce female fitness success of this narrow endemic shrub. This, jointly to the highly specific requirements for growth, and adaptation to soil nutrient deficiency, constitutes a very specific niche that may constrain the species distribution

reinforcing the complex scenario to reproduce and maintain over time in rupestrian grasslands areas.

Acknowledgements

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Appendix 1. Pearson's correlation coefficients for floral attractiveness traits of *C. cipoensis*.

$* P < 0.05; **P < 0.01.$

Vellozia	Banner width	Banner length	Keel length	Wing length	Floral display
Banner width	-	0.404	0.305	0.350	0.390
Banner length		-	0.555**	0.491*	-0.169
Keel length			-	0.911**	-0.276
Wing length				-	-0.230
Floral display					-
Travessão					
Banner width	-	0.289	0.232	0.265	0.155
Banner length		-	0.861**	0.873**	-0.371
Keel length			-	0.965**	-0.393
Wing length				-	-0.338
Floral display				-0.338	-