Research Article

Interaction between Bees and the Tristylous Flowers of *Oxalis cytisoides* Mart. & Zucc. (Oxalidaceae)

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The interaction of bees with the tristylous flowers of *Oxalis cytisoides* Mart. & Zucc. (Oxalidaceae) was evaluated. The study was conducted in a semideciduous forest at the Fritz Plaumann State Park in Concórdia, Santa Catarina state. Two *Oxalis cytisoides* aggregations were found and the flower visiting bees were observed. The 3 floral morphs were found at the following proportions: 16 long-, 37 mid-, and 34 shortstyled individuals ($n = 87$). Anthesis lasted one day (6:30 AM to 3 PM). No fruit was formed in the autogamy test; thus, pollination was dependent on the visitors. The pollen grain size varied between the stamens and morphs and formed subsets in accordance with the stigma height (long/mid/short). We collected 165 bees from 30 species visiting the flowers. *Hypanthium divaricatum* was the most abundant bee species (34%) and the males were often observed patrolling the flowers in search of females for mating. Analysis of the pollen loads from 34 females showed that 27 carried *O. cytisoides* pollen. The most frequent bees that carried *O. cytisoides* pollen grains on their bodies were considered pollinator agents, responsible for transferring pollen grains among the floral morphs.

1. Introduction

Heterostyly is a rare phenomenon among plants that has been observed in 28 angiosperm families [1]. Heterostylous species have flowers with different morphs in their populations and may be distylous or tristylous. Each plant holds just one type of flowers. Tristyly is a more complex and rare type of heterostyly, which has been reported in seven botanical families, including Oxalidaceae [1–5].

Charles Darwin formulated an explanation for the adaptive function of heterostyly in 1877, which suggested that the anthers and stigma were positioned to promote cross-pollination between the floral morphs. Darwin [6] also reported observations from Fritz Müller in Santa Catarina for the genus *Oxalis*, who found that flowers do not produce seeds at sites with only one floral morph. However, when the three morphs were planted in a garden, many seeds were produced.

The tristyly species comprises three floral morphs that differ in stamen filament height, pistil style position, pollen grain size and self-compatibility systems. Cross-pollination mediated by insect visitors is favored for this type of flowers [2, 6–9]. The three floral morphs in tristyly populations likely represent the maximum number of sexual polymorphisms in plants that promote cross-pollination through pollinator contact geometry [10]. For legitimate cross-pollination plant requires vectors able to transfer pollen from flowers with long, mid, or short-level anthers to flowers with long-, mid-, or short styles, respectively [7, 9, 11]. All other filament-style length combinations result in little or no seed set [12].

The family Oxalidaceae has radial bisexual flowers with five free sepals and five distinct petals that are slightly connate and often convoluted [13]. There are typically ten stamens with connate filaments at the base; the external filaments are shorter than the internal filaments; and nectar is produced at the base of them or in glands that may alternate with

the petals. Typically, the flowers have five styles and stigma that are often globular or punctate [14]. Three Oxalidaceae genera have been observed in Brazil; one of them, *Averrhoa*, (which includes starfruit) is native to Asia, but it is often cultivated in Brazil [13]. *Oxalis* is the largest genus in the family, with approximately 800 species; it is cosmopolitan, and Africa and the Americas are the centers for its diversity. There are approximately 114 *Oxalis* species in Brazil [14]. According to Lourteig [13], *Oxalis cytisoides* Mart. & Zucc. is widely distributed from the northeastern (Ceará) to south regions (Rio Grande do Sul) in Brazil and in Argentina. *Oxalis cytisoides* has an erect herbaceous or shrub habit that can grow up to 1 m high. This species is discontinuously distributed almost entirely across the state of Santa Catarina (southern Brazil), and it is characterized as a heliophile or with a preference for diffuse light and selective hygrophytes that often develop in altered areas, including tropical rain forests, mixed ombrophilous forests on the plateau, and the Upper Uruguay River forest. *O. cytisoides* flowers primarily in spring, which lasts until summer [13].

The frequency of the morphs in the populations and compatible system of many American *Oxalis* species were investigated by Mulcahy [15], Ornduff [2], Weller [16, 17], and Weller et al. [18], demonstrating that in some populations morphs are not in equilibrium and also showing the process of losing styled forms. Recently, Turketti [5] presented an extensive work on the expression of the tristyly in the genus *Oxalis* of South Africa, where it was found that most populations (of 58 different species) were at isopletic equilibrium, which means equal representation of style length morphs. The same thesis [5] studied a special case of two species of *Oxalis* section sagittate which have a different arrange and orientation of the anthers and stigma compared to other *Oxalis*. In these species self-compatibility was more expressive, but the dependence on the pollinator still needs to be tested.

The objective of this work was to study the interactions between the visiting bees and the tristyly flowers of *Oxalis cytisoides*, evaluating the pollination in natural conditions. Our questions were as follows: are there seeds that have been produced in natural conditions? If yes, which flower visitors are potentially responsible for the legitimate transference among the morphs? Additionally, the pollen grains of the different morphs were described.

2. Material and Methods

The study was conducted in the Concórdia municipality between September 2008 and April 2009 at the Fritz Plaumann State Park, 27°16′18′′S, 27°18′57′′S, 52°04′15′′W and 52°10′20′′W, which is predominantly composed of a semideciduous forest. In the study area, we observed two *Oxalis cytisoides* aggregations with clustered distribution of individuals. These aggregations were separated by approximately 1000 m, with sparsely individuals occurring along them. The plants in these aggregations were evaluated together. The three floral morphs of *O. cytisoides* were observed. The morphs did not exhibit remarkable structural
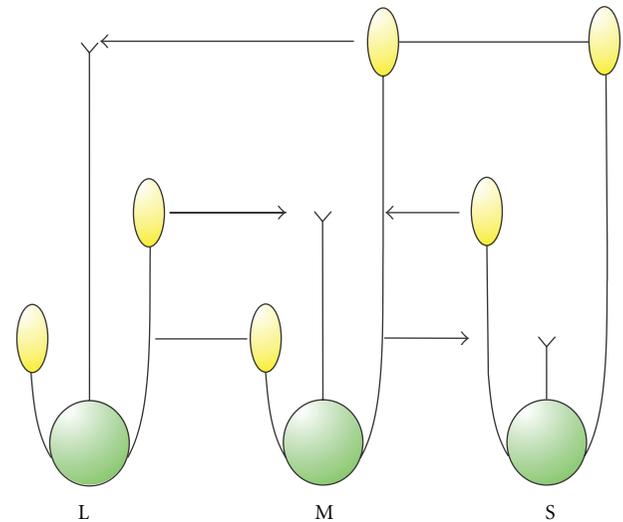

FIGURE 1: Scheme of *Oxalis cytisoides* Zucc flower morphology. L: long-styled flower with medium and short filaments. M: mid-styled flower with short and long filaments. S: short-styled flower with medium and long filaments. The arrows indicate the legitimate cross-pollination, according to the system of the heretostyly plants.

differences except for the relative stamen positions, which had different filament and style heights, as schematized in Figure 1.

A self-fertilization treatment was used to evaluate *Oxalis cytisoides* self-pollination. In this treatment, 191 preanthesis flower buds from the three floral types were protected with bags made from voile fabric. This sample included 76 long-styled (L) flowers, 63 mid-styled (M), and 52 short-styled (S) flowers. The open flowers and fruits were removed from the branches when the flower buds were bagged. These buds were followed for 1-2 months to assess fruit formation. Pollination in a natural condition was analyzed through examining the fruit formation with seeds. At least 20 fruits of each floral morph were evaluated from each aggregation.

During the flowering period for *O. cytisoides* (September to April), we collected bees visiting the flowers once a month for two consecutive days for a total of 96 hours of sampling; the three flowers morphs were observed equally in time. The collected bees were killed and identified. In addition to collection, we observed the behavior of the bees at the flowers from the three morphs for 30 hours to assess the contribution of bees to *O. cytisoides* pollination. The bee specimens were deposited in the Entomological Collection Paulo Nogueira Neto (CEPANN) of the Bee Laboratory at the Institute of Biosciences in the University of São Paulo.

There are morphological differences in pollen grains from anthers of different filament sizes in *Oxalis* flowers [19]. Based on this information, the *O. cytisoides* pollen grains were analyzed separately to investigate the pollen morphology in the three morphs, samples with stamen anthers that had medium (m) and long filaments (l) in S flowers, short (s) and long (l) filaments in M flowers, and short (s) and mid (m) filaments in L flowers. Pollen grains were removed from the anthers of the three stamen types
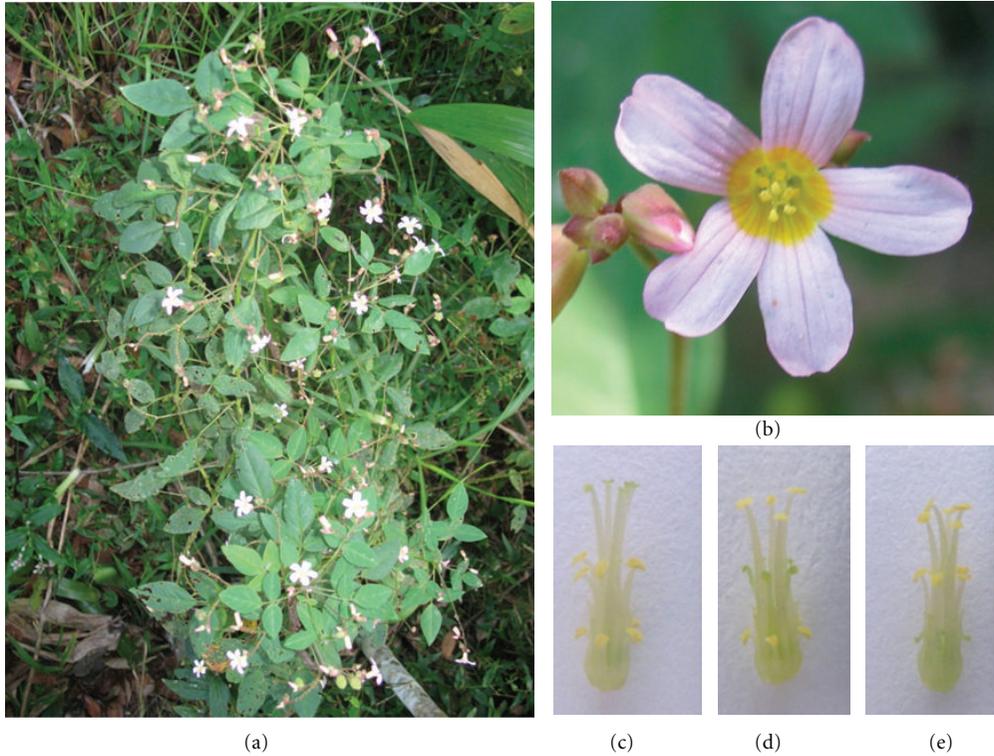
Figure 2: *Oxalis cytisoides*. (a) Plant habit. (b) Flower and buds. (c)–(e): Stamens and pistils from L (c), M (d), and S (e) morphs. Photo scales (c)–(e) = 5 mm.

with different size filaments; they were described separately, and the grain size, and shape were considered. The pollen grains were acetolyzed following the method proposed by Erdtman [20] and subsequently mounted on slides following Barth [21]. For each sample, twenty pollen grains were measured along the polar and equatorial axes in accordance with Silva et al. [22]. A single factor analysis of variance (ANOVA) was used to evaluate the difference in size between the pollen grains [23]. The size of pollen grains is given by the measure from the longest axis in the equatorial view. The data on the pollen grain size were analyzed using the median from boxplots, which were plotted using the program R [24], graphics package version 2.13.0.

Samples of the pollen load from the scopae of the visiting female bees on the *O. cytisoides* flowers were collected for analysis. The pollen grains were subjected to the acetolysis process described above, and they were qualitatively analyzed for the presence or absence of *O. cytisoides* pollen.

This work was performed with the authorization (number 13486-2) for collection and transportation of biological material by IBAMA/SISBIO (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/Sistema de Autorização e Informação em Bioversidade).

3. Results

A total of 87 flowering individuals were observed: 42 at the first plant aggregation (11 long-, 13 mid-, and 18 short-styled

Table 1: Means (±standard deviation) for pollen grain sizes from the three *Oxalis cytisoides* floral morphs at the equatorial and polar perspectives.

Morph	Filaments	Equatorial	Polar	P/E
M	s	24.52 (±1.00)	32.49 (±1.36)	1.32 (±0.06)
L	s	23.89 (±1.17)	33.30 (±1.98)	1.39 (±0.06)
S	m	27.59 (±1.42)	37.30 (±1.65)	1.35 (±0.06)
L	m	26.51 (±0.90)	37.90 (±1.10)	1.43 (±0.05)
S	l	28.89 (±1.13)	40.63 (±1.96)	1.41 (±0.09)
M	l	27.74 (±1.28)	40.59 (±2.67)	1.46 (±0.06)

S: short, M: medium, and L: long.

morphs) and 45 at the second aggregation (5 long-, 24 mid-, and 16 short-styled morphs) (Figure 2). Anthesis began at dawn (6:30), and flower abscission began at approximately 15:00.

In self-fertilization test with the three morphs, no fruit was formed in the 191 buds analyzed. In contrast, all those fruits sampled in natural conditions presented seeds.

The pollen grains from *O. cytisoides* (Figure 3) are monads, small and medium size, radial, isopolar, subtriangular in area, subprolate to prolate in shape (Table 1), and tricolpate and have colpate furrows, lolongate endoapertures, and reticulated exine.

The pollen grain size varied between the stamens and morphs (Table 1). Significant differences were observed for
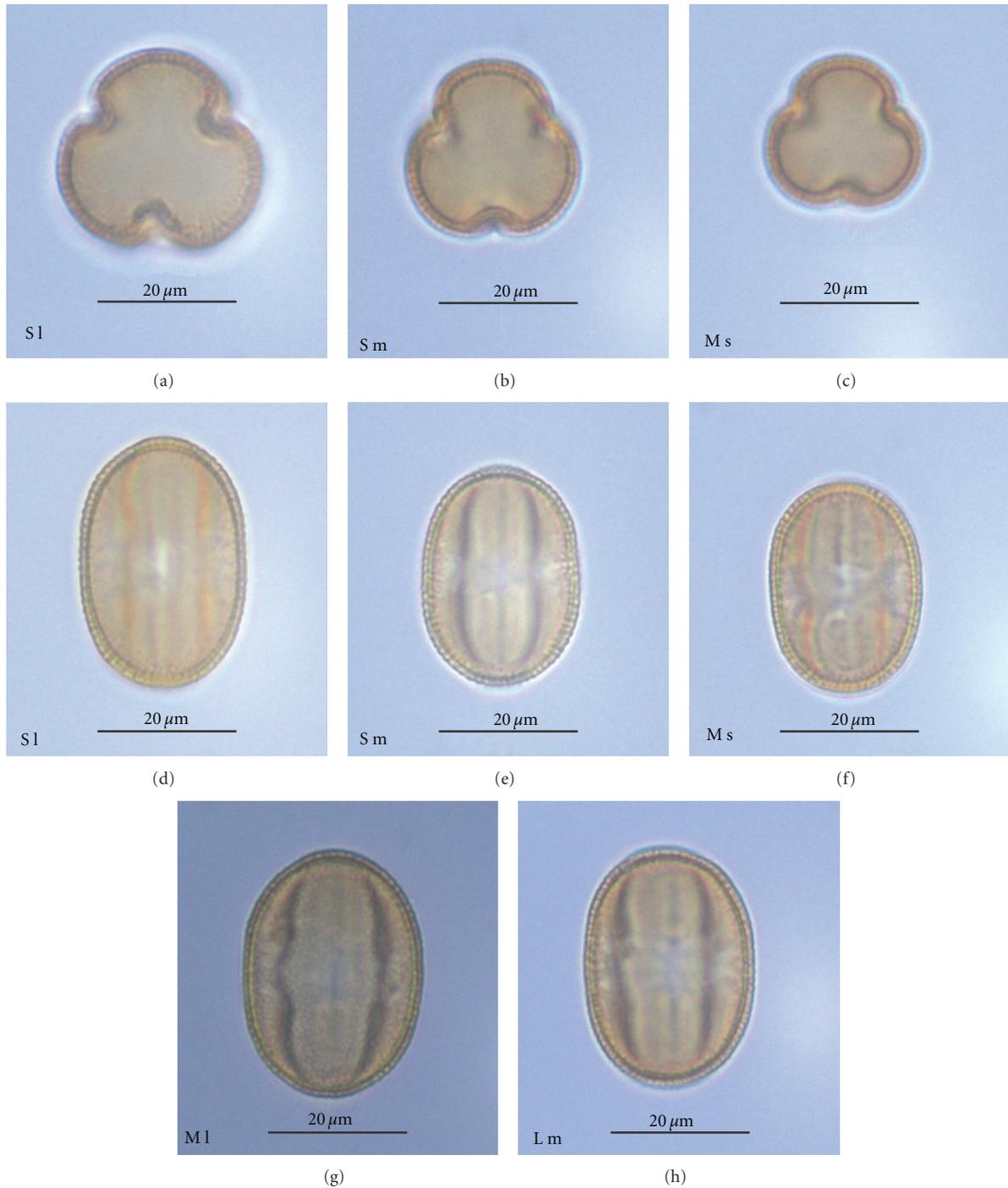
Figure 3: Pollen grains from *Oxalis cytisoides*. ((a)–(c)) Polar perspective for pollen grains from long (a), mid (b), and short (c) anthers. ((d)–(i)) Equatorial perspective for pollen grains in long ((d) and (g)), mid ((e) and (h)) and short ((f) and (i)) anthers. Capital letters are the floral morph (style height) and lower letters are the anthers from the pollen grain origin, for example: S l = pollen came from the long filaments (l) of the short styled morph (S).

the pollen grain sizes in polar view from the six samples (mean square, 3.4609; degrees of freedom, 114.00, $P < 0.05$). We also observed that the pollen from short-, mid-, and long-stamen anthers was grouped (Figure 4), forming subsets in accordance with the stigma height.

3.1. Floral Visitors.

A total of 165 individuals were sampled and distributed over 30 species and four bee families (Table 2). The visitors were more abundant in December and February, although the number of flowering plants was practically the same throughout the flowering months.

TABLE 2: Visiting bees of *Oxalis cytisoides* flowers in Concordia, Santa Catarina, southern Brazil.

Family	Species	Ind.	Month
Andrenidae	*Anthrenoides meridionalis* (Schrottky, 1906)*	2 (2 F)	Nov
	Psaenythia bergii Holmberg, 1884*	2 (2 F)	Nov
Apidae	*Ceratina* (*Calloceratina*) sp. 2	1 (1 F)	Dec
	Ceratina (*Crewella*) sp. 12	1 (1 F)	Sep
	Ceratina (*Crewella*) sp. 16*	15 (12 F, 3 M)	Feb-Mar, Sep–Dec
	Lophopedia nigrispinis (Vachal, 1909)*	23 (16 F, 7 M)	Feb, Sep, Nov-Dec
	Odyneropsis sp.	1 (1 F)	Dec
	Paratetrapedia (*Paratetrapedia*) sp. 1	5 (1 F, 4 M)	Feb
	Paratetrapedia (*Paratetrapedia*) sp. 2*	7 (6 F, 1 M)	Nov–Mar
	Paratetrapedia (*Paratetrapedia*) sp. 4	6 (6 M)	Feb, Nov
	Tetrapedia diversipes Klug, 1810*	13 (13 F)	Feb-Mar, Nov-Dec
	Trigona spinipes (Fabricius, 1793)	1 (1 F)	Sep
Halictidae	*Augochlora* (*Augochlora*) sp. 1[†]	1 (1 F)	Nov
	Augochlora (*Augochlora*) sp. 3	1 (1 F)	Oct
	Augochlora (*Augochlora*) sp. 4	1 (1 M)	Dec
	Augochlora (*Augochlora*) sp. 6	1 (1 F)	Dec
	Augochlora (*Oxystoglossela*) sp. 4	1 (1 F)	Feb
	Augochlorella sp. 2	7 (7 F)	Feb, Nov-Dec
	Augochlorella sp. 5	5 (4 F, 1 M)	Oct–Jan
	Augochloropsis sp. 1	1 (1 F)	Jan
	Augochloropsis sp. 2	1 (1 F)	Dec
	Augochloropsis sp. 12	1 (1 F)	Feb
	Neocorynura sp.	1 (1 F)	Dec
Megachilidae	*Anthidulum mourei* Urban, 1993	1 (1 M)	Nov
	Hypanthidium divaricatum (Smith, 1854)*	57 (17 F, 40 M)	Nov–Apr
	Hypanthidium obscurius Schrottky, 1908[†]	3 (1 F, 2 M)	Nov–Jan
	Megachile (*Leptorachina*) sp. 1	2 (2 M)	Jan, Mar
	Megachile (*Austromegachile*) *susurrans* Haliday, 1836	2 (2 F)	Dec
	Moureanthidium paranaense Urban, 1995	1 (1 M)	Nov
	Moureanthidium subarenarium (Schwarz, 1933)	1 (1 M)	Nov
Total		165	Sep–Apr

Ind.: number of individuals, month: collection month, *O. cytisoides* pollen grains in the scopae pollen load, and [†] number of *O. cytisoides* pollen grains in the scopae. F: female and M: male.

In general the bees visited more than one flower on the same plant and different plants in the same aggregation with no observed preference for a particular morph. *Lophopedia nigrispinis* (Vachal, 1909) (Apidae), *Tetrapedia diversipes* Klug, 1810 and *Ceratina* sp. remained for a few seconds in the flowers and visited between 2 and 5 flowers, preferentially collecting nectar. *Hypanthidium divaricatum* (Smith, 1854) (Megachilidae) and *Ceratina* sp. (Apidae) actively collected pollen and nectar.

Among the sampled bees, the most abundant in the flowers were *H. divaricatum* (Figures 5(a) and 6(a)–6(c)) and *L. nigrispinis* (Figures 6(d)–6(f)), which corresponded to 34% and 14% individuals, respectively. Pollen grains were often observed adhered to the mouthparts of the sampled bees (Figures 6(c) and 6(f)). *H. divaricatum* males were frequently observed patrolling *O. cytisoides* flowers searching

for females, and a mate was recorded on certain occasions (Figure 5(b)).

Oxalis cytisoides pollen was observed in 25 samples of pollen material removed from the scopae of 32 females that belonged to nine bee species, which indicates that these females effectively collect this floral resource (Table 2).

4. Discussion

The three floral morphs of *Oxalis cytisoides* were clearly distinguished in the studied area. The self-fertilization (bagged flowers) tests showed no fruit production, even in the mid- and short-styled flowers, which would be easily contaminated with pollen from the above anthers. On the other hand flowers left under natural conditions produced fruits, which demonstrated participation by pollinators. In the studied
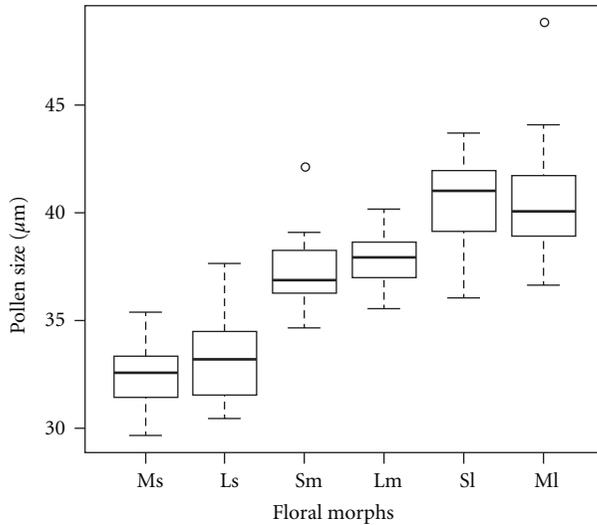
FIGURE 4: Box plot of pollen grain sizes for the three flower morphs measured from the polar perspective. Box plots show the medians as well as 1° and 2° quartiles and ranges; the circles indicate extreme values (outliers).

area we sampled 30 bee species visiting the flowers of *O. cytisoides*. Among them there were some very frequent in the visits and that were flying 4–6 months during the whole flowering period of the plant species, like *Hypanthidium divaricatum*, *Lophopedia nigrispinis*, and *Ceratina* sp. 16. These three species are potential candidates for pollinators, due to the frequency and abundance in the flowering period, and also because the females collected actively pollen from *Oxalis* flowers (demonstrated by behavior and the analysis of the pollen load of the scopae). Pollen collection requires more time and ability at the flowers during the visits. This increases the permanence time at the flower and contact with the reproductive parts, assisting in pollen transference to the stigma. Simultaneously, during the visits, the pollen can adhere to the visitor bodies, like to the forehead, abdomen, and thorax. These pollens adhered to the hairs are likely more important for pollen transfer than the pollens in the scopae, because of the position they will touch into the next flower. Therefore, the bee species with these characteristics were most likely to promote cross-pollination for *O. cytisoides* in the studied area, guarantying the verified fruit set and also the maintenance of the three morphs in the populations. It is known that the trimorphic condition tends to break down the polymorphism and evolves a homostylous condition in populations with a deficit of effective pollinators [4, 12].

Hypanthidium divaricatum shows a special behavior and preference to the flowers of *O. cytisoides*. The males patrol and seek for females to copulate in the plants. This behavior indicates that males recognize this plant as a preferred species of their females, showing specialization. A similar fact was also reported for *Ancyloscelis* bees and plants of the Pontederiaceae family [25] and for *Cephalurgus anomalus* Moure and Oliveira and plants of the Malvaceae family [26, 27]. Further results reinforcing the specialization were attested by the pollen load of the females of *H. divaricatum*,

(a)

(b)

FIGURE 5: *Hypanthidium divaricatum* visiting (a) and mating (b) at *Oxalis cytisoides* flowers.

which were carrying most grains of *O. cytisoides*. This bee species was found visiting other plants in Concórdia, but of 86 individuals collected in the region 46 (53%) were visiting *O. cytisoides* flowers [28].

According to our results *H. divaricatum* is flying between November and April covering the most period of flowering of *O. cytisoides*. Since the flower season for this species starts in September, probably the 2 other bee species (*Ceratina* sp. 16 and *L. nigrispinis*) with high frequency in the flowers may act as effective pollinators at the beginning of the season.

Luo et al. [29] studied the South American species *Oxalis debilis* Kunth, which was introduced in China and found that it does not predominantly reproduce vegetatively, as was previously assumed for this species. The pollination of *O. debilis* was performed by bees that collected nectar (*Apis cerana* Fabricius, 1793) as well as pollen (*Ceratina* (*Pithitis*) *smaragdula* Fabricius, 1787, and *Ceratina* sp.). According to Björkman [30], disc-shaped and small flowers allow for
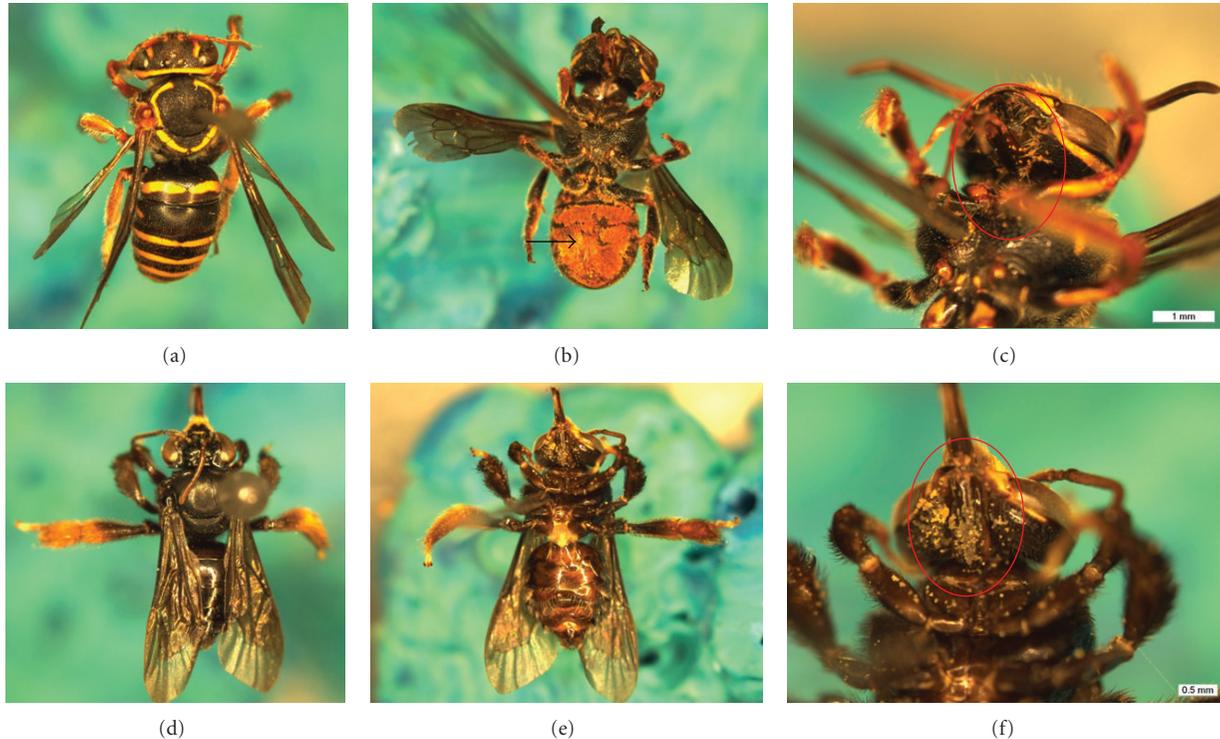
FIGURE 6: (a)–(c): *Hypanthidium divaricatum* ((a): male dorsal view, (b): female ventral view with loaded abdominal scopae (arrow) and (c): close-up of the male mouthparts with pollen grains attached to the hairs). (d)–(f): Male *Lophopedia nigrispinis* (d: dorsal view, e: ventral view and f: close-up of mouthparts with pollen grains attached to the hairs).

nectar access by several species, which facilitates the cross-fertilization of the plant. This is probably the case of *O. cytisoides* studied herein which received a spectrum of 30 bee species, including short and long tongued bees. But, it is important to emphasize that to the plant visitors with high frequency and carrying pollen grains are likely more effective for the cross-pollination than those sporadically visitors. However, future studies on stigmatic receptivity and specificity in *O. cytisoides* are necessary for a more accurate conclusion about the role of each visitor.

The morphology and ornamentation of the pollen grains from *O. cytisoides* are consistent with the description for nine species in the *Oxalis* genus by Rosenfeldt and Galati [19]. The largest pollen grains were produced in the high level anthers (long stamens), intermediate size pollen was produced by mid-level anthers, and smaller grains were produced by low level anthers, as has been reported for other *Oxalis* species [14, 19, 31] and would be expected in a tristyly species. At the load of the bees it was not possible to distinguish or quantify precisely the anther origin of the pollen grains, because of some overlap in their size. But the three types of pollen were presented in the samples of the seven bee species carrying *Oxalis* pollen (Table 2). We believe that female bees are able to collect the pollen grains from all the anther levels without a problem, since the flowers of *Oxalis* do not have special morphology to hide the resource, like it is known for the tristyly *Eichhornia* species [9, 12].

Compatibility tests between morphs of species of the genus *Oxalis* studied by Ornduff [2] and Pacheco and Coleman [31] revealed that legitimate pollination (following heterostyly scheme) is more successful than illegitimate pollination for frequency and seed production. Although we did not conduct cross-pollination tests, we found that under natural conditions fruits were produced, demonstrating the importance of the local visiting bees as agents for the correct pollen transference.

Finally, to the bee perspective, it is necessary to highlight that *O. cytisoides* seems to be an important source of floral rewards for the local bee fauna. Certain studies refer to *Oxalis* as a flower resource for honeybees [32, 33]. But at the study area we verified that it deals with a relevant source of nectar and pollen for many native bee species, especially solitary bees. According to Krug [28], about 27% of the known *Apiformes* species occurring in the Fritz Plaumann Park in Concordia were visiting the flowers of *O. cytisoides*.

Acknowledgments

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