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INSTITUTO DE BIOLOGIA

NATHÁLIA SUSIN STREHER

FLOWERING PHENOLOGY AND REPRODUCTIVE BIOLOGY
IN SUBTROPICAL GEOPHYTES: CASE STUDIES WITH
SYMPATRIC SPECIES OF AMARYLLIDACEAE

FENOLOGIA DA FLORAÇÃO E BIOLOGIA REPRODUTIVA EM
GEÓFITAS SUBTROPICAIS: ESTUDOS DE CASO COM
ESPÉCIES SIMPÁTRICAS DE AMARYLLIDACEAE

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RESUMO

As plantas com flores exibem diversos mecanismos que interferem e podem otimizar seus processos reprodutivos. Nesse sentido, a fenologia da floração e a biologia da polinização podem contribuir para o entendimento das estratégias reprodutivas das plantas e de suas interações com os agentes abióticos e bióticos. Nesta dissertação, buscamos entender os padrões reprodutivos de três espécies de Amaryllidaceae que co-ocorrem em uma região subtropical do Brasil. No primeiro capítulo, mostramos evidências de hercogamia e autoincompatibilidade gametofítica numa população de *Habranthus gracilifolius*, demonstrando, portanto, que tanto a presença de polinizadores como florescer em sincronia com a população são requisitos fundamentais para o sucesso reprodutivo dos indivíduos desta espécie. No segundo capítulo, nas três espécies simpátricas, *Habranthus tubispathus*, *Habranthus gracilifolius* e *Zephyranthes mesochloa*, o gatilho da floração se dá por um conjunto de variáveis ambientais, salientando que tanto a precipitação como a temperatura e o fotoperíodo estão envolvidos nesta resposta. O período de floração destas espécies está relacionado com os sinais climáticos locais, enquanto que os visitantes florais parecem não exercer fortes pressões sobre o tempo de floração. Este é o primeiro trabalho a apresentar como estas espécies se comportam sob condições naturais e agrega este conhecimento aos estudos filogenéticos, contribuindo, assim, para o entendimento da história evolutiva do grupo.

ABSTRACT

Flowering plants display several mechanisms that interfere and can optimize their reproductive processes. In this sense, flowering phenology and pollination biology can contribute to understand the reproductive strategies of plants and their interactions with the abiotic and biotic agents. In this work, we seek to understand the reproductive patterns of three species of Amaryllidaceae that co-occur in a subtropical region of Brazil. In the first chapter, we show the presence of herkogamy and evidence of gametophytic self-incompatibility in a population of *Habranthus gracilifolius*. Thus, we demonstrated that both presence of pollinators and blooming in synchrony with the population play key roles for reproductive success of this species. In the second chapter, mass flowering in the three sympatric species, *Habranthus tubispathus*, *Habranthus gracilifolius* and *Zephyranthes mesochloa*, is triggered by a set of environmental variables, pointing out that precipitation, temperature and photoperiod are involved in this response. The flowering season in these three species is related to local weather cues while the floral visitors do not seem to exert strong pressure on the flowering time. This is the first work to explore how these species behave under natural conditions and adds knowledge to phylogenetic studies, thereby contributing to the knowledge of the evolutionary history of the group.

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Introdução Geral

No livro “*Pollination and floral ecology*”, Pat Wilmer (2011) diz que a biologia da polinização é uma área tão fascinante porque além de nos dar ideias sobre os mecanismos evolutivos por trás das interações entre plantas e polinizadores, ainda nos dá artifícios para entender a ecologia das estratégias reprodutivas das plantas. O período de floração para as plantas pode ser crucial, pois ao abrir suas flores podem determinar seu fracasso ou sucesso reprodutivo e o fluxo gênico dentro e entre as suas populações (Otárola & Rocca, 2014). Por serem organismos sésseis, muitas vezes hermafroditas e dependerem de vetores para transferência dos grãos de pólen, as plantas exibem complexos padrões de cruzamento (Barrett & Harder, 1996), e, portanto, devem florescer de uma maneira que maximize a aptidão de seus indivíduos (Wilmer, 2011). A seleção natural que age sobre os padrões de cruzamento e fertilidade é a influência mais poderosa na evolução floral e responsável pelos diversos sistemas sexuais e de polinização que ocorrem nas plantas com flores (Barrett et al., 2000).

A fenologia reprodutiva das plantas pode ser modulada por diversos fatores ambientais (como temperatura, fotoperíodo e precipitação), por fatores históricos (filogenia) e por interações bióticas mutualísticas (polinizadores e dispersores de sementes) e antagonísticas (herbívoros) (Elzinga et al., 2007; Rathcke & Lacey, 1985; van Schaik et al., 1993; Wright & Calderón, 1995). A fenofase da floração tem a capacidade de englobar tanto questões ecológicas como evolutivas, visto que as flores são importantes recursos para os seus visitantes no tempo ecológico e podem providenciar um mecanismo de isolamento reprodutivo ou especiação sobre o tempo evolutivo (Kearns & Inouye, 1993). Geralmente, fenologia e biologia da polinização são estudadas de maneira dissociada, mas apenas uma visão integrada de ambas pode permitir o entendimento das estratégias reprodutivas das plantas e de suas interações com os polinizadores (Otárola & Rocca, 2014).

As ervas perenes e geófitas da família Amaryllidaceae são amplamente distribuídas pelo mundo (Stevens, 2015) com a maioria das espécies ocorrendo no Hemisfério Sul (Arroyo & Cutler, 1984). Dentro da família, os gêneros *Habranthus* Herb. e *Zephyranthes* Herb. se distribuem desde o sudoeste dos Estados Unidos até o sul da América do Sul (Meerow & Sinjman, 1998). Além da difícil separação morfológica entre os dois (Arroyo and Cutler, 1984; Dutilh, 2005), estudos moleculares demonstram que estes fazem parte de um complexo (*Habranthus-Sprekelia-Zephyranthes*) da subtribo Hippeastrinae (García et al., 2014), o que indica que a distinção atual dos dois gêneros é taxonomicamente artificial. Dados filogenéticos

suportam a hipótese de evolução por um evento antigo de hibridização precedente à radiação do grupo (“*deep reticulation*”) (García et al., 2014). A formação de híbridos artificiais e naturais inter e intragenéricos é conhecida nas espécies desta subtribo (Dutilh, 1996; Flory, 1977; RoyChowdhury & Hubstenberger, 2000), o que sugere que as barreiras genéticas entre as linhagens são facilmente cruzadas no grupo, provavelmente como uma consequência da reticulação durante a sua diversificação (García et al., 2014). Ao que tudo indica, a hibridização teve e, provavelmente, ainda tem um papel importante na evolução das Amaryllidaceae (Flory, 1977).

Conhecer os mecanismos reprodutivos e padrões de floração das espécies deste grupo são importantes passos para começarmos a entender como as interações com seus polinizadores e o ambiente explicam sua história evolutiva. Este tipo de abordagem é particularmente interessante para as espécies de *Habranthus* e *Zephyranthes* pois apresentam um florescimento efêmero com flores que, em geral, duram apenas um dia (Dutilh, 2005). Assim, estudamos a fenologia e biologia reprodutiva de três espécies simpátricas de Amaryllidaceae no Pampa brasileiro. No primeiro capítulo, damos continuidade ao trabalho de Streher (2014), que descreveu o sistema de cruzamento de *Habranthus gracilifolius* através de polinizações manuais. Aqui, os objetivos foram verificar qual o tipo de autoincompatibilidade que esta espécie apresenta e caracterizar seus visitantes florais. No segundo capítulo, buscamos entender os padrões de floração de três espécies co-ocorrentes, *Habranthus gracilifolius*, *Habranthus tubispathus* e *Zephyranthes mesochloa*, e quais variáveis ambientais estão relacionadas com as suas florações.

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CHAPTER 1

Self-incompatibility in *Habranthus gracilifolius* (Amaryllidaceae): spatial and physiological evidences

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Abstract

A wide array of mechanisms are used by plants to interfere and optimize their reproductive processes. The evolution of self-incompatibility seems to be responsible for great part of the success of flowering plants by preventing self-fertilization and, hence, the effects of inbreeding. Here we show evidence of herkogamy and gametophytic self-incompatibility in a population of *Habranthus gracilifolius* Herb.. The existence of its gametophytic self-incompatibility nature indicates that spatial separation between stigma and anthers in the flowers of this species may function to reduce sexual self-interference. Our results support that both presence of pollinators and flowering with conspecifics are required for reproductive success of individuals.

Keywords: Herkogamy, Mating system, Xenogamy, Outcrossing, Flower visitors, Pampa Biome.

Introduction

Flowering plants are essentially sessile maters (Richards, 1997), but they display a great diversity of structural, phenological and physiological adaptations whereby they can control sexual reproduction and optimize the choice of partners (Oliveira and Maruyama, 2014). Such reproductive structures and processes that affect fecundity and genetic composition of offspring are usually used to define plant breeding systems (Sage et al. 2005; Wyatt, 1983). The major importance of reproductive systems is related to the role they play in determining the pattern and extent of population responses to natural selection, since they exert a direct impact on the amount and distribution of genetic variation within and between populations (Holsinger, 2000). They are a key trait in plant life history that define individual fitness (Barrett, 2011) and so, knowing them is an essential preliminary step in the investigation of floral biology of any species (Percival, 1965).

One important thing to recognize when studying reproductive biology in a population is who mates with whom and how often it happens (Barrett, 2014). For hermaphroditic organisms, this means the relative frequency of self-fertilization and cross-fertilization (Barrett, 2014). Outcrossing rates can be affected by diverse demographic and enviromental factors, but it substantially depends on whether species are self-compatible or self-incompatible (Barrett, 2013).

It is thought that a great part of the success of flowering plants is due to the evolution of self-incompatibility, which is the most important mechanism used to prevent self-fertilization and, consequently, its harmful effects on progeny fitness (Franklin-Tong and Franklin, 2003). Self-incompatibility systems are determined according to (1) whether the mating types are morphologically distinct (heteromorphic) or not (homomorphic), (2) the genetic mode of action (gametophytic or sporophytic) and (3) in what part of the pistil self-pollen tubes stop growing (stigma, style or ovary) (Barrett, 2013).

The morphology of reproductive organs in hermaphrodite plants can also affect the probability in occurrence of cross against self-fertilization (Barrett, 2014). The spatial separation of pollen presentation and pollen receipt within a flower, a mechanism known as herkogamy, is pretty common among Amarillydaceae species (Webb and Lloyd, 1986). Two common hypotheses used to explain the function of this condition in angiosperms are: (1) the reduction of self-fertilization and hence promotion of outcrossing; and (2) avoidance of self-interference between the female and male functions (Barrett, 2002; Webb and Lloyd, 1986).

Amaryllidaceae comprises about 73 genera and 1600 species (Stevens, 2015). This family displays three main centers of diversity: South America, Southern Africa and the Mediterranean region (Meerow, 2004). In general, members of Amaryllidaceae have their reproductive systems poorly documented, particularly in the Southern Hemisphere, where most species are located (Kiepiel and Johnson, 2014). The genus *Habranthus* Herb. is part of this group of which we still know little about the reproductive patterns and pollination mechanisms. This happens partly because of its problematic taxonomy, but also due to the ephemeral flowering of the very delicate flowers that usually last just one day. (Dutilh, 2005). To our knowledge, only *Habranthus tubispathus* (L'Hér.) Traub has had its reproductive biology investigated and turned out to be an apomictic and self-compatible species (Brown, 1951; Fernández et al., 2013). Evidence for its preferential outbreeding was suggested because of the higher fruit set by cross-pollinated plants compared to self-pollinated and the presence of herkogamy (Fernández et al., 2013).

There are at least 18 species of *Habranthus* that occur in Brazilian territory (Dutilh and Oliveira, 2015) and, since there are no studies involving their reproductive mechanisms, little is known about their ecology and evolution. This kind of approach is also important because it helps to understand the limits of a species and, therefore, it is helpful for taxonomists too (Stace, 1989). On trying to fulfill this gap on the knowledge of this group, our work unveils the mating system of *Habranthus gracilifolius*, its self-incompatibility system and the significance of herkogamy in this species. Since we evidence the need of pollen vectors for reproductive success of the studied species, we also provide the first records of its flower visitors.

Materials and Methods

This study was carried out in a grassy field of Irmão Teodoro Luís Botanical Garden (31°48'00.5" S; 52°25'06.3" W), municipality of Capão do Leão, Southeast of Rio Grande do Sul state, Brazil. The region is part of the coastal plain of the Pampa biome, where the main vegetational coverage found is characterized by shrubs and herbaceous pioneer formations, typical of the lagoon complex (IBGE, 2004).

Habranthus gracilifolius Herb. is a bulbous herb with leaves that shed months before flowering time and are not produced until after the flowers decay (hysteranthous). The scape is up to 20 cm high and usually has one or two flowers, pale purplish pink colored, scentless, that close at night. The style of the flower is longer than the filaments, which are

arranged in four heights (Herbert, 1824). This species occurs in Uruguay and Argentina (Arroyo, 1990) and in Brazil has only been found in Rio Grande do Sul state (Dutilh and Oliveira, 2015).

We conducted field work during species flowering time in the studied area. Usually, there are two peaks of flowering, one in February and another in March, and both last about just a week (unpublished results). In order to describe the mating system of *H. gracilifolius*, we performed pollination tests in 2013, while the investigation of self-incompatibility and herkogamy was made in 2015. In these two years we also observed flower visitors. The population sampled is distributed over an area of around 12,000 m². It is difficult to average the real size of the population because of its geophyte life form and hysteranthous foliage, but there are at least 300 individuals at the site (Fig. 1A). No evidence of vegetative reproduction underground was found. Botanical material was collected both in fertile and vegetable stages and incorporated into the PEL Herbarium collection of the Department of Botany of Federal University of Pelotas (n° 25402; 25403; 25434; 25435; 25437; 25438; 25439; 25443; 25445; 25454).

For pollination treatments we selected 30 individuals for each treatment of sexual reproductive systems, including a control group, and 20 individuals for asexual reproduction (agamospermy) treatment (total n=140). We chose randomly pre-flowering bulbs that were at least two meters away from each other so we could assume that they were different individuals. The buds were previously bagged to avoid any contact with possible pollinators and, by hand pollination, we performed (1) spontaneous self-pollination, (2) manual self-pollination, (3) cross-pollination and (4) agamospermy. For the latter, buds were emasculated before anthesis. Unbagged floral buds were marked and observed in order to estimate the success of pollination in natural conditions and used as a control group (Dafni, 1992; Dafni et al., 2005).

Fruit set was established per treatment as the ratio of the number of developed fruits/number of flower treated. We applied the chi-square test (χ^2) to investigate whether there were significant differences in the formation of fruits among treatments. We calculated the index of self-incompatibility (ISI) as the ratio of the percentage of fruit set after manual self-pollination and cross-pollination (Bullock, 1985). We also calculated the reproductive efficiency as the ratio of fruit set after natural pollination and cross-pollination (Zapata and Arroyo, 1978).

In order to characterize the nature of the incompatibility system, we observed pollen tube growth. Pistils were self (n=20) and cross-pollinated (n=15) and after 24 and 48 hours of

manual pollination they were collected and fixed in 50% FAA. We adapted Martin's (1959) technique to clear the material (pistils were cleared with NaOH 9N and stoved at 60 °C for 20 mins) and followed the usual method for staining with blue aniline. Then, using a fluorescent microscopy we observed in which portion of the pistils pollen tubes were arrested.

To access the degree of herkogamy, we collected flowers in the field and preserved them in 70% ethanol. Flowers at similar stages (n=22) were photographed against calibrated graph paper and, using ImageJ (Schneider et al., 2012), we measured the distance between stigmatic surface and the higher anthers.



Figure 1 *Habranthus gracilifolius* in the study field Irmão Teodoro Luis Botanical Garden, Rio Grande do Sul, Brazil. A. View of the population mass flowering. B. Flower with a large

distance between stigma and higher anthers (arrow). C. Flower with a short distance between stigma and higher anthers (arrow).

Observations on flower visitors occurred from 7h30 to 14h30 for five days in the first year and eight days in the second one. The weather conditions were not the same during all days. Whenever possible, we captured the visitors using insect nets so we could obtain additional accurate identifications (which were made with the help of specialists). For every visitor observed, we recorded what resource were they looking for (pollen or nectar) and if they contacted the stigmatic area of flowers. We calculated the relative frequency for each family of visitors for the two years, separately, as well as in total.

Results

Our treatments outcomes (Table 1) show that *Habranthus gracilifolius* is a self-incompatible species ($ISI = 0.037$) and pollination treatments were significantly different between themselves ($\chi^2_{0,05} = 124.89$; df: 4, $p < 0.001$). The largest fruiting rates were registered for cross-pollination experiments and under natural conditions and there were no significant differences between these two treatments ($\chi^2_{0,05} = 3.1579$, df: 1, $p = 0.075$). The reproductive efficiency was 1.11.

Table 1 Results of controlled pollination experiments and natural pollination in *Habranthus gracilifolius* Herb. (Amaryllidaceae) at Irmão Teodoro Luis Botanical Garden, Rio Grande do Sul, Brazil. N° Fl. = number of flowers; N° Fr. = number of mature fruits.

Treatment	N° Fl./N° Fr.	% Fruit Set
Spontaneous self-pollination	30/1	3.333
Manual self-pollination	30/0	0
Cross-pollination	30/27	90
Agamospermy	20/0	0
Natural pollination (control)	30/30	100

Pollen tube growth after self-pollination showed that pollen grains germinate in the stigma surface and are arrested at different portions of the style, but mostly at the beginning (Fig. 2A-B). Just in one self-pollinated pistil (24h) it was possible to observe a few pollen tubes reaching the ovules (Fig. 2C-E). In all cross-pollinated pistils pollen tubes penetrated the ovules within 24 hours (Fig. 2F-H).

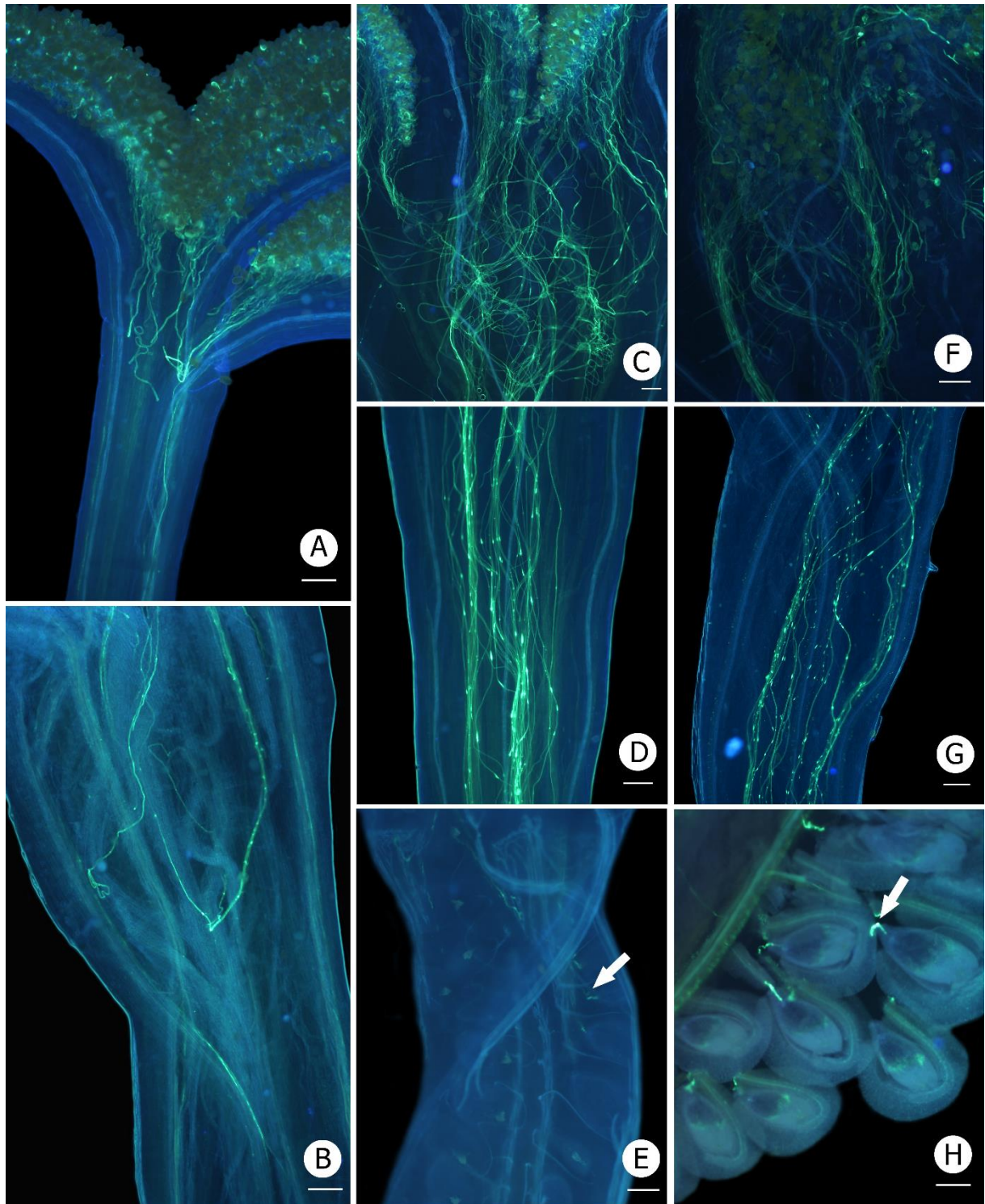


Figure 2 Pollen tube growth in *Habranthus gracilifolius* Herb. (Amaryllidaceae). A-E. After 24h of self-pollination. A. Pollen grains germinate in the stigma and pollen tubes are arrested at the beginning of the style. B. Only a few pollen tubes grow into the medial portion of the style. C-E. The single self-pollinated pistil where pollen tubes reached the ovules. C. Pollen tubes growing in the stigma and D. in the style. E. Pollen tubes reach the firsts ovules of the

ovary (arrow). F-H. After 24h of cross pollination. F. Pollen grains germinate at the stigma, G. grow in the style and H. reach the ovules (arrow). Bar: 200 μm in A-G and 100 μm in H.

Habranthus gracilifolius showed approach herkogamy (Fig. 1B-C). Stigmas are projected beyond the anthers with a degree of 2.13 ± 1.4 mm (mean $\pm$ standard deviation). The minimum value found was 0.82 mm, while maximum was 5.9 mm.

We observed a total of 34 individuals of insects visiting flowers in the first year and 67 in the second (Fig. 3). Syrphidae were the most frequent during both years (43.56% of the visits), followed by Curculionidae (15.84%), Nitidulidae (13.86%), Halictidae (7.93%) and Bombyliidae (4.95%). Individuals of Hesperidae were only registered visiting flowers in 2015, while *Xylocopa augusti* and *Apis mellifera* (the latter was observed just once) were recorded just in 2013. The visitors foraged looking for both nectar and pollen, except bee flies and skippers, which collected only nectar, and hover flies, that were foraging for pollen. Bombyliidae (*Poecilognathus* sp.) and Hesperidae were the only flower visitors that never touched the stigma (Table 2). . In most of the situations here observed, small bees foraged for pollen, however, few individuals entered the flowers only in search of nectar, going straight to the corolla basis and, hence, not touching stigmas or anthers.

Among visitors *Xylocopa augusti* presented the biggest body size (2cm) that seems to fit floral reproductive organs arrangement (herkogamy). Stigma was the first floral part to be contacted by *X. augusti* hairy body, which was covered with pollen from the preceding visit. This carpenter bee performed very quick visits (about four seconds each) and kept visiting other *H. gracilifolius* flowers in sequence.

The hairs in beetles bodies (especially curculionids) enabled them to carry a great amount of pollen grains. They showed an up-and-down movement between lower and higher anthers, so its possible that part of pollen adhered to their body was deposited on the stigma of the same flower.

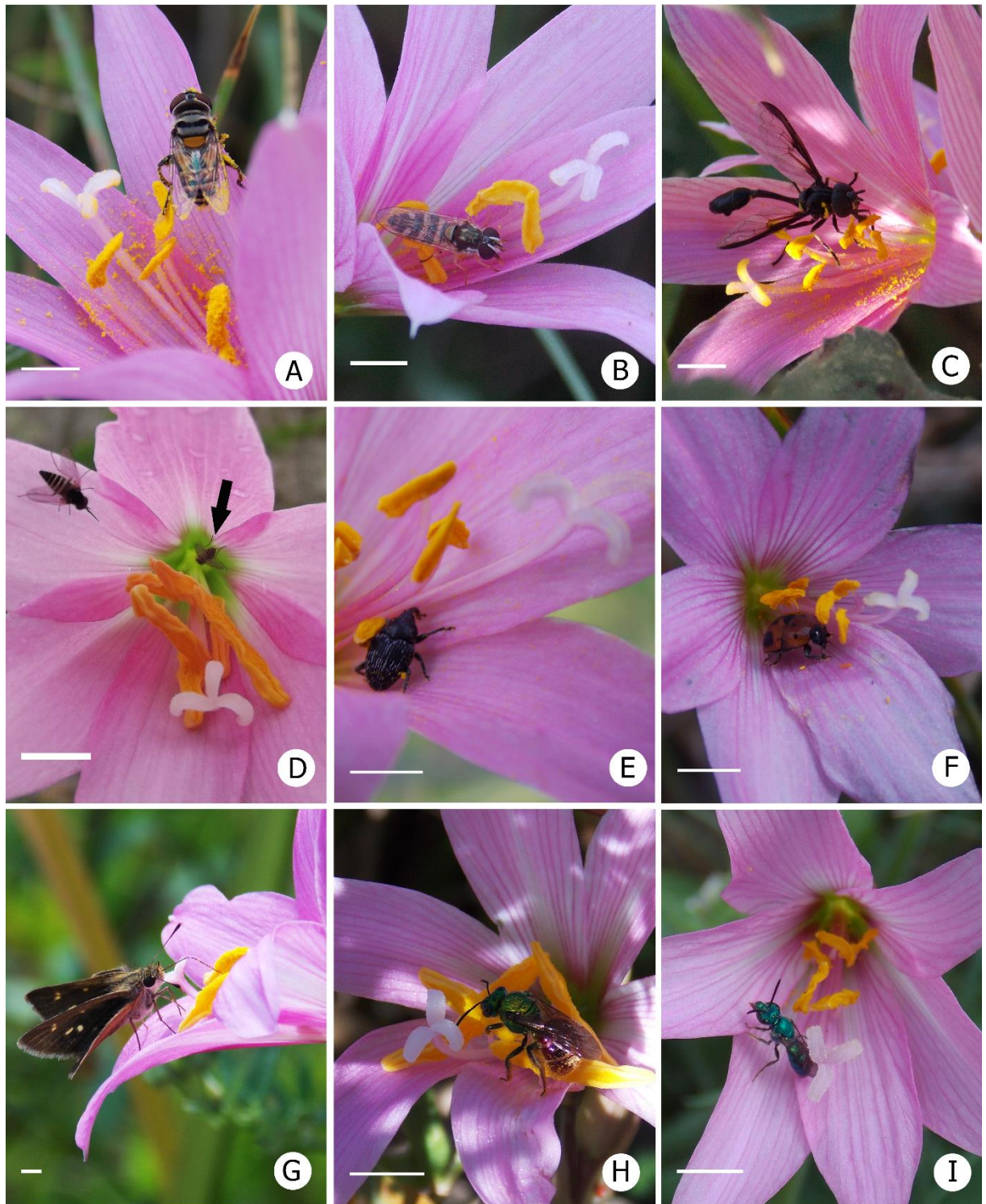


Figure 3 Flower visitors of *Habranthus gracilifolius* at Irmão Teodoro Luis Botanical Garden, Rio Grande do Sul, Brazil. A-C. Syrphidae. A. *Palpada* sp.. B. *Toxomerus* sp.. C. *Pseudodoros clavatus*. D. Two *Poecilognathus* sp. (Bombyliidae). E. Curculionidae. F. *Camptoides* sp. (Nitidulidae). G. HesperIIDae. H-I. Halictidae. H. *Augochloropsis* sp.. I. *Pseudaugochlora* sp.. Bar: 1 cm.

Table 2 Relative frequency of floral visitors of *Habranthus gracilifolius* at the Irmão Teodoro Luis Botanical Garden, Rio Grande do Sul, Brazil, the resource they were collecting (N: nectar and/or P: pollen) and contact with stigma while visiting (X: yes or -: no).

Flower visitors	Relative Frequency (%)			Resource	Stigma touch
	2013	2015	2013 and 2015		
COLEOPTERA					
Curculionidae	17.65	14.93	15.84	N/P	X
Baridinae					
Nitidulidae	11.77	14.93	13.86	N/P	X
<i>Camptoides</i> sp.					
DIPTERA					
Bombyliidae (Bee flies)	5.88	4.48	4.95	N	-
<i>Poecilognathus</i> sp.					
Syrphidae (Hover flies)	47.65	41.79	43.56	P	X
<i>Allograpta neotropica</i>					
<i>Allograpta obliqua</i>					
<i>Palpada agrorum</i>					
<i>Palpada distinguenda</i>					
<i>Pseudodoros clavatus</i>					
<i>Toxomerus basalis</i>					
<i>Toxomerus difficilis</i>					
<i>Toxomerus dispar</i>					
<i>Toxomerus watsoni</i>					
<i>Toxomerus</i> sp.					
HYMENOPTERA					
Apidae	11.76	1.49	4.95	N/P	X
<i>Apis mellifera</i>					
<i>Xylocopa (Neoxylocopa) augusti</i>					
Halictidae (Small bees)	5.88	8.95	7.93	N/P	X
<i>Augochloropsis</i> sp.					
<i>Pseudaugochlora</i> sp.					
LEPIDOPTERA					
Hesperiidae (Skippers)	0	13.43	8.91	N	-
<i>Hylephila</i> cf. <i>phileus</i>					

Discussion

According to the pollination experiments, *Habranthus gracilifolius* is - at least this population - self-incompatible and reproduces only through cross-pollination. So the presence of a pollinator becomes mandatory for the plant to obtain reproductive success. The high reproductive efficiency recorded points out the high efficiency of the pollinators in the studied area (Zapata and Arroyo, 1978). Along with this, since natural fructification (pollinator driven) was very similar to that of cross pollination we can suggest that this population does not experience pollen limitation (Ashman et al., 2004) in the studied period.

In obligate outcrossing plants, just the presence of pollinators is not enough to ensure sexual reproduction, it is also necessary that an individual flowers at the same time as its conspecifics (Augspurger, 1981). Considering that all natural pollinated flowers set fruits, mass flowering seems to be a successful strategy for these species, probably due to the fact that presenting more flowers at the same time might attract more pollinators.

Morran et al. (2009) address that the prevalence of outcrossing in nature appears to be an evolutionary puzzle, given the inherent advantages of self-pollination as, for example, reproductive assurance. Thus, they demonstrated that this prevalence occurs because outcrossing prevents attachment of deleterious mutations and facilitates rapid adaptation in relation to selfing. So, cross-pollination would be, at least conditionally, favored by selection.

Cross-pollination is often forced by a self-incompatibility system that allows a plant individual to recognize and reject their own pollen (Franklin-Tong, 2008). Its main benefit is to avoid inbreeding depression (Porchet and Lande, 2005), while its biggest disadvantage is to limit the ability of an individual to reproduce when there is no available pollen from another plant (Igić and Kohn, 2006). Like many other Amaryllidaceae species (Arroyo et al., 2002; Kiepiel and Johnson, 2014; Navarro et al., 2012; Parolo et al., 2011; Pérez-Barrales et al., 2006; Sage et al., 1999; Vaughton et al., 2010), *H. gracilifolius* is self-incompatible due to the low ISI (<0.25) (Bullock, 1985). The arrest of self-pollen tubes in the style suggests a gametophytic self-incompatibility system. It is a cell-cell recognition system that regulates the acceptance or rejection of pollen landing on the stigma of the same individual, as inhibition occurs only after incompatible pollen tube grows for some distance in the style (Franklin-Tong and Franklin, 2003). In this study, pollen tube walls from self-pollinated flowers were thicker than those from the cross-pollinated ones, indicating that the reaction of incompatibility starts right after pollen grains germination on stigma.

We believe that the formation of a single fruit by self-pollination may be due to some failure in the self-incompatibility reaction. We discarded faults during experiments, because this fruit was set from spontaneous self-pollination treatment, where no handling was performed after anthesis and, therefore, hardly any pollen contamination would be possible. According to this, in one of the self-pollinated pistils in which we could observe pollen tube growth, it was possible to see the arrival of a few pollen grains at the ovules. So, we think it is possible that the incompatibility does not work fully in some cases and, hence, self-fertilization can occur. This may explain the formation of this self-pollinated fruit, but it is a rare event and our pollination tests shows that it is not significant for this population.

Herkogamy is one of the floral characteristics that evolved to prevent the disadvantages of being hermaphrodite (Webb and Lloyd, 1986). Approach herkogamy is by far the most common type and allows pollinators to contact stigmas before their entry into flowers (Barrett, 2003; Endress, 1994). As *Habranthus gracilifolius* is gametophytic self-incompatible, we expected that this species should be already protected from the negative effects of inbreeding by its inability of sexually self-reproducing. Therefore, the main selective force promoting herkogamy in this case should be related to preventing pollen self-interference (Barrett, 2003; Harder and Barrett, 1996; Medrano et al., 2005; Webb and Lloyd, 1986). The major advantage of decreasing sexual interference between the male and female function is that the flower reduces waste of gametes and increases the opportunity to perform cross pollination through a more efficient pollen dispersal (Barrett, 2002; Cesaro et al., 2004).

Habranthus is a paraphyletic group within *Zephyranthes* Herb. (García et al., 2014; Merrow et al., 2000) and this last group has had more investigations of its reproductive biology. Relationships of herkogamy and breeding systems in this genus have indicated that species with long styles are self-incompatible, while those with styles located below anthers are self-compatible (Raina and Khoshoo, 1972). The species with flowers where style and stamens are of equal length can be self-compatible or self-incompatible, but in these cases self-interference can not be avoided (Khoshoo, 1981; Raina and Khoshoo, 1972). We found plants of *H. gracilifolius* for which the distance of style and anthers were agreeing with the long style category proposed by Raina and Khoshoo (1972). But we also found individuals where the approach herkogamy was minimal. It would be interesting to observe if the latter suffer more from pollen self-interference compared to long styled individuals (Navarro et al., 2012). Since this is a self-incompatible species, we would expect a lower fitness in flowers where there is a small degree of herkogamy.

Visitors and pollinators patterns in Amaryllidaceae were most studied in Europe and Africa (Arroyo and Dafni, 1995; Dafni and Werker, 1982; Marques et al., 2007; Navarro et al., 2012; Pérez-Barrales et al., 2006; Vaughton et al., 2010) whereas about Southern American species, little is known. There are a few records for species in the Chilean Andes, that are visited by insects (Hymenoptera, Lepidoptera and Diptera) and hummingbirds (Arroyo et al., 1982; Ladd and Arroyo, 2009) and for some Brazilian *Hippeastrum*, that are also visited by hummingbirds (Buzato et al., 2000; Piratelli, 1997). Regarding *Zephyranthes*, there is just the mention that species are bee visited (Khoshoo, 1981). Even though we did not collect direct information on the legitimate pollinators of *H. gracilifolius*, our data of flower visitors can give us a clue about it. Matches between floral and visitor morphologies seem to be an important aspect to consider when indentifying effective pollinators.

Syrphidae is considered the most significant anthophilous family of Diptera (Larson et al., 2001). Even though they might not be very efficient pollinators as bees, their sheer abundance offset in numbers what they lack in skill (Irshad, 2014). In this sense, we expect that they exert an important impact on pollination of the studied species. Hover flies were also considered main pollen vectors of *Sternbergia clusiana* (Amaryllidaceae) given their high frequency of visits (Dafni and Werker, 1982).

The behavior presented by the beetles was also described for Nitidulidae visits in *Narcissus serotinus* (Marques et al., 2007). But, unlike *H. gracilifolius*, this plant owns a mixed mating system and, hence, is not negatively affected by Coleoptera movements within flowers. Again, *H. gracilifolius* flowers with a major degree of herkogamy may avoid self-pollen deposition by the visitors.

The mismatch morphology between *H. gracilifolius* flowers and bombyliids body size results in nectar thieving (Inouye, 1980). This may also be the case of the halictids that did not touch the stigmas during visits. Yet, since in most of the situations they foraged for pollen too, this increased the likelihood of getting in contact with the female floral parts, with the occurrence of pollination. Disparity also occurs among flowers and skippers, as their long and thin proboscis allow them to reach nectar without touching the stigma and stamens. Skippers have already been reported as nectar thieves in some Amaryllidaceae species (Venables and Barrows, 1985).

Although *Xylocopa augusti* was rarely seen visiting, its morphology and behavior indicates that this insect can be an efficacious pollinator of *H. gracilifolius*. The absence of

more than one individual of *Apis mellifera* in two years of observation is a good indicator that *H. gracilifolius* is important for providing food resource to native pollinators of the studied area.

This study shows that *Habranthus gracilifolius* is an outcrossing species with approach herkogamy and a gametophytic self-incompatibility system. Such findings highlight the need of pollinators for the plants to achieve its reproductive success. In this way we take the first step towards assessing them by showing who are the flowers visitors. For pollinator effectiveness it would be necessary to evaluate the total contribution to plant reproductive success, such as pollinator efficacy and intensity of visitation (Freitas, 2013). Even with only a few studies regarding the reproductive biology of *Habranthus*, data indicate that this is a versatile genus, as its related group *Zephyranthes* (Raina and Khoshoo, 1972), which contains both self-compatible (Fernández et al., 2013) and self-incompatible species.

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CHAPTER 2

Mass flowering triggered by abiotic factors in three species of geophytes in the subtropics

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Abstract

Understanding which biotic and abiotic factors are behind the flowering strategy can help the analysis of phylogenetically related species coexistence patterns. In this study we evaluated which signals trigger the flowering of three Amaryllidaceae species in a subtropical region. *Habranthus tubispathus*, *Zephyranthes mesochloa* and *Habranthus gracilifolius* display a short and massive flowering in the warmer periods of the year and have a high degree of intraspecific synchrony. The flowering of the species is controlled by the combination of the climatic factors such as temperature, photoperiod and precipitation. This multiple bang flowering pattern attracts generalist flower visitors, such as flies, beetles and solitary bees. Temporal overlap in flowering between species did not differ from the expected by chance. This can be explained by the opportunistic behavior of the floral visitors indicating that they do not impose sufficient strong selective pressures on the flowering time.

Keywords: Amaryllidaceae. Competition. Facilitation. Pampa. Simpatry.

Introduction

Time and pattern of flowering are crucial features of the life history of flowering plants as they strongly influence reproductive processes such as pollination and seed dispersal (Johnson, 1992; Kudo, 2006). Several different factors influence the ecology and evolution of flowering phenology, as its patterns can be the result of selective pressures imposed by both biotic interactions and the physical environment (Elzinga et al., 2007; Kudo, 2006; Rathcke & Lacey, 1985; van Schaik et al., 1993).

Several hypothesis have emerged in an attempt to understand the phenological patterns of flowering, especially of sympatric species that are visited by the same pollinators. The *competition hypothesis* predicts that species that segregate temporally the flowering should decrease the interspecific flowering time overlap and reducing the effects of competition for pollinators (Ashton et al., 1988; Levin & Anderson, 1970; Pleasants, 1980; Stiles, 1977; Waser, 1978). On the other hand, the *facilitation hypothesis* predicts that an aggregated flowering pattern of species that are pollinated by the same animals can result in greater attraction of pollinators increasing the fitness of individual plants (Janzen, 1967; Moeller, 2004; Rathcke, 1983; Schemske, 1981).

Competition and facilitation processes, however, are not the only ones shaping the reproductive phenology of species. According to the *phylogenetic hypothesis*, closely related species should exhibit similar phenological patterns by virtue of a recent common ancestor (Kochmer & Handel, 1986; Wright & Calderón, 1995). This latter hypothesis and that of competition generates conflicting predictions about the evolutionary persistence of phylogenetic patterns (Wright & Calderon, 1995). For related species to bloom in similar dates, regardless of their geographical distribution, phylogenetic constraints must be stronger than local selection pressures (e. g. pollinators) (Kochmer & Handel, 1986). Thus, taking into account the shared influence of phylogenetic and ecological factors allows a better understanding of the phenological responses of species (Staggemeier et al., 2010).

Plants of different species occurring in the same place can share phenological patterns as they are under the same climatic conditions. The *climatic hypothesis* foretell that the time of phenological activity is correlated to the variation of abiotic factors (Rathcke & Lacey, 1985). The main climatic driver that stimulate flowering are temperature (Arroyo et al. 1981), precipitation (Opler et al., 1976) and daylength (Wright & van Schaik, 1994). Nonetheless, the role of each signal as a trigger of phenological phenomena can covary with latitude (ter Steege

& Persaud, 1991). In tropical regions with marked seasonality, phenology seems to be controlled more by water availability so that flowering is induced by rain (Borchert, 1994; van Schaik et al., 1993). Moving away from the Equator, daylength and temperature start to vary more during the year and so they tend to influence flowering more in the subtropics (Marchioretto et al., 2007; Marques & Oliveira, 2004; Marques et al., 2004). Abiotic factors may limit the flowering period either directly, affecting the capacity of plants to produce flowers, or indirectly by affecting pollen vectors (Rathcke & Lacey, 1985).

Life form also influence species flowering patterns since morphological and physiological adaptations reflect how water and nutrients are absorbed and used by plants (Sarmiento & Monasterio, 1983; Smith-Ramírez & Armesto, 1994). It is expected that species with the same life form present significantly similar flowering periods, regardless of their phylogenetic relationship (Kochmer & Handel, 1986). The geophytic habit arose in climatic areas with marked seasonal changes where periods of very high or low temperatures and/or water restriction occur (Rees, 1966; de Hiertogh & Le Nard, 1993). When under adverse conditions, plants with bulbs have the advantage of going into dormancy until an external spur signals favorable conditions for resumption of development (de Hiertogh & le Nard, 1993; Fidelis et al., 2009). The flowering process of geophytes involves several stages, from the formation of flower buds within the bulbs until the emission of the scape and flower anthesis. (de Hiertogh & le Nard, 1993). Therefore, each stage can be triggered by different environmental cues (Rees, 1966).

The Amaryllidaceae family, composed by bulbous herbs, display as main centers of diversity the Mediterranean region, southern Africa and South America (Meerow, 2004). *Habranthus* and *Zephyranthes* species, which occur mostly in South America, are commonly known as "rain lilies" due to their tendency to quickly flower after a rainy season (de Hiertogh & le Nard, 1993; Damián-domínguez et al., 2009; Dutilh 2005). Although these genera are accepted taxonomically, morphological (Arroyo & Cutler, 1984) and phylogenetic studies (García et al., 2014) do not support their separation. Evidence of reticulate evolution in the group that *Habranthus* and *Zephyranthes* are part of highlights the importance of hybridization events along their diversification (García et al., 2014). Thus, flowering patterns of species that occur in sympatry have strong evolutionary implications, since they may affect the degree of pre-zygotic reproductive isolation between them. Understanding which biotic and abiotic signals are behind the flowering strategy can help analyze phylogenetically related species coexistence patterns (Frankie et al., 1974).

The species *Habranthus tubispathus*, *H. gracilifolius* and *Zephyranthes mesochloa* co-occur in an area in southern Brazil. As they are phylogenetically close and present the same life form, they are expected be visited by the same floral visitors and to respond similarly to the local climate. Thus, this study examined (1) if they are visited by the same groups of insects; (2) the pattern, synchrony and seasonality of flowering of each species; (3) if segregation or aggregation occur in their flowering times; and (4) which climatic factors trigger the anthesis process of each species.

Materials and methods

Study site

The Irmão Teodoro Luis Botanical Garden (31°48'00.5" S; 52°25'06.3" W and 13m of altitude) is a permanent conservation area located at Capão do Leão, Southeast of Rio Grande do Sul state, Brazil. It consists of a Restinga Forest fragment surrounded by wetlands (marsh) and low vegetation (South Brazilian Campos). It is part of the coastal plain of the Pampa biome, where the vegetation is characterized as shrub-herbaceous pioneer formations typical of a lagoon complex (IBGE, 2004). The study was conducted in a grassland area of Irmão Teodoro Luis Botanical Garden (Fig. 1), where the three species of interest co-occur.

The weather in Capão do Leão region is Cfa - according to Köppen - humid subtropical with defined seasons, warm summers and well distributed rainfall during the year (Moreno, 1961; Alvares et al., 2013). Mean temperatures for the seasons are 22.9 ° C in summer, 16.4°C in the fall, 13.2 ° C in the winter and 19°C in spring. Mean seasonal of rainfall is 333.5mm in summer, 289.7mm in the fall, 356.3mm in winter and 286.1mm in spring (Estação Agroclimatológica de Pelotas, 2015) (Fig. 2).

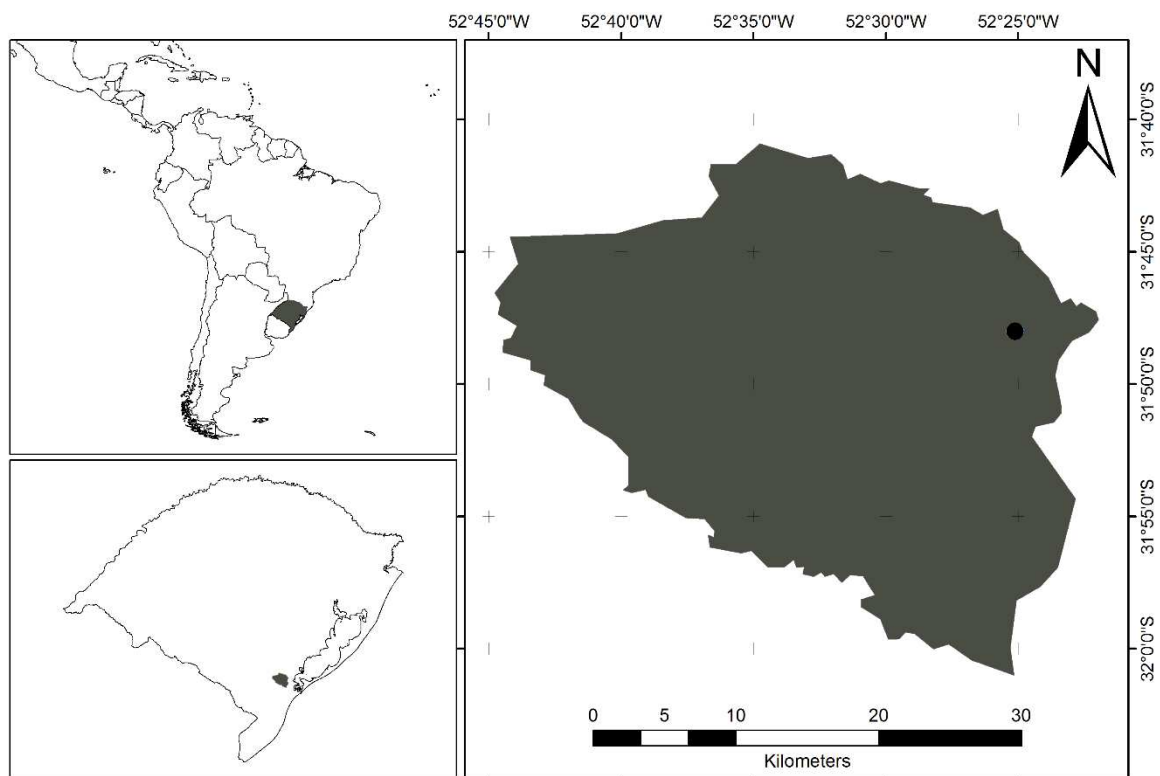


Figure 1 Study area indicated by the black dot at Irmão Teodoro Luis Botanical Garden, Southeast Rio Grande do Sul state, Brazil.

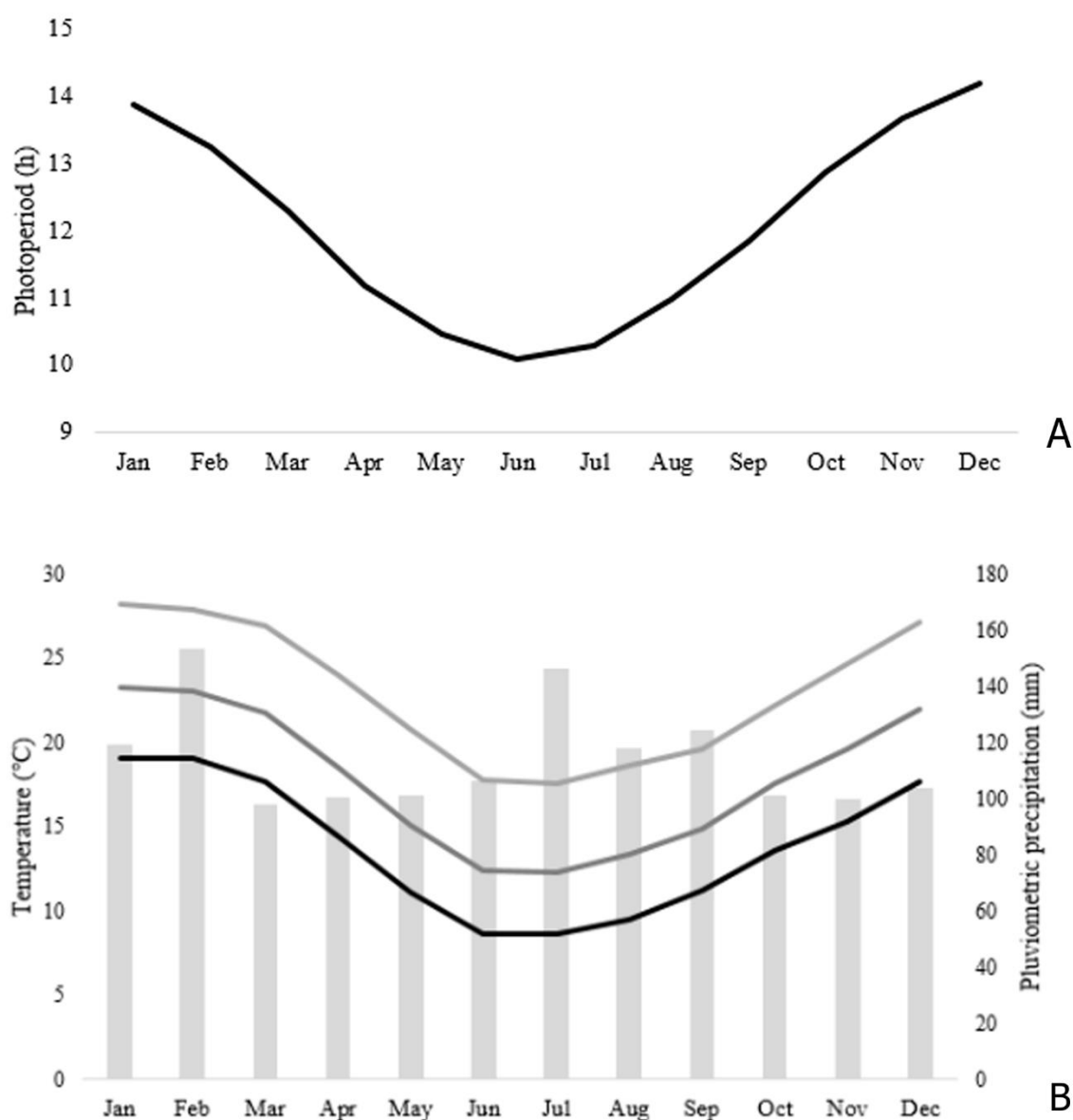


Figure 2 Annual distribution of means of A. photoperiod (h), B. temperature (°C) and precipitation (mm) in the region of Capão do Leão, Rio Grande do Sul, Brazil. In the graph B, the black, dark gray and light gray lines refer to the means of minimum, mean and maximum temperatures, respectively. The bars indicate the monthly mean of rainfall. Means calculated for the period between 1971 and 2000 (Estação Agroclimatológica de Pelotas, 2015).

Studied species

The three species studied, *Habranthus gracilifolius* Herb., *Habranthus tubispathus* (L'Hér.) Traub and *Zephyranthes mesochloa* Herb. ex Lindl. possess some similar floral features like the larger length of the style in relation to the filaments, flowers that close at night and no perceptible odor to human olfaction. Another important characteristic is that these plants lose their leaves months before flowering and only produce them again after the floral senescence (hysteranthous leaves) (Shmida & Dafni, 1989).

Habranthus tubispathus (L'Hér.) Traub is a self-compatible species (Fernández et al., 2013) whose flowers can be orange, yellow or pale pink (Fig. 3A-C) and have a mean of 2.74 cm (CV = coefficient of variation = 0.06) of length. Native in Argentina, Chile, Paraguay, Uruguay and in the state of Rio Grande do Sul in Brazil (Arroyo, 1990; Dutilh & Oliveira, 2015), and supposedly naturalized in the United States of America (Holmes & Wells, 1980).

Habranthus gracilifolius Herb. has flowers that can range in color from pale pink to very intense pink and rare albinos (Fig. 3D-F) (Arroyo, 1990). The size of its flowers is of 3.81 cm on average, but they can be greater or smaller (CV = 0,22). This species occurs in Uruguay, Argentina (Arroyo, 1990) and Brazil in Rio Grande do Sul state (Dutilh & Oliveira, 2015), where it was reported to be self-incompatible (Capítulo 1).

Zephyranthes mesochloa Herb. ex Lindl. has white flowers stained with red on the outside (Fig. 3G-H). On average, the flowers can reach 3.9cm (CV = 0.09) of length. This species occurs in Argentina, Paraguay, Uruguay and in Brazil where it is recorded in Santa Catarina and Rio Grande do Sul states (Dutilh & Oliveira, 2015).

Voucher specimens were deposited in the herbarium collection of the Federal University of Pelotas (PEL n° 25332, 25376 and 25434) and in the University of Campinas (UEC n° 188415, 188416 and 188418).

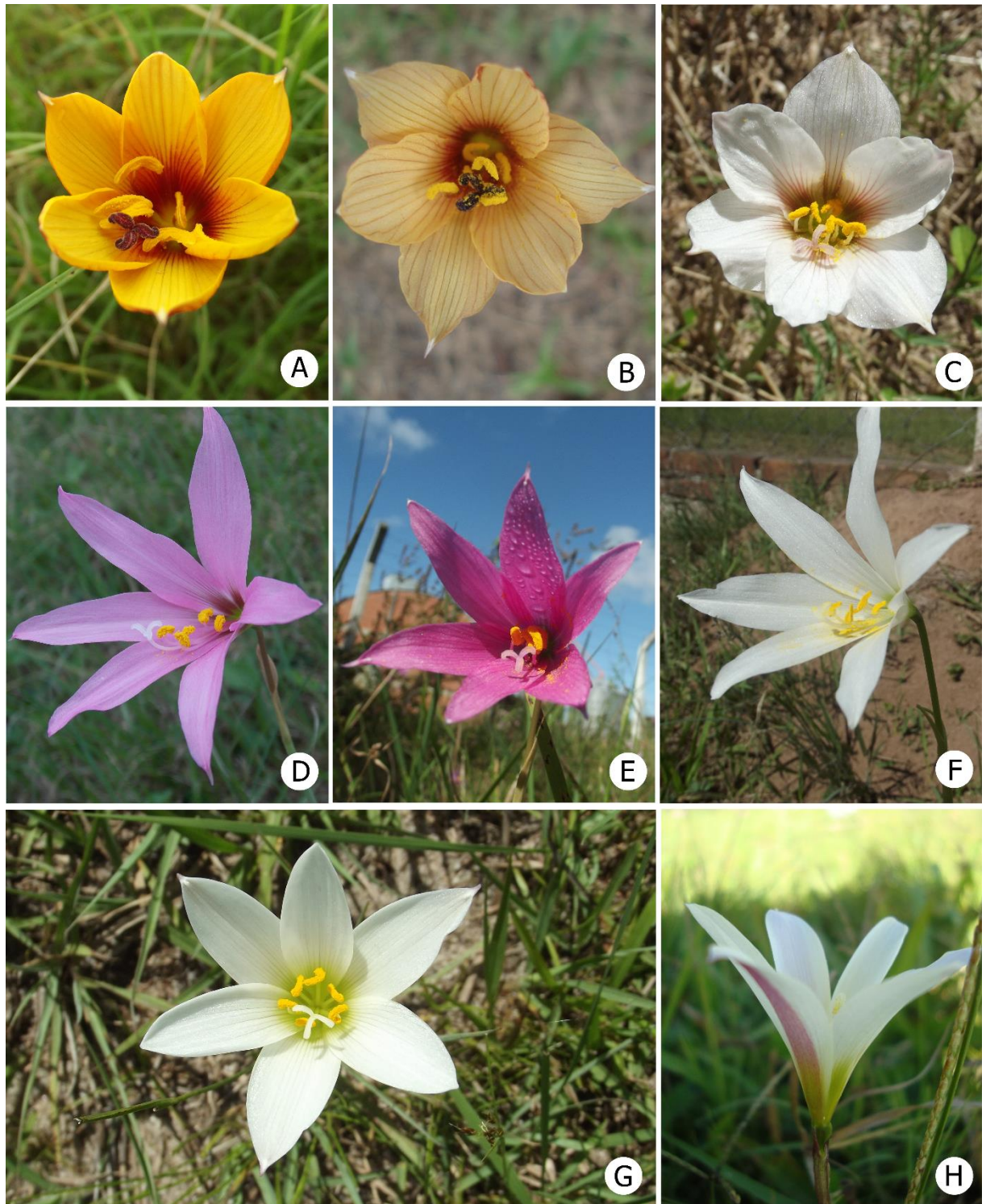


Figure 3 Species studied of Amaryllidaceae in the Southeast of Rio Grande do Sul state, Brazil. A-C. *Habranthus tubispathus*. D-F. *Habranthus gracilifolius*. G-H. *Zephyranthes mesochloa*. Note their morphology and color variations.

Flowering phenology

The study area was monitored from 2010 to 2013 so that we could identify the reproductive period of the three species. Based on these observations, we conducted fieldwork during the flowering period of the species from October 2014 to April 2015. Due to the quick emission of the flower buds and brief lifespan of the flowers, the flowering phenology data was collected daily counting the number of open flowers of each species.

We describe the pattern of flowering according to Gentry (1974). Flowering peak of each species was defined by highest number of individuals with flowers in a day. Due to the nature of the data, we applied circular statistics to test the seasonality and the degree of concentration of flowering of each species. Therefore, we converted days in angles so that one day of the year corresponds approximately 1° (January 1st = 0° or 360°). The mean angle represents the mean date of phenological activity of each species and the Rayleigh test (Zar 2010) was applied to verify if the sampled populations are uniformly distributed around the circle. The length of the mean vector r represents how much the data is concentrated around the estimated mean angle. The value of r ranges from zero (when there is too much dispersion) to one (when all the data are concentrated in the same direction) indicating the degree of reproductive intraspecific synchrony (for details on the methods used for circular statistics see Morellato et al., 2000; Morellato et al., 2010).

Temporal overlap of flowering

Since this study was conducted in the subtropics, with low temperatures which favor frost formation we restricted the flowering overlap analysis for the warmer periods of the year in which at least one individual was flowering (November to March). The amount of temporal overlap of flowering of the three species was estimated pairwise (*H. tubispathus* x *Z. mesochloa*, *H. tubispathus* x *H. gracilifolius* e *Z. mesochloa* x *H. gracilifolius*) via Pianka (Pianka, 1973) and Czechanowski (Feinsinger et al., 1981) indexes. In order to evaluate whether the observed amount of overlap between species was greater (aggregation) or less (segregation) than expected by chance, we used null model analysis using the randomization algorithm Rosario (Castro-Arellano et al., 2010). The significance was determined by comparing the values of randomized overlap with the amount of empirical overlap. These analysis were conducted in the program TimeOverlap (I. Castro-Arellano et al.; available from the authors on request).

Abiotic factors as a trigger of flowering

To evaluate the effect of different environmental factors on the onset of flowering of the studied species we built Generalized Linear Models using binomial distributions in the *bbmle* package (Bolker, 2008) in R (R Development Core Team, 2015). The response variable of interest was the daily occurrence of flowering and so the data abundance of flowers were binarized. To avoid the noise coming from individuals that flowered anomalously for each species we considered flowering events the days where there were at least three open flowers. In general, plants exhibit a delay between the abiotic trigger and the flowering and thus the predictors of the occurrence or not of daily flowering were: 1) immediate precipitation – summing the amount of precipitation of the four previous days, 2) remote precipitation – sum of the amount of precipitation of the fifth, sixth, seventh and eighth previous days, 3) photoperiod of the observed day and 4) accumulated temperature of the previous eight days. The variable "precipitation" came twice in the models selection due to the existence of previous data suggesting that these species flower after rain (de Hiertogh & le Nard, 1993; Damián-Domínguez et al.; 2009, Dutilh 2005). Climatic data of mean precipitation and minimum temperature were obtained by Agrometeorological Station of Pelotas (Estação Agroclimatológica de Pelotas, 2015) located in the municipality of Capão do Leão (31° 52' 00" S; 52° 21' 24" W; 13,24 m), while the photoperiod was obtained from the National Observatory (Observatório Nacional, 2015).

The models built were composed by the following effects: a) full: immediate precipitation, remote precipitation, photoperiod and temperature b) full less rPrec: immediate precipitation, photoperiod and temperature c) full less iPrec: remote precipitation, photoperiod and temperature d) iPrec: immediate precipitation e) rPrec: remote precipitation f) Phot: photoperiod g) Temp: temperature h) iPrecPhot: immediate precipitation and photoperiod i) iPrecTemp: immediate precipitation and temperature j) rPrecPhot: remote precipitation and photoperiod k) rPrecTemp: remote precipitation and temperature l) PhotTemp: photoperiod and temperature m) null model: with just one interceptum. To assess the predictive ability of each model we use the Akaike Information Criterion corrected for small samples (AICc) to compare them and select the most suitable. Since models with $\Delta AICc \leq 2$ exhibit substantial support (Burnham and Anderson 2004) we considered the simplest(s) model(s) among those who had $\Delta AICc \leq 2$.

Flower visitors

We recorded the flower visitors of the plants of interest to check if they are visited by insects that belong to the same functional groups (i. e. family). Observations were carried out from 7:30 to 15:00 for 17 non-consecutive days between December 2014 and March 2015. The sampling effort for each species depended on the number of open flowers and climatic conditions in the days of the observations, since in nine of those 17 days it rained. For each plant species, we calculated the relative frequency of legitimate visits of the insect families.

Results

Flowering phenology

The three species flowered in a massive way (Fig. 4) and had two short flowering peaks each (Fig. 5 and 6). This fact together with the high degree of intraspecific synchrony (vector r) shows that the flowering strategy of these plants follows the "multiple bang" pattern. Mean dates indicate that the sampled populations have a mean direction to flower and the Rayleigh test (Z) proved the seasonality of flowering. (tab. 1).

Table 1 Results of the circular statistics based on the number of flowers per day of the studied species in Southeast Rio Grande do Sul state, Brazil. Peak dates and maximum amount of individuals in flowering (n); Synchrony degree (vector r); Mean date of flowering and Rayleigh test.

Species	1st peak		2nd peak		Vector r	Mean date	Rayleigh test	
	Date	n	Date	n			Z	P
<i>Habranthus tubispathus</i>	12/04/2014	129	12/13/2014	146	0.981	12/10/2014	511.25	< 1E-12
<i>Zephyranthes mesochloa</i>	12/14/2014	1659	01/12/2015	1111	0.967	12/28/2014	4438.72	< 1E-12
<i>Habranthus gracilifolius</i>	02/28/2015	178	03/24/2015	308	0.983	03/17/2015	1507.89	< 1E-12



Figure 4 Mass flowering of the Amaryllidaceae species studied in Southeast Rio Grande do Sul state, Brazil. A. *Habranthus tubispathus*, B. *Zephyranthes mesochloa* and C. *Habranthus gracilifolius*.

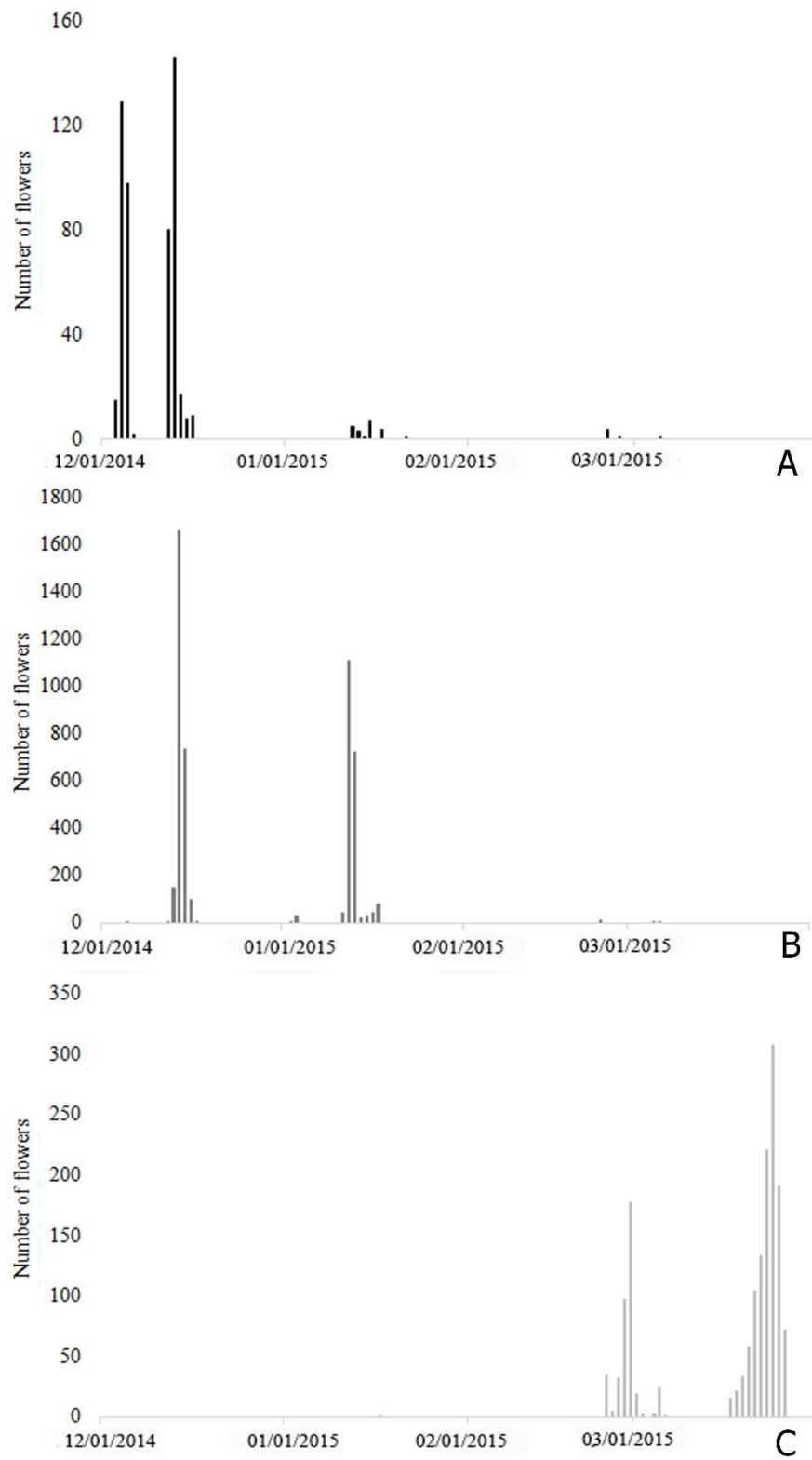


Figure 5 Number of open flowers per day from December 2014 to March 2015. A. *Habranthus tubispatus*, B. *Zephyranthes mesochloa* and C. *Habranthus gracilifolius*.

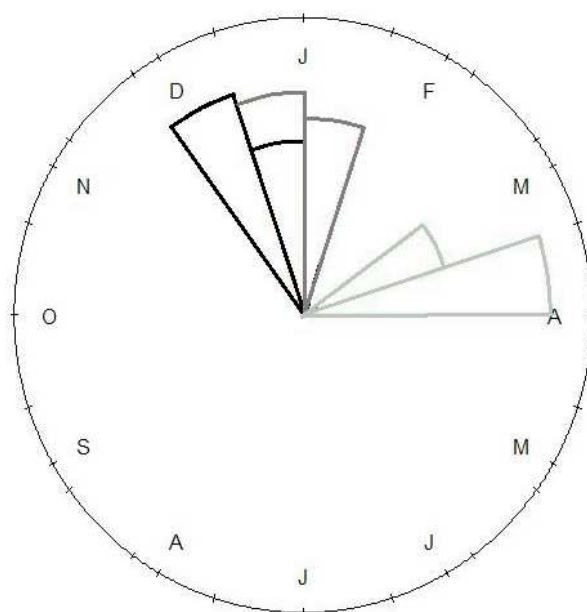


Figure 6 Flowering pattern of the studied species in Southeast Rio Grande do Sul, Brazil. The circle represents a year where the letters represent each month. The two flowering peaks of *Habranthus tubispathus* are represented in black, those of *Zephyranthes mesochloa* in dark gray and of *Habranthus gracilifolius* in light gray.

Temporal overlap of flowering

For all pairs of tested species, the observed amount of flowering overlap was not different than expected by chance. Thus, flowering peaks follow random distributions (tab. 2).

Table 2 Results for the test of flowering overlap pairwise. Means of overlap of Pianka and Czechanowski indexes. *p*-value corresponding to randomization.

Species	Pianka Index		Czechanowski Index	
	Mean overlap	<i>p</i> -value	Mean overlap	<i>p</i> -value
<i>H. tubispathus</i> X <i>Z. mesochloa</i>	0.12316	0.9294	0.12878	0.9239
<i>H. tubispathus</i> X <i>H. gracilifolius</i>	0.00167	0.4883	0.01193	0.5847
<i>Z. mesochloa</i> X <i>H. gracilifolius</i>	0.00038	0.4993	0.00316	0.6142

Abiotic factors as a trigger of flowering

For *Habranthus tubispathus* the model that best explained the probability of flowering was the “full less rPrec”, that includes the variables: immediate precipitation, accumulated temperature and photoperiod. For *Zephyranthes mesochloa*, the model that considers all environmental variables tested was the most suitable, while for *H. gracilifolius* the best model was the “full less iPrec” which includes remote precipitation, temperature and photoperiod (Tab. 3) (Fig. 7).

Table 3 Results of the model selection of the possible variables responsible for triggering flowering of the studied species in southern Brazil. AICc – Akaike Information Criterion; Δ AICc = support of each model. df = degrees of freedom. Weight of each model. The most suitable model for each species is shown in bold.

Model	<i>H. tubispathus</i>				<i>Z. mesochloa</i>				<i>H. gracilifolius</i>			
	AICc	Δ AICc	df	Weight	AICc	Δ AICc	df	Weight	AICc	Δ AICc	df	Weight
full	71.4	0.0	5	0.3233	51.2	0.0	5	0.7908	60.2	0.2	5	0.4405
full less rPrec	71.5	0.0	4	0.3168	57.3	6.2	4	0.1572	68.4	8.4	4	0.0074
full less iPrec	74.0	2.6	4	0.0878	54.4	3.2	4	0.1572	60.0	0.0	4	0.4866
iPrecPhot	73.6	2.2	3	0.1080	73.9	22.7	3	<0.001	70.8	10.8	3	0.0022
PhotTemp	73.9	2.5	3	0.0915	59.9	8.8	3	0.0099	68.4	8.4	3	0.0075
rPrecPhot	74.9	3.5	3	0.0553	62.1	11.0	3	0.0033	64.4	4.4	3	0.0539
Phot	79.4	7.9	2	0.0061	80.8	29.6	2	<0.001	71.1	11.1	2	0.0019
iPrecTemp	79.5	8.1	3	0.0055	65.8	14.6	3	<0.001	107.1	47.1	3	<0.001
iPrec	80.2	8.8	2	0.0039	79.1	28.0	2	<0.001	105.2	45.2	2	<0.001
rPrec	83.5	12.0	2	<0.001	69.1	18.0	2	<0.001	112.3	52.3	2	<0.001
rPrecTemp	83.7	12.2	3	<0.001	63.0	11.8	3	0.0022	114.3	54.2	3	<0.001
Temp	86.4	15.0	2	<0.001	73.0	21.8	2	<0.001	112.3	52.3	2	<0.001
null	89.2	17.7	1	<0.001	89.2	38.0	1	<0.001	110.3	50.3	1	<0.001

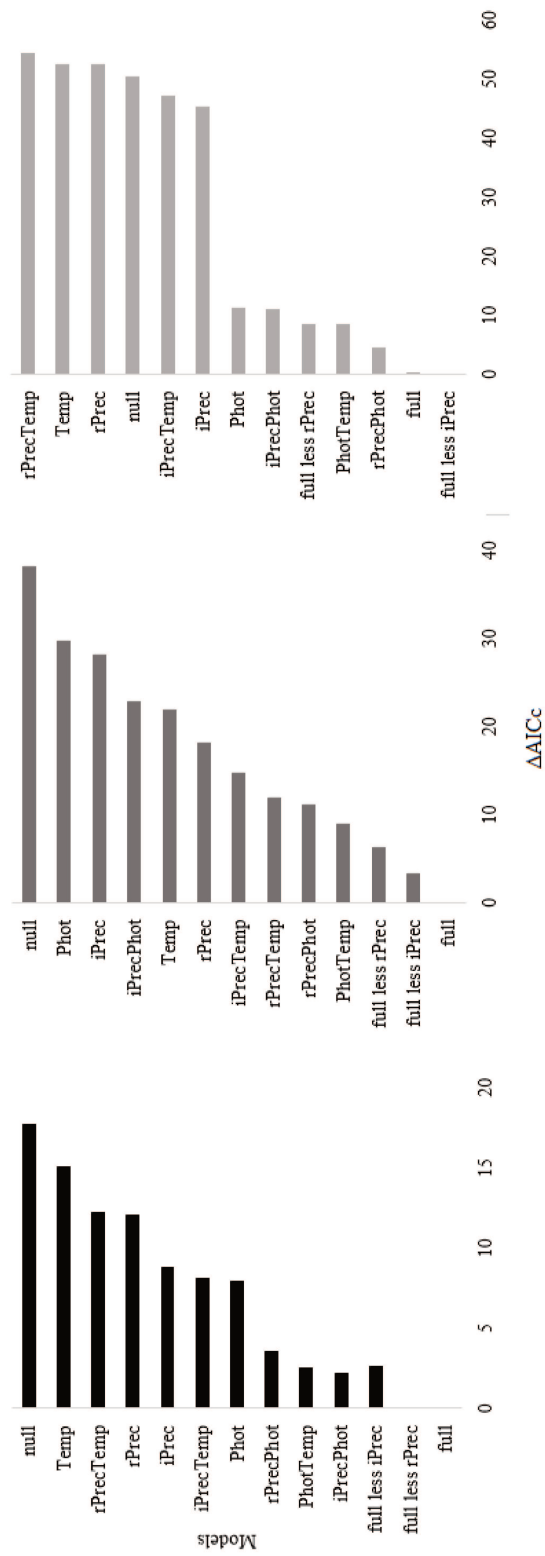


Figure 7 Comparisons of the $\Delta AICc$ of each model. Black bars = *Habranthus tubispathus*; Dark gray bars = *Zephyranthes mesochloa*; Light gray bars = *Habranthus gracilifolius*.

Flower visitors

Habranthus tubispathus was visited by Bombyliidae (66.67%), Syrphidae (28.57%) and Halictidae (4.76%) (Fig. 9A-C), while *Zephyranthes mesochloa* was visited by Nitidulidae (46.15%), Bombyliidae (27.35%), Syrphidae (11.97%), Curculionidae (7.69%), Halictidae (5.12%), Apidae and Hesperidae (0.86% each) (Fig. 9D-F). The flowers of *H. gracilifolius* were visited by Syrphidae (41.79%), Nitidulidae and Curculionidae (14.93% each), Hesperidae (13.43%), Halictidae (8.95%), Hesperidae (13.43%), Bombyliidae (4.48%) and Apidae (1.49%) (Fig. 9G-I). The relative frequency of each family of insects to the flowers of Amaryllidaceae species is shown in figure 8.

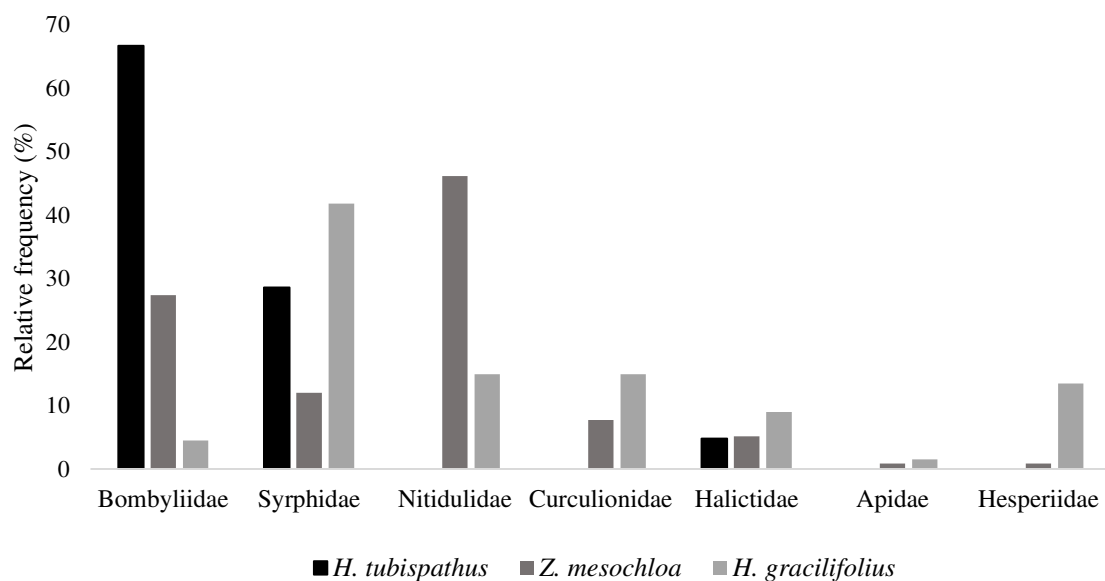


Figure 8 Relative frequency of the floral visitors of *Habranthus tubispathus*, *Zephyranthes mesochloa* and *Habranthus gracilifolius* in the study area, Southern Brazil, in 2014 and 2015.

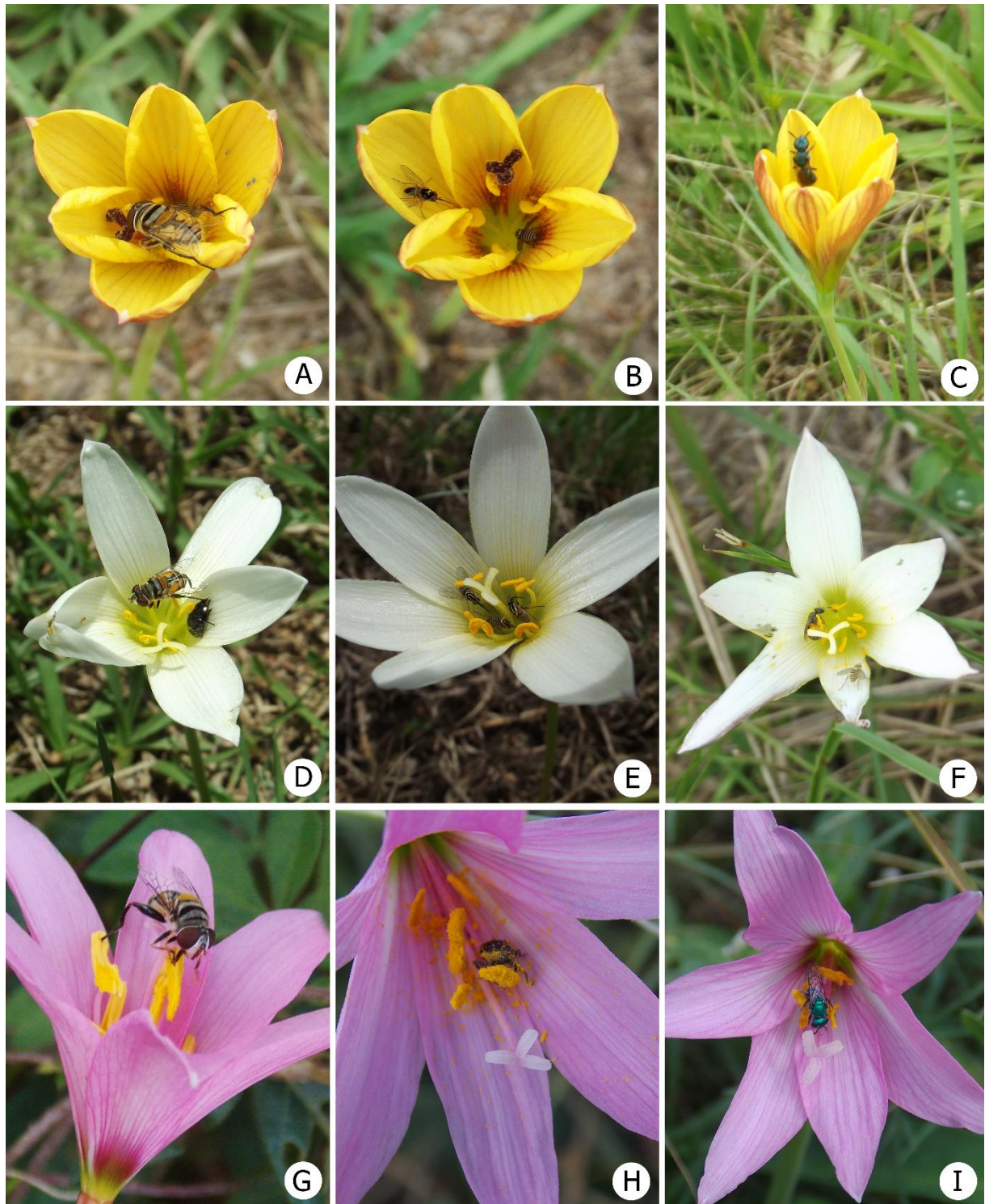


Figure 9 Floral visitors of the studied species in Southeast Rio Grande do Sul state, Brazil. A-C. *Habranthus tubispatus* visitors. A. *Palpada* sp. (Syrphidae). B. *Poecilognathus* sp. (Bombyliidae). C. *Pseudaugochlora* sp. (Halictidae). D-F. *Zephyranthes mesochloa* visitors. D. *Palpada* sp. (Syrphidae) and *Camptoides* sp. (Nitidulidae). E. *Poecilognathus* sp. (Bombyliidae). F. Halictidae and *Poecilognathus* sp. (Bombyliidae). G-I. *Habranthus gracilifolius* visitors. G. *Palpada* sp. (Syrphidae). H. Curculionidae. I. *Pseudaugochlora* sp. (Halictidae).

Discussion

Flowering of the three Amaryllidaceae species studied is seasonal, massive and with a high degree of intraspecific synchrony. This similarity in flowering patterns is in accordance with the expected for phylogenetically related plant species with the same life form. The randomness of the flowering overlap between species suggests that flower visitors do not exert strong pressure on the flowering time. In contrast, flowering is subject to local climatic conditions, so that the presence of flowers is explained by the set of environmental cues tested.

The fact that species flowered in the warmer periods, between the months December and April, explains the sazonality found. The restriction of flowering in the rest of the year probably happens due to the low temperatures and daylength, typical winter phenomena of subtropic regions. In these colder periods, it is expected that the bulbs exhibit low or no activity (de Hiertogh & le Nard, 1993; Fidelis et al. 2009) and therefore do not bloom. Flowering only in the warm season makes sense from the point of view of plants since as the temperature increases, the activity of pollinators also increases, resulting in more frequent visits to the flowers (Kameyama & Kudo, 2009).

Population flowering patterns influence pollinators attraction (Kudo, 2006). High intraspecific synchrony increases floral display and may attract more pollinators since they ordinarily behave in a manner dependent on flower density (Augspurger, 1981; Rathcke & Lacey, 1985). The species studied may not suffer with problems associated with mass flowering, as geitonogamy (Gentry, 1978), because usually each individual emits only one floral scape at a time (with one flower per scape). In this case, by increasing the number of potential reproductive partners the synchrony is probably related to the promotion of cross-pollination (Kudo, 2006). Synchronous flowering encourage visitors to exchange flowers is especially important for *H. gracilifolius* because of its self-incompatible mating system (Chapter 1). This reliance on pollinators that switch flowers may not be so significant in self-compatible species like *H. tubispathus* (Fernández et al., 2013) and *Z. mesochloa* (personal observations). Spatial separation of anthers and stigmas in these species also highlights the need of pollen vectors to effect pollination (Webb & Lloyd, 1986).

Failure to detect processes of competition (segregation) or facilitation (aggregation) in relation to flowering of the three species may be caused by the absence of selective pressure of the biotic agents (e. g. pollinators). The flowers of *Habranthus* and *Zephyranthes* were mostly visited by flies, beetles and small bees. Many members of these groups of insects are

usually seen as generalist foragers that can visit flowers opportunistically (Weiss, 2001). This kind of visit is expected in plants with massive flowering (Gentry, 1974; Frankie et al. 1974). Due to the quick flowering, these plants end up depending on the opportunistic behavior of potential pollinators that eventually quit foraging other species flowers to take advantage of a conspicuous, generous and ephemeral source of resource (Gentry, 1974). Species visited by generalist pollinators are more likely to show this kind of pattern of random flowering (Kudo, 2006).

Usually flowering is a physiological response (Fenner, 1998) to a variety of environmental factors that may interact to determine the start of the breeding season (Rathcke & Lacey, 1985). The selection of models showed that the process of flower anthesis is associated with a set of signals, including temperature, daylength and rainfall. We expected that the best model to explain the flowering were simpler (with less variables) and that the precipitation would be included, as studies mention the reliance on rain to stimulate the output of the floral scapes of the bulbs in these genus (de Hiertogh & le Nard, 1993; Damián-Domínguez et al., 2009; Dutilh, 2005). However, the results indicate that rainfall alone is not sufficient to explain the presence of flowers, as until now was suggested, which may be a reflect of the latitude where this study was conducted. The rain, in fact, seems to play an important role in triggering flowering because for all species the first or second model attempting to explain the phenomenon included one of the precipitation variables. The immediate precipitation seems to have greater weight in the flowering of *H. tubispathus* while for *H. gracilifolius* and *Z. mesochloa* the remote precipitation has a stronger weight. The main factors that seems to be related to the absence of flowering in the colder months are temperature and photoperiod. When we look at these two variables alone they are among the models that explain less the presence of flowers, especially the temperature for *Habranthus* species and photoperiod for *Zephyranthes*. This reinforces the idea that for flowers onset there is the need of combined environmental factors.

The results agree with the idea that climate conditions associated with latitude control the time of flowering of theses species while the pollinators generally not impose consistent selections on flowering period (Munguía-Rosas et al., 2011). The fact that the studied species require a combination of abiotic variables to trigger the flowering process indicates that they can bloom at different times in other places that have different climatic conditions.

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Considerações Finais

- A autoincompatibilidade de *Habranthus gracilifolius* é do tipo gametofítica, o que indica que a separação entre estigma e anteras nas suas flores ajuda a prevenir a autointerferência polínica.
- A presença de diferentes graus de hercogamia nas flores da população que estudamos pode ter reflexos na aptidão dos indivíduos, de modo que flores com menor grau de hercogamia devem sofrer mais com a deposição do próprio pólen no estigma.
- Durante o estudo do sistema de cruzamento de *H. gracilifolius* percebemos que houve variação de visitantes florais entre os dois anos de acompanhamento, o que também pode afetar o sucesso reprodutivo dos indivíduos de ano para ano.
- Com relação aos visitantes florais, *Xylocopa augusti* (Apidae) parece ser o polinizador mais eficiente de *Habranthus gracilifolius*, mas não o mais efetivo.
- A floração sazonal de *Habranthus gracilifolius*, *Habranthus tubispathus* e *Zephyranthes mesochloa* deve se dar pelas baixas temperaturas do inverno, que podem provocar a dormência dos seus bulbos sob essa condição estressante.
- A floração massiva das três espécies é desencadeada pelo conjunto de temperatura, fotoperíodo e precipitação. Entretanto, para *H. tubispathus* a precipitação imediata parece ter um peso maior para estimular a floração, enquanto que para *H. gracilifolius* e *Z. mesochloa* a precipitação remota tem uma influência mais forte.
- Os visitantes florais visitam estas espécies de maneira oportunista, aproveitando a fonte conspícua e efêmera de recurso que é a floração massiva e, portanto, não exercem pressões seletivas consistentes sobre o tempo de floração.

Anexos



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DECLARAÇÃO

Em observância ao §5º do Artigo 1º da Informação CCPG-UNICAMP/001/15, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada ***“Fenologia da floração e biologia reprodutiva em geófitas subtropicais: estudos de caso com espécies simpátricas de Amaryllidaceae”***, desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Profa. Dra. Rachel Meneguello
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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Fenologia da floração e biologia reprodutiva em geófitas subtropicais: estudos de caso com espécies simpátricas de Amaryllidaceae**, não infringem os dispositivos da Lei n.º 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 12 de janeiro de 2016.

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