Biologia Reprodutiva e Sistemas de Polinização de Iridaceae da tribo Tigridieae

Tese de Doutorado

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ORGANIZAÇÃO

Esta tese está estruturada em uma introdução geral, três capítulos referentes aos manuscritos redigidos, um capítulo com considerações finais e material complementar composto por um manuscrito de revisão bibliográfica.

Introdução geral

Capítulo I: The specialization–generalization continuum in oil-bee pollination systems: a case study of six Brazilian species of Tigridieae (Iridaceae).

(Publicado na revista Biological Journal of the Linnean Society).

Capítulo II: Pollination failure in *Tritoniopsis*: a case of breakdown between oil bees and oil- producing flower interaction.

(Será submetido à revista South African Journal of Botany).

Capítulo III: Do pollen and oil-collecting bees differ on pollination performance? The case of three oil-producing Iridaceae in Brazilian Pampa.

(*Será submetido à Revista American Journal of Botany).*

Considerações finais

Material complementar: Elucidating plant-pollinator interactions in South Brazilian grasslands: What do we know and where are we going?

(Submetido à Acta Botanica Brasilica).

SUMÁRIO

Foto: Suiane Oleques

INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

As flores são consideradas estruturas chave na evolução das angiospermas e as variações entre os atributos destas estruturas reprodutivas foram muitas vezes atribuídas como forma de se adaptar aos diferentes tipos de polinizadores. Sendo assim, tanto as variações inter quanto intra-específicas podem ser resultantes, dentre outros fatores, de interações com diferentes vetores em distintos sistemas de polinização (Barret 2011).

Mudanças nas características reprodutivas das plantas podem representar o passo inicial para o isolamento reprodutivo de linhagens e a subsequente especiação, promovendo diversificação de linhagens (Anderson *et al.* 2002; Foxe *et al.* 2009). Tais mudanças podem estar relacionadas a dois traços reprodutivos principais que são altamente diversificados nas Angiospermas: o sistema de polinização e o sistema reprodutivo (Fenster *et al.* 2004). Ambos os sistemas, de polinização e reprodução, são reconhecidos por apresentarem inúmeras transições e frequentemente linhagens de plantas podem apresentar mudanças nos estados de caracteres relacionados a tais funções (Smith *et al.* 2008). Desta forma, repetidas mudanças em características florais e reprodutivas ao longo da evolução de um grupo podem ser a chave para o entendimento do processo de diversificação mediado por adaptação aos polinizadores (Goldblatt e Manning 2006).

No entanto, para um avanço neste campo, é necessário que tenhamos acesso a dados sobre a biologia reprodutiva e ecologia da polinização das espécies vegetais. A maioria dos ecossistemas ainda carecem de estudos sobre polinizadores e tal carência impossibilita não só o acesso a informações valiosas sobre aspectos reprodutivos das espécies, como o entendimento sobre processos ecológicos e evolutivos, tanto de grupos de plantas quanto de polinizadores (Wolowski *et al.* 2016). Atualmente a região Sul do Brasil é considerada uma das regiões com maior carência de estudos sobre sistemas de polinização (Imperatriz-Fonseca *et al*. 2012). Embora haja alguns estudos importantes sobre inventários faunísticos de abelhas nos ecossistemas campestres no Sul do país (Mouga e Neto 2012), os estudos com abordagem sistemática das comunidades ainda são pontuais (Imperatriz-Fonseca *et al.* 2012). Alguns inventários florísticos realizados nos campos sulinos citam a necessidade de estudos sobre aspectos reprodutivos das espécies vegetais para a conservação, porém ainda há uma grande lacuna sobre polinização dos campos (Imperatriz-Fonseca *et al.* 2012). Nesse sentido, um dos capítulos desta tese se deteve em apresentar uma revisão sistemática sobre os estudos relacionados à polinização nos campos sulinos.

1.1. Interações entre plantas produtoras de óleo floral e abelhas especializadas

Em sistemas considerados especializados, como as interações entre espécies de plantas com flores secretoras de óleos e abelhas especializadas na coleta deste recurso, mudanças nas características reprodutivas e florais são potencialmente passíveis de seleção (Fenster *et al.* 2004; Ramírez *et al.* 2011). Outro ponto importante de se salientar é o fato de que sistemas especializados tendem a ser mais suscetíveis a processos estocásticos ambientais. Sendo assim, plantas que apresentam sistema de polinização especializado e que possuem altos índices de limitação polínica tendem, ao longo do tempo, a experimentar uma generalização em termos de polinizadores a fim de diminuir os riscos de extinção (Knight *et al.* 2005). No entanto, para investigar se as variações nas características reprodutivas e morfológicas apresentadas pelas flores são resultado de pressões seletivas exercidas pelos agentes polinizadores, é preciso estabelecer claramente os caracteres reprodutivos a serem analisados. Da mesma forma, necessita-se coletar dados precisos sobre ecologia da polinização e biologia reprodutiva do grupo em questão. Atualmente, o conhecimento evolutivo sobre grupos de plantas que possuem árvores filogenéticas bem resolvidas, aliado ao conhecimento sobre ecologia da polinização, tem nos possibilitado evidenciar quando transições evolutivas nos traços florais e no sistema reprodutivo podem ter ocorrido em resposta a mudanças no mecanismo de polinização (Armbruster 1996; Mitchel 2009, Alcantara e Lohmann 2010, Castañeda-Zaráte *et al.* 2020).

Plantas que possuem variações funcionais e morfológicas nas estruturas da flor responsáveis pela secreção de óleos, e são polinizadas por abelhas especializadas na coleta deste recurso, representam um ótimo sistema de estudo. Além disso, alguns destes grupos vêm sendo alvo tanto de estudos filogenéticos (Renner e Schaefer 2010; Chies *et al.* 2012; Chauveau *et al.* 2011, 2012; Martins *et al.* 2015) quanto sobre biologia da polinização (Sigrist e Sazima 2004; Goldblatt e Manning 2006; Anderson *et al.* 2009; Martins *et al.* 2013).

A produção de óleos florais como recompensa aos polinizadores é uma característica encontrada em apenas 11 famílias de Angiospermas (Renner e Schaefer 2010, Martins *et al.* 2015). Estas plantas apresentam glândulas secretoras de óleo denominadas elaióforos e foram descritas pela primeira vez por Vogel em 1974. No Brasil, as principais famílias de plantas produtoras de óleos florais são: Malpighiaceae Juss., Krameriaceae Dumort., Plantaginaceae Juss., Solanaceae A. Juss., Orchidaceae A. Juss. e Iridaceae Juss. Destas, Malpighiaceae (Sigrist e Sazima 2004; Bezerra *et al.* 2009) e Orchidaceae (Singer *et al.* 1999; Pansarin *et al.* 2009) concentram a maior parte dos estudos sobre biologia da polinização. As variações encontradas na estrutura dos elaióforos, como posição e tamanho, e outras características da flor, estão relacionadas principalmente a adaptações morfológicas e comportamentais das abelhas coletoras de óleo (Cocucci *et al.* 2001; Sigrist e Sazima 2004). Da mesma forma, as espécies de abelhas especializadas na coleta deste recurso (principalmente abelhas da tribo Tapinotaspidini) apresentam uma variedade de adaptações, como cerdas e pêlos em diferentes posições como nas pernas anteriores e/ou médias ou externos das fêmeas (Cocucci et al. 2000).

A produção de óleo em estruturas secretoras especializadas surgiu ao menos 28 vezes de forma independente entre as Angiospermas e foi perdida entre 36-38 vezes (Renner e Shaefer 2010). Entre as monocotiledôneas, nas famílias Iridaceae e Orchidaceae, as estruturas surgiram em vários momentos distintos da história evolutiva. Da mesma forma, a coleta deste recurso surgiu ao menos sete vezes em cinco linhagens distintas e não relacionadas de abelhas (Renner e Shaefer 2010). Sendo assim, ao longo da evolução das Angiospermas, a produção de óleo foi por mais vezes perdida do que adquirida. Este fato tem sido atribuído a duas hipóteses principais: limitação de abelhas coletoras de óleo em algumas regiões geográficas, favorecendo a perda desta característica no momento da diversificação dos grupos ou altos custos envolvidos na produção deste recurso (Renner e Shaefer 2010). Um estudo filogenético realizado com a sub-família Iridoideae (Iridaceae) por Chauveau *et al.* (2012) demonstrou várias transições quanto ao surgimento e perda dos elaióforos. Esta instabilidade na presença/ausência de tricomas glandulares (tanto secretores de óleos quanto néctar) pode estar relacionada a frequentes mudanças nos polinizadores durante a diversificação do grupo, algo bastante comum e estudado em espécies de Iridaceae sul-africanas (Goldblatt e Manning 2006). No entanto, a carência de estudos sobre a biologia da polinização em espécies de Iridaceae americanas, principalmente no Sul do Brasil, faz com que a relação entre essas transições na presença/ausência de tricomas elaióforos e mudanças nos sistemas de polinização sejam uma lacuna no conhecimento deste grupo o tornando um excelente sistema de estudo.

1.2. Iridaceae: diversidade e aspectos evolutivos

A família Iridaceae apresenta cerca de 2.000 espécies distribuídas em aproximadamente 70 gêneros, sendo a África subsaariana a região de maior diversidade com estimativa de 1190 espécies distribuídas em 37 gêneros (Goldblatt *et al.* 2008). Para o Brasil são descritas 190 espécies, distribuídas em 23 gêneros, todas elas pertencentes à subfamília Iridoideae (Eggers *et al.* 2020). Iridoideae é uma das subfamílias mais diversas e representa cerca de 40% do total de espécies de Iridaceae, sendo dividida em cinco tribos: Diplarreneae, Irideae, Sisyrinchieae, Trimezieae e Tigridieae (Goldblatt e Manning 2006). Dentre estas, Tigridieae, Sisyrinchieae e Trimezieae são as mais representativas no Sul do Brasil. Atualmente são descritas 106 espécies para a região Sul do país, sendo que destas, aproximadamente 70 ocorrem no estado do Rio Grande do Sul (Eggers *et al.* 2020. Em termos de diversidade e abundância, os gêneros mais importantes encontrados no Sul do país são *Sisyrinchium* L. *Calydorea* Herb., *Cypella Her*b. e *Herbertia* Sweet (Chies *et al.* 2012).

Com número de gêneros e espécies incertos, a tribo Tigridieae é considerada taxonomicamente complexa por consequência da ampla variação morfológica e de suas flores efêmeras. Atualmente são considerados de 15 a 20 gêneros e 172 espécies, sendo estes filogeneticamente organizados em dois clados: **A** (*Calydorea*, *Catila* Ravenna, *Cipura* Aubl., *Cypella*, *Herbertia*, *Nemastylis* Nutt., *Larentia* Klatt, *Kelissa* Ravenna e *Onira* Ravenna) e **B** (gêneros restantes da subtribo Cipurineae e todos da subtribo Tigridiinae) (Chauveau *et al.* 2012). O clado A possui maior representatividade de espécies ocorrentes no Sul do Brasil, sendo os principais gêneros: *Cypella* - 21 spp., *Calydorea* – 11 spp., *Herbertia* – 7 spp., *Kelissa* – 1 espécie e *Onira* – 1sp (Eggers *et al.* 2020). O clado A de Tigridieae é filogeneticamente bem suportado, no entanto, de acordo com a filogenia proposta por Chauveau *et al.* (2012), existem algumas inconsistências na circunscrição dos gêneros *Calydorea* e *Cypella*, pois as espécies pertencentes a estes gêneros não formam um grupo monofilético. A espécie *Cypella hauthalli* (Kuntze) R.C.Foster, por exemplo, forma um clado fortemente suportado juntamente com espécies de dois outros gêneros: *Kelissa brasiliensis* (Baker) Ravenna e *Onira unguiculata* (Baker) Ravenna. O gênero *Herbertia* é moderadamente suportado e apresenta espécies agrupadas com linhagens dos gêneros *Calydorea* e *Cypella* (Chauveau *et al.* 2012). Em relação à evolução de caracteres florais ocorreram ao menos quatro transições entre ausência e presença de elaióforos nas espécies da tribo, sendo a ausência dos mesmos o estado ancestral para ambos os clados (Chauveau *et al*. 2012).

As espécies da tribo Tigridieae apresentam variações em atributos florais, como cor, forma, tipo de recurso ofertado e morfologia das estruturas reprodutivas. Tais características podem ser atribuídas principalmente à adaptação aos polinizadores (Goldblatt e Manning 2006). Porém, poucas espécies são conhecidas em relação à biologia reprodutiva, sendo esta característica importante tanto para estudos genéticos e populacionais, quanto para estudos sobre biologia da polinização. As principais espécies que vêm sendo estudadas no Sul do Brasil são pertencentes aos gêneros *Cypella* (Pastori 2014) e *Herbertia* (Stiehl-Alves *et al.* 2016, 2017, Alencar *et al.* 2017), evidenciando importantes variações morfológicas e citogenéticas (Chies *et al.* 2012).

Entender aspectos da biologia reprodutiva e da ecologia da polinização é importante para a compreensão dos fatores que influenciaram na diversificação das espécies. Além disso, estudos sobre biologia da polinização são essenciais para a compreensão de aspectos populacionais e de vulnerabilidade de determinadas espécies.

1.3. Interações planta-polinizadores na família Iridaceae

Apenas na África Subsaariana, são conhecidos 17 tipos diferentes de sistemas de polinização para espécies da família Iridaceae (Goldblatt e Manning 2008). A família Iridaceae é considerada uma das maiores em termos de número de espécies na região Sul da África do Sul. Além da grande diversidade de espécies, esta família é conhecida pela espetacular diversidade de tipos florais (Johnson 2010). Dentre os distintos sistemas de polinização, muitos são exclusivos de espécies Sul-Africanas, como o caso de espécies polinizadas por moscas de língua longa (long-tongued flies) e espécies polinizadas por aves (Sun Birds) (Goldblatt *et al.* 1999; Goldblatt e Manning 2000). Na região Sul da África do Sul, Iridaceae é considerada uma das famílias com maior número de espécies polinizadas por apenas um agente polinizador, o que ressalta o alto nível de especialização nos sistemas de polinização das espécies (Jonhson 2010). Estudo feito por Goldblatt e Manning (2006) demonstrou que apenas 2% das espécies de Iridaceae Sul-Africanas possuem sistema de polinização generalistas e 95% são consideradas altamente especializadas. Dentre os recursos florais ofertados pelas espécies de Iridaceae, pólen e néctar são os mais comuns, sendo o óleo o terceiro tipo de recurso mais importante dentro da família (Chauveau *et al*. 2012). Porém, *Tritoniopsis parvilfora* (Jacq.) G.J.Lewis, uma espécie sul-africana, oferta ambos, néctar e óleo (Manning e Goldblatt 2002). Oferta de óleo e néctar como recursos florais em uma mesma flor é considerado algo raro nas angiospermas (Ferreiro *et al.* 2017). Os mecanismos evolutivos envolvidos na oferta de ambos os recursos ainda são pouco conhecidos e isso torna este sistema particularmente interessante, principalmente pelo fato de *Tritoniopsis parviflora* ser a única espécie que oferta óleo floral dentre todas as espécies de Iridaceae não americanas. Neste sentido, um dos capítulos desta tese teve por objetivo descrever a anatomia floral e das estruturas secretoras de *T. parviflora*, além de elucidar alguns aspectos importantes da sua biologia reprodutiva.

O vasto número de estudos sobre biologia reprodutiva e ecologia da polinização de espécies de Iridaceae Sul-Africanas vêm permitindo uma compreensão cada vez melhor sobre a evolução e diversificação da família no continente africano (Goldblatt *et al.* 2008; Johnson 2010; Johnson *et al*. 2017). No entanto, existe um baixo número de estudos sobre os sistemas de polinização de espécies de Iridaceae nas Américas, principalmente no que se refere à América do Sul. Desta forma, ainda há a necessidade de um grande esforço em estudos de biologia reprodutiva e ecologia da polinização de espécies de Iridaceae do novo mundo.

Dentre os principais sistemas de polinização conhecidos nas Américas estão as espécies polinizadas por abelhas coletoras de óleo (Cocucci 2000). No entanto, a determinação do sistema de polinização de grande parte destas espécies se dá apenas através da identificação da presença de estruturas secretoras de óleo (Chauveau *et al.* 2012). Poucas espécies foram efetivamente observadas em campo ou foram investigadas em relação à biologia floral, biologia reprodutiva ou ecologia da polinização.

2. Objetivos gerais

Os objetivos gerais dessa tese foram responder as seguintes questões:

Quais são os principais polinizadores das espécies alvo do estudo e como estes variam em relação à frequência de visitas, recurso coletado e comportamento?

O primeiro passo para a compreensão do papel dos polinizadores na diversificação de um grupo de plantas é identificá-los. Por isso, a primeira etapa desta tese consistiu em observar quais os visitantes florais de cada espécie, quais deles são polinizadores efetivos e como o comportamento dos mesmos pode variar.

Como se relacionam a morfologia floral, comportamento dos polinizadores, biologia reprodutiva e sistemas de polinização nas espécies estudadas?

Buscamos compreender como as diferenças morfológicas entre as espécies, bem como as diferenças na presença e ausência de estruturas secretoras podem determinar quais são os polinizadores efetivos de cada espécie e o comportamento dos mesmos. Da mesma forma, descrevemos o sistema reprodutivo de espécies selecionadas e as taxas de frutificação.

Como cada grupo de polinizador contribui em termos de deposição de pólen nos estigmas das espécies visitadas?

Para responder esta pergunta, investigamos a contribuição de uma única visita na deposição de pólen nos estigmas, uma vez que este dado pode ser usado como forma de estimar a eficiência dos polinizadores.

2.1. Objetivos específicos

- Observar e identificar o conjunto de possíveis polinizadores de cada espécie e caracterizar o comportamento dos mesmos;
- Descrever a biologia reprodutiva e os sistemas de polinização de espécies chave representantes da Tribo Tigridieae ocorrentes no Rio Grande do Sul;
- Compreender como os principais atributos florais importantes para biologia da polinização, como posicionamento das estruturas secretoras de recurso, tipo de recurso ofertado e fenologia e associam-se com aspectos da biologia reprodutiva, dos sistemas de polinização e eficiência de abelhas coletoras de pólen e óleo;
- Investigar aspectos da anatomia floral e da biologia reprodutiva da espécie Sul-Africana *Tritoniopsis parviflora*, a única espécie produtora de óleo floral fora das Américas.
- Por último, no material suplementar apresentamos uma revisão sistemática sobre sistemas de polinização dos campos sulinos.

THE SPECIALIZATION–GENERALIZATION CONTINUUM IN OIL-BEE POLLINATION SYSTEMS: A CASE STUDY OF SIX BRAZILIAN SPECIES OF TIGRIDIEAE (IRIDACEAE).

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The specialization–generalization continuum in oil-bee pollination systems: a case study of six Brazilian species of Tigridieae (Iridaceae)

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Several South American species of Iridaceae, especially those of Tigridieae, produce floral oils as rewards to oil-bee pollinators. The present study aimed to contribute to a deeper understanding of the reproductive biology, pollination ecology and level of specialization of the interactions of species encompassed in Tigridieae. Data on breeding and pollination systems were acquired from six species native to Southern Brazil. The visitation frequency and pollen load of pollen- and oil-collecting bees were also investigated. The results strongly suggest that the studied species are distributed along a specialization–generalization continuum. Three oil-producing taxa, *Cypella herbertii*, *Cypella pusilla* and *Cypella amplimaculata*, were pollinated effectively by oil-bees, whereas in the other two studied species, *Kelissa brasiliensis* and *Herbertia pulchella*, the oil-bees appeared to function as oil thieves, owing to failure to contact the plant reproductive parts during oil-foraging behaviour. New insights into aspects of the specialization–generalization continuum of pollination systems, differences in pollinator behaviour during oil and pollen foraging, and reproductive outputs of the studied species are provided. Taken together, our results provide a significant contribution towards a better understanding of reproductive biology and plant–pollinator interactions between Iridaceae and oil-collecting bees.

ADDITIONAL KEYWORDS: elaiophores – Iridoideae – oil-collecting bees – oil-producing flowers – pollination system – specialization.

INTRODUCTION

The production of floral oils as a reward to pollinators has been reported for 11 angiosperm families and was more often lost than acquired during the evolution of angiosperms (Renner & Schaefer, 2010; Martins *et al.*, 2015). Among these plant families, Iridaceae is considered one of the most species rich in the Western Hemisphere. It encompasses ~2030 species among 65–75 genera worldwide (Goldblatt *et al.*, 2008), and shifts in pollination system are frequently associated with diversification in this florally diverse group. Floral oils are the third type of reward collected by pollinators in Iridaceae, besides pollen and nectar. The 1190 species from South African Iridaceae exhibit a wide range of pollination systems; however, epithelial elaiophores and pollination by oil-collecting bees have been recorded for only a single species, *Tritoniopsis parviflora* (Jacq.)

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G.J. Lewis, in Crocoideae (Manning & Goldblatt 2002). In contrast, floral oil secretion and pollination by oil-collecting bees occur within numerous South American species of Iridoideae. Brazil harbours 204 species of Iridaceae, all of the subfamily Iridoideae. These species are divided into three tribes, Sisyrinchieae, Tigridieae and Trimezieae, with the first two being the most species-rich Iridaceae in South Brazil (Souza-Chies *et al.*, 2012; Eggers *et al.*, 2015). A recent phylogenetic study showed that the distribution of oil-producing trichomes among species of Iridoideae evolved repeatedly in association with the pollination system, which suggests that these glandular structures might have played a significant role in the diversification of the subfamily in the Western Hemisphere (Chauveau *et al.*, 2012).

Tigridieae currently includes 172 species distributed among 15–20 genera, which are divided into two main clades that are formally named Clade A and Clade B (Chauveau *et al.*, 2012). Most members of Clade A occur in South Brazil, where they are distributed among the following predominant genera: *Cypella* Herb. (20 spp.), *Calydorea* Herb. (12 spp.) and *Herbertia* Sweet (seven spp.) (Eggers *et al.*, 2015).

Species of Tigridieae are separated from each other by different combinations of floral attributes, the diversity of which is presumably driven by distinct functional groups of pollinators (Goldblatt & Manning, 2006); however, this has never been tested empirically, and the reproductive biology and pollination ecology of most of these species are still poorly known. A particularly intriguing aspect of floral diversity in Tigridieae is the variation in the nature and presentation of floral rewards. Chauveau *et al.* (2012) documented two types of pollinator resources for species of Clade A in Southern Brazil: flowers offering only pollen (*Calydorea*) and flowers offering pollen and oil (*Cypella*, *Herbertia*, *Kelissa* Ravenna and *Onira* Ravenna), with various arrangements of oil-producing trichomes occurring among these genera.

Lability of traits related to reproductive organs during evolution is often considered a major driver for lineage diversification and is attributed to adaptation to different pollinators (Anderson *et al.*, 2002; Foxe *et al.*, 2009; Barret, 2013). Two essential trends in plant reproduction have occurred during the evolution of angiosperm breeding systems: the transition from selfing to outcrossing, and the evolution of animal pollination (Fenster & Martén-Rodríguez, 2007; Barrett, 2010; Armbruster, 2014). Different pollinators are expected to act as agents of divergent selection pressures on floral traits. Thus, knowledge of the foraging behaviour, the morphological matching between plant and pollinators and the plant fitness are crucial to understand the role of shifts in pollination systems in evolutionary transitions related to floral rewards.

In addition to the evidence that plant–pollinator interactions can strongly influence evolutionary processes, these interactions also provide essential ecosystem services by enabling the reproduction of flowering plants (Thébault & Fontaine, 2010; Wolowski *et al.*, 2016). Moreover, studies of pollination biology are crucial to estimate population dynamics and the vulnerability of species, especially in highly threatened and poorly studied environments, such as the grasslands of South Brazil, where Iridaceae is considered to be one of the most species-rich families (Overbeck *et al.*, 2007).

The aim of the present study was to investigate the reproductive biology and pollination ecology of seven taxa belonging to Clade A of Tigridieae, which represent the different arrangements of pollination rewards observed in this clade. More specifically, our aims were as follows: (1) to identify pollinators and characterize their foraging behaviour; (2) to characterize differences in breeding system to assess self-compatibility and the extent of reliance on pollinators for reproduction; and (3) to use visitation frequencies and pollen loads of flower visitors to determine pollination systems and the associated degree of specialization for the seven studied taxa.

Given that the species studied present two combinations of resources, oil + pollen and only pollen, our hypothesis about pollination system was that *Calydorea alba* Roitman & J.A.Castillo would be pollinated exclusively by pollen-collecting bees, whereas the *Cypella* spp., *Herbertia pulchella* Sweet and *Kelissa brasiliensis* (Baker) Ravenna would be pollinated mainly by oil-collecting bees, with pollen-collecting bees acting, to different extents, as less important pollinators. Unlike *Cypella* spp. and *K. brasiliensis*, *H. pulchella* offers oil in both inner and outer tepals; therefore, we hypothesized that *H. pulchella* would be visited more by oil-bees and, as a consequence, more dependent on oil-bee pollination. Concerning the breeding system, our hypothesis was that taxa with more specialized pollination systems, i.e. more dependent on oil-bee pollination, would present higher indexes of self-compatibility. This is based on other selective forces, because an increase of pollen limitation in the absence of specialized pollinators, then high outcrossing rates could be considered, contradicting the notion that specialization reflects only selective pressures to increase outcrossing rates (Fenster & Martén-Rodríguez, 2007).

MATERIAL AND METHODS

PLANT SPECIES AND STUDY AREA

The seven taxa studied for pollination and breeding system were *Cypella herbertii* subsp. *herbertii* (Lindl.)

Herb, *Cypella herbertii* subsp. *brevicristata* Ravenna, *Cypella amplimaculata* Chauveau & L. Eggers, *Cypella pusilla* (Link & Otto) Benth. & Hook.f.ex B.D. Jacks, *K. brasiliensis*, *H. pulchella* and *Calydorea alba*. The species *Cypella pusilla* and *K. brasiliensis*, both endemic to the State of Rio Grande do Sul (Brazil), are considered Critically Endangered and Vulnerable, respectively, according to the Red List of threatened species of the State of Rio Grande do Sul (SEMA, 2014). Vouchers of all plant populations studied were deposited in institutional herbaria, and information is provided in the Supporting Information (Appendix S1).

These taxa were chosen in order to sample the different combinations of pollination rewards and the different locations of trichomatic elaiophores observed among taxa within Clade A of Tigridieae. Specifically, the following combinations of floral reward and location of secretory structures were considered: elaiophores located on inner tepals (taxa of *Cypella* and *K. brasiliensis*); floral oil secreted from both inner and outer tepals (*H. pulchella*); and pollen as the only reward available (*Calydorea alba*) (Chauveau *et al.*, 2012). The studied taxa bloom mainly from spring to early summer, except for *Cypella pusilla*, which presents a second flowering period in March and April. Most species usually have synflorescences that expose one open flower at a time, with the exception of *Cypella herbertii* and *Cypella amplimaculata*, which could eventually expose more than one open flower at the same time.

Field observations were conducted in the state of Rio Grande do Sul, Brazil, and mainly in the Serra do Sudeste region in the South Brazilian Campos (Pampa biome, grasslands) (Schlindwein, 1998; Overbeck *et al.*, 2007). This region has an average elevation of 568 m (Supporting Information, Appendix S2), was formed mostly during the Precambrian and is characterized by a mosaic of native forest and rocky grasslands (Rambo, 1956). The area is of high conservation interest owing to the beauty of the original landscape and the endemicity of much of its flora (MMA, 2007; Overbeck *et al.*, 2007). Furthermore, the region is distinguished by a high representation of oil-producing species of Iridaceae (Schlindwein, 1998; Chauveau *et al.*, 2012; Eggers *et al.*, 2015).

FLORAL VISITORS AND BREEDING SYSTEM

The study was conducted in 2016 and 2017 from September to April, in order to encompass the entire flowering season of the different plant species, and further observations were achieved in April 2018. Floral visitors were monitored on sunny days, from the time of anthesis to closure, during periods of 15–30 min throughout the entire flowering season (approximately 3 months). Plant–insect interactions were studied methodically in two geographically distinct populations per plant taxon (Supporting Information, Appendices S1 and S2). Multiple randomly chosen flowers were surveyed in each population, and the total number of visits, in addition to the type and behaviour of visitor, were consistently recorded. Each taxon was observed for ≥ 20 h (21.0 ± 3.40 h per plant taxon, mean ± SD) during the flowering season, with the exception of *Calydorea alba*, which was observed for only 15 h, considering that the flower opening time for this species was considerably shorter (from 07.00 to 10.30 h). Floral visitors were divided into two functional groups: pollen-collecting bees and oil-collecting bees. Visitation frequency was calculated as the number of visits per minute of observation. All bee species collected were identified by a specialist using taxonomic keys, and specimen vouchers were deposited at the Museu de Zoologia (Universidade de São Paulo, Brazil).

The breeding system was determined by controlled pollination experiments, including hand cross-pollination (hand pollination with pollen from another plant); hand self-pollination (hand pollination with pollen from the same individual, usually the same flower because the majority of taxa presented one flower per individual); spontaneous self-pollination (flowers were maintained bagged, without further treatment); emasculation (anthers were removed, and flowers were bagged to verify fruit formation), which was conducted only for self-compatible taxa; and natural pollination (flowers were not bagged and were exposed to pollinators) (Dafni, 2005). We calculated the index of self-incompatibility (ISI) and ranked it into three states: (1) self-incompatible, ISI ≥ 0.8; (2) partly self-incompatible, 0.2 < ISI < 0.8; and (3) self-compatible, ISI ≤ 0.2 (Raduski *et al.*, 2012). To calculate the ISI, we used the following formula, as proposed by Lloyd (1965):

$$\mathrm{ISI} = 1 - \frac{\text{fruit set from hand self-pollination}}{\text{fuit set from hand cross-pollination}}$$

POLLEN LOAD

Initially, a library of pollen grains collected from each studied taxa of Iridaceae was prepared to identify pollen grains at the genus level, after which the number and ratio of pollen grains carried by each visitor was determined. The average pollen load for each functional group of bees was determined from a sample of 20 individuals per functional group of insect visitors.

Bees were invariably collected during foraging, and their pollen load was removed and maintained in test tubes with 4 mL of 70% alcohol. Pretreatments and analyses of pollen samples were performed according to Erdtman (1952). After chemical treatment, slides were deposited in the Pollen Library of the Laboratório de Palinologia [Universidade Luterana do Brasil (ULBRA), Brazil]. The proportions of pollen types were classified in classes of occurrence for each functional group according to Louveaux *et al.* (1978): abundant (> 45% of total grains), common (15–45%), uncommon (3– 14%) and rare pollen (< 3%). The contribution of each functional group to pollination systems was assessed using an approach that considered both the frequency of visits and the pollen load of the bee (Krakos & Fabricant, 2014). Pollen transfer was calculated for each *Cypella* spp., *H. pulchella* and *K. brasiliensis* by applying the formula $\sum(\mathrm{VR}_x \times \mathrm{PL}_x)$, where VR is the total visitation frequency by any given functional group (x) and PL is the average pollen load carried by that group. The main pollination system was defined for each plant genus according to the pollinator functional group that reached > 75% of the total pollen transfer (Fenster *et al.*, 2004; Krakos & Fabricant, 2014).

STATISTICAL ANALYSES

Bees were systematically assigned to one of the two functional groups defined above. The frequencies of legitimate visits (*f*; visitors that came into contact with anthers and stigmas) were estimated for each of the two functional groups and compared using general linear mixed-effects models (GLMMs) with a Poisson distribution, with plant species, bee functional group and the interaction between them as fixed effects. To take into account the variation in observation times, the duration of observation was included as a $\log_{10}$-transformed offset. Differences in visitation by oil- and pollen-collecting bees among and between plant taxa were assessed with Tukey's post-hoc test using the function glht() from *multcomp* package in R (R Core Team, 2015).

To analyse binary data related to fruit set obtained from the different pollination treatments, data were fitted to GLMMs, considering pollination treatments as fixed effects and individual plants as random effects. The fit of logistic regression models was assessed using maximum likelihood analysis (Akaike information criterion). Finally, a deviance analysis was performed to test whether model deviance was greater than expected by chance and whether each model as a whole was better than the null model (Bolker *et al.*, 2008).

We performed a Wilcoxon rank-sum test to assess the difference between the numbers of pollen grains from each plant genus carried by pollen- and oil-collecting bees (Rhodes *et al.*, 2017).

RESULTS

FLORAL VISITORS

We recorded 975 flower visits during a total of 142 h of observations and identified 16 species of pollen-collecting bees and four species of oil-collecting bees (Table 1). Pollinator diversity (Fig. 1) was highest for *H. pulchella* (14 species: 13 pollen-bees and one oil-bee), followed by *K. brasiliensis* (seven species: six pollen-bees and one oil-bee), *Cypella amplimaculata* (two pollen-bees and two oil-bees), *Cypella herbertii* subsp. *herbertii* (one pollen-bee and three oil-bees), *Cypella herbertii* subsp. *brevicristata* (two pollen-bees and two oil-bees), *Cypella pusilla* (one pollen-bee and two oil-bees) and *Calydorea alba* (three species, all pollen-bees).

All oil-producing taxa were pollinated by oil-collecting bees belonging to the tribe Tapinotaspidini. *Arhysoceble picta* was observed on flowers of all six oil-producing taxa studied, and *Chalepogenus muelleri* was recorded on three species with oil flowers: *Cypella herbertii* subsp. *herbertii*, *Cypella herbertii* subsp. *brevicristata* and *H. pulchella*. *Caenonomada brunerii* and *Chalepogenus goeldianus* were found exclusively on *Cypella herbertii* subsp. *brevicristata* and *Cypella pusilla*, respectively (Fig. 1).

Arhysoceble picta and *Chalepogenus muelleri* did not contact fertile parts during their oil-foraging activities in *H. pulchella*, nor did *Arhysoceble picta* when it visited flowers of *K. brasiliensis*. Legitimate visits in these plant taxa were observed only when oil-collecting bees collected both oil and pollen. Pollen-collecting behaviour by oil-collecting bees was observed only in combination with oil-collecting behaviour (Figs 2, 3). Oil-collecting bees visited *H. pulchella* and *K. brasiliensis* exclusively to collect oil or to collect both oil and pollen, but never to collect pollen only (Table 1).

Oil-collecting bees first landed on the inner tepals of *Cypella* flowers, heading towards the distal part of the inner tepals, where the trichomes are localized. The bees contacted the reproductive organs while gathering the oil resource; consequently, pollen was attached on their anterior head and sometimes removed by bees and deposited in the scopes. Pollen-collecting bees (Halictidae) landed directly on reproductive organs of *Cypella* and collected pollen actively from the anthers. We recorded two different behaviours of oil-collecting bees on *H. pulchella* and *K. brasiliensis*: first, they landed on the outer or inner tepals of the species, respectively, gathering only the oil resource without making contact with the reproductive organs. However, during some visits, immediately after oil foraging they landed on the reproductive structure to collect pollen actively from the anthers, behaving exactly like the pollen-collecting bees we observed (Fig. 2).

Table 1. Visitation frequency for each bee species and floral reward collected for the seven taxa of Iridaceae studied

Taxa/species (authors)		Floral reward	*Cypella herbertii* subsp. *herbertii*	*Cypella herbertii* subsp. *brevicristata*	*Cypella amplimaculata*	*Cypella pusilla*	*Kelissa brasiliensis*	*Herbertia pulchella*	*Calydorea alba*
Apinae*/ Tapinotaspidini*	*Arhysoceble picta* (Friese, 1899)	Oil	**0.242 ± 0.054**	**0.800 ± 0.198**	**0.133 ± 0.036**	0.200 ± 0.021	0.052 ± 0.012*	**0.293 ± 0.081***	–
	Arhysoceble picta	Oil + pollen	**0.443 ± 0.078**	**0.260 ± 0.091**	**0.225 ± 0.104**	–	0.050 ± 0.016	**0.119 ± 0.044**	–
	Chalepogenus muelleri (Friese, 1899)	Oil	0.088 ± 0.029	**0.133 ± 0.021**	–	–	–	0.100 ± 0.000	–
	Chalepogenus muelleri	Oil + pollen	–	–	–	–	–	–	–
	Chalepogenus goeldianus (Friese, 1899)	Oil	–	–	–	**0.149 ± 0.016**	–	–	–
	Caenonomada bruneri (Ashmead, 1899)	Oil	**0.333 ± 0.000**	–	–	–	–	–	–
Apinae	*Apis mellifera* (Linnaeus, 1758)	Pollen	–	–	–	–	**0.233 ± 0.100**	**0.529 ± 0.196**	**0.316 ± 0.023**
	Trigona spinipes (Fabricius, 1793)	Pollen	–	–	–	–	–	**0.102 ± 0.026**	–
	Mourella caerulea (Friese, 1900)	Pollen	–	–	–	–	–	**0.178 ± 0.070**	–
Halictinae	*Augochlorella urania* (Smith, 1853)	Pollen	**0.162 ± 0.067**	**0.244 ± 0.058**	–	–	–	–	–
	Augochlorella iopoecila (Moure, 1950)	Pollen	–	–	–	–	**0.133 ± 0.000**	0.033 ± 0.000	–
	Augochlorella ephyra (Schrottky, 1910)	Pollen	–	–	0.067 ± 0.000	–	0.017 ± 0.000	–	–
	Augochlora amphitrite (Schrottky, 1909)	Pollen	–	**0.117 ± 0.083**	–	–	–	–	**0.258 ± 0.240**
	Augochloropsis sp. 1	Pollen	–	–	**0.100 ± 0.000**	–	–	0.067 ± 0.000	–
	Dialictus sp. 1	Pollen	–	–	**0.133 ± 0.000**	–	–	**0.133 ± 0.000**	–
	Dialictus sp. 2	Pollen	–	–	–	–	–	0.067 ± 0.000	–
	Dialictus sp. 3	Pollen	–	–	–	**0.107 ± 0.031**	–	–	**0.583 ± 0.083**
	Pseudagapostemon aff. *pruinosus*	Pollen	–	–	–	–	**0.142 ± 0.034**	0.067 ± 0.016	–
Colletinae	*Bellopria* sp. 1	Pollen	–	–	–	–	0.079 ± 0.020	0.053 ± 0.008	–
Andreninae	*Anthrenoides micans* (Urban, 1995)	Pollen	–	–	–	–	0.033 ± 0.000	0.044 ± 0.011	–
	Rhophitulus sp. 1	Pollen	–	–	–	–	–	0.091 ± 0.024	–
	Rophitulus guaraniticus (Schlindwein & Moure, 1998)	Pollen	–	–	–	–	–	**0.125 ± 0.075**	–

Data are means ± SE. Visitation rates > 0.1 are in bold.
*Bee did not contact the reproductive structures during foraging behaviour.

Figure 1. Network representation of plant–pollinator interactions observed. Plant species are indicated on the left by green bars and pollinators on the right by blue bars for oil-collecting bees and yellow bars for pollen-collecting bees. Grey lines represent species interactions, and the line thickness indicates the relative frequency of each interaction.

The frequency of visitation differed significantly, not only between bee functional groups for a given plant species, but also among plant species of a given functional group ($\chi^2 = 402.11$, d.f. = 7, $P < 0.001$). The frequency of flower visitation was significantly different between oil-collecting bees and pollen-collecting bees for *Cypella herbertii* subsp. *herbertii* ($Z = -15.53$, $P < 0.01$; Fig. 4), *Cypella herbertii* subsp. *brevicristata* ($Z = -10.80$, $P < 0.01$), *Cypella amplimaculata* ($Z = -8.35$, $P < 0.01$) and *H. pulchella* ($Z = 3.67$, $P < 0.01$). Among plant species, the visitation frequency of oil-collecting bees was higher for *Cypella herbertii* subsp. *brevicristata* when compared with *Cypella herbertii* subsp. *herbertii* ($Z = -9.04$, $P < 0.01$),

Figure 2. Illustration of distinct behaviour of *Arhysoceble picta*. A, inefficient oil-collecting foraging in *Herbertia pulchella* (left) and pollen-collecting foraging (right). Note that only during pollen-collecting activity does the bee contact the reproductive organs of plant species. B, efficient oil-collecting foraging in *Cypella herbertii*.

Cypella amplimaculata ($Z = -7.35$, $P < 0.01$), *Cypella pusilla* ($Z = -14.34$, $P < 0.01$), *K. brasiliensis* ($Z = -9.62$, $P < 0.01$) and *H. pulchella* ($Z = -12.19$, $P < 0.01$).

The proportion of flowers that set fruit was dependent on pollination treatment for four taxa (*Cypella herbertii* subsp. *herbertii*, $\chi^2 = 7.36$, d.f. = 2, $P = 0.02$; *Cypella pusilla*, $\chi^2 = 37.94$, d.f. = 4, $P < 0.01$; *K. brasiliensis*, $\chi^2 = 13.73$, d.f. = 1, $P < 0.01$; *H. pulchella*, $\chi^2 = 4.61$, d.f. = 1, $P = 0.031$). In contrast, pollination treatment did not significantly impact fruit set in *Cypella herbertii* subsp. *brevicristata* ($\chi^2 = 3.43$, d.f. = 2, $P = 0.179$), *Cypella amplimaculata* ($\chi^2 = 4.91$, d.f. = 2, $P = 0.08$) and *Calydorea alba* ($\chi^2 = 3.63$, d.f. = 1, $P = 0.05$; Table 2).

Fruit set from natural pollination (Fig. 5) differed significantly among plant taxa ($\chi^2 = 40.801$, d.f. = 6, $P = 0.003$). The highest value of fruit set from natural pollination was observed for *Cypella amplimaculata* (61.3%), which differed significantly from that found for *Cypella herbertii* subsp. *herbertii* (50.9%), *Cypella herbertii* subsp. *brevicristata* (30%), *Cypella pusilla* (17.4% in April and 29% in November), *H. pulchella* (40%) and *Calydorea alba* (20%).

The results of controlled pollination experiments showed that all taxa of *Cypella* studied were partly self-incompatible ($0.20 < \text{ISI} < 0.80$). However, all plant species sampled were pollinator dependent, and no evidence of spontaneous self-pollination was

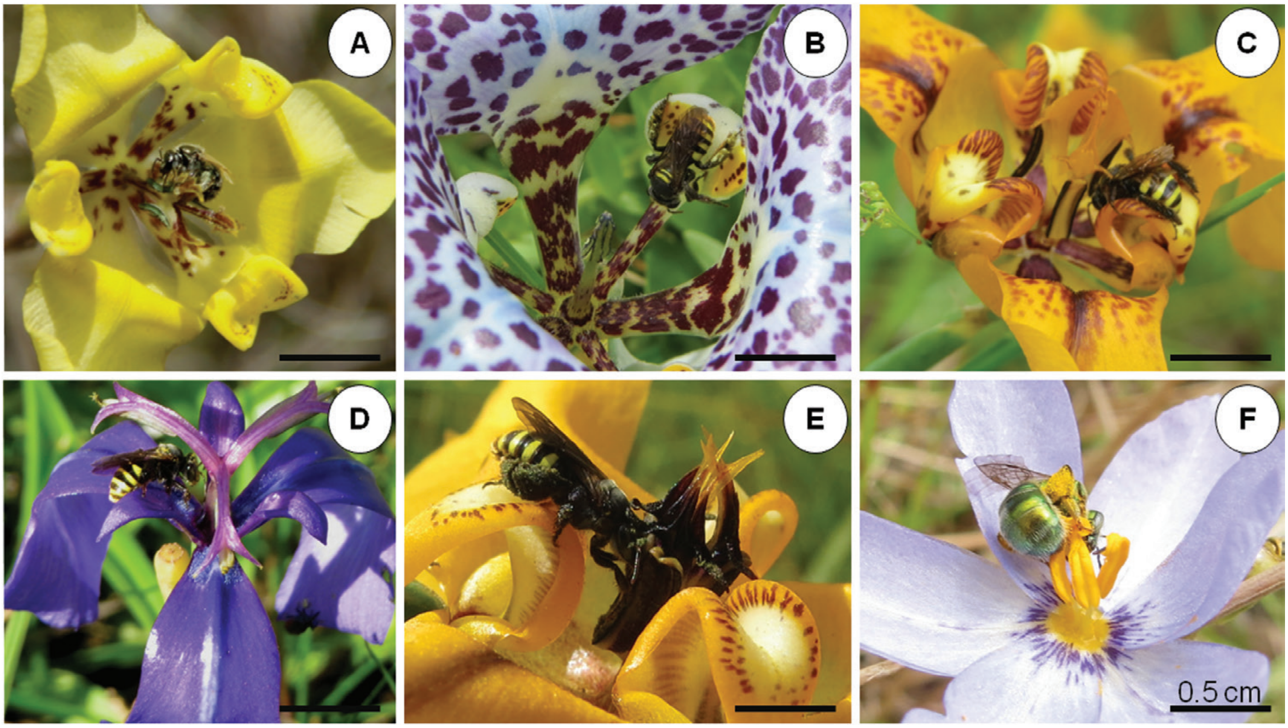

Figure 3. Plant–bee interactions observed. A, *Dialictus* sp. collecting pollen on *Cypella pusilla*. B–E, *Arhysoceble picta* collecting oil on *Kelissa brasiliensis*, *Cypella amplimaculata*, *Herbertia pulchella* and *Cypella herbertii*, respectively. F, *Augochlora amphritite* collecting pollen on *Calydorea alba*. Note that in pictures B and D, the oil-collecting bee *Arhysoceble picta* does not contact the reproductive organs of *K. brasiliensis* and *H. pulchella*.

found for any species included in our study (Table 3). Fruit set from hand self-pollination was significantly different among species ($\chi^2 = 12.529$, d.f. = 3, $P = 0.005$; Table 3; Supporting Information, Appendix S3). The highest values of fruit set from hand self-pollination were observed for *Cypella herbertii* subsp. *herbertii* (65.2%) and *Cypella amplimaculata* (42.4%), whereas *K. brasiliensis*, *H. pulchella* and *Calydorea alba* were strongly self-incompatible (ISI > 0.80).

POLLEN LOAD

A total of 83 pollen types were identified in pollen loads of visitors to Iridaceae; 29 pollen types were identified from oil-collecting bees and 54 from pollen-collecting bees (Supporting Information, Appendices S4, S5 and S6). Among oil-collecting bees, 53.57% of the pollen load was from *Cypella* (dominant pollen type), 19.16% from *Sisyrinchium* (accessory pollen type), 14.07% from *Kelissa* (isolated important), 4.85% from *Herbertia* (isolated occasional) and 8.37% from other plant families. Pollen from *Herbertia* (49.18%) was predominant in the pollen load recovered from pollen-bees, followed by *Kelissa* (21%), *Cypella* (5.7%), *Calydorea* (2.6%) and *Sisyrinchium* (1.1%); however, 24.1% of the pollen load was from other plant families (Fig. 6). There was no significant difference between the number of pollen grains carried by oil- and pollen-collecting bees of *Cypella* ($W = 38$, $P = 0.201$), *Kelissa* ($W = 10$, $P = 0.90$) and *Sisyrinchium* ($W = 37$, $P = 0.164$). However, the number of *Herbertia* pollen grains carried by pollen-collecting bees was significantly higher ($W = 46$, $P = 0.004$).

According to *F*-score analysis, *Cypella* spp. revealed a specialized pollination system based on oil-bees, which contributed 86.48% of the pollen transfer, whereas pollen-collecting bees were responsible for only 13.52%. For *K. brasiliensis*, 73.33% of the pollen transfer was from pollen-collecting bees and 26.66% from oil-collecting bees (Fig. 6), with an *F*-score value indicating contributions to total pollen transfer from both functional groups. Pollen transfer for *H. pulchella* was dependent on pollen-collecting bees for 95.49% and oil-collecting bees for 4.51%, indicating a greater contribution by generalist pollen-collecting bees (Fig. 7).

DISCUSSION

In the present study, the breeding system, visitation frequency, pollinator behaviour and pollen load were

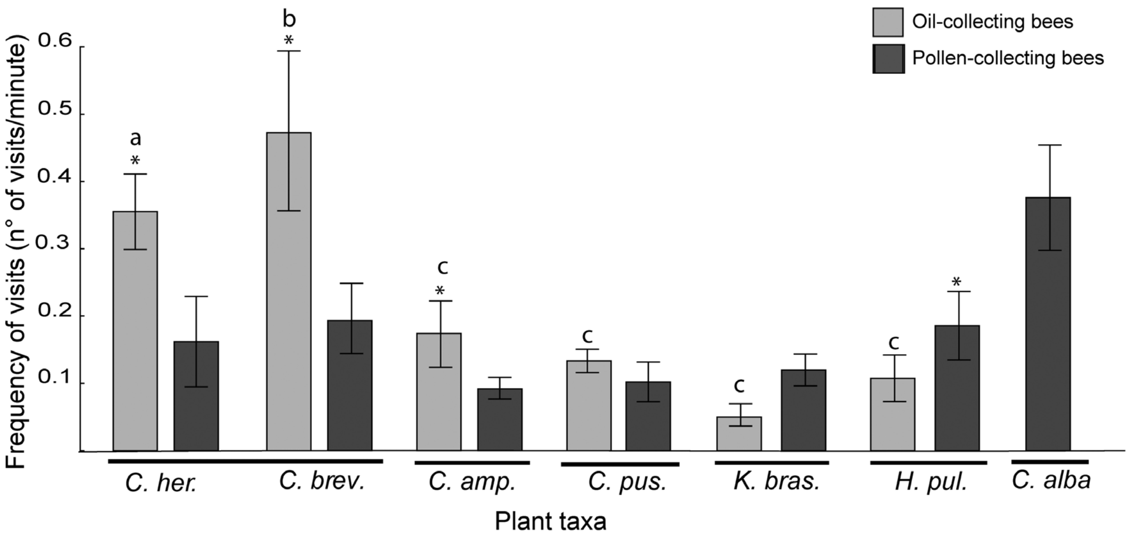

Figure 4. Visitation frequency (mean ± SE) of oil- and pollen-collecting functional groups of bees for each taxa studied. Light and dark grey bars represent oil- and pollen-collecting bees, respectively. Species names are abbreviated as follows: *C. amp.*, *Cypella amplimaculata*; *C. brev.*, *Cypella herbertii* subsp. *brevicristata*; *C. her.*, *Cypella herbertii* subsp. *herbertii*; *H. pul.*, *Herbertia puchella*; *K. bras.*, *Kelissa brasiliensis*. *Significant differences in visitation frequency between functional groups on *C. herbertii* subsp. *herbertii* ($W = 138$, $P = 0.034$). Letters indicate significant differences in visitation frequency of oil-collecting bees between *Cypella herbertii* subsp. *herbertii* and *H. pulchella* ($\chi^2 = 20.6$, d.f. = 5, $P < 0.01$). Significant differences among cross-pollination and natural pollination of two flowering periods in *Cypella pusilla*.

used to improve knowledge about plant–pollinator interactions among taxa of Tigridieae in South Brazil. New insights into aspects of the pollination systems in this tribe are provided and suggest a continuum of the importance of oil-collecting bees as pollinators of the studied taxa.

Oil is considered a specialized type of reward, because other animals are not attracted by this reward and it is collected by only a few groups of bees in Mellitinae and Apinae (Armbruster, 2017). In contrast to the expected strong functional-group specialization between oil-producing plants and oil-collecting bees, the findings of the present study suggest a more flexible pollination system and a continuum of specialization. Contrary to our expectations, this continuum emerges from highly specialized taxa to generalized taxa as follows: *Cypella* spp., mainly pollinated by three species of oil-collecting bees; *K. brasiliensis*, with a bimodal pollination system having contributions from both oil- and pollen-collecting bees; *H. pulchella*, mainly pollinated by multiple species of pollen-collecting bees although it secretes oil; and *Calydorea alba*, which is pollinated exclusively by pollen-collecting bees.

FLORAL VISITORS

This study provides the first record of specialized oil-collecting bees as pollinators of *Cypella pusilla* and *K. brasiliensis*. Previous studies have reported low frequencies or a complete absence of specialized oil-collecting bees visiting Iridaceae (Schlindwein, 1998; Devoto & Medan, 2004; Pinheiro *et al.*, 2008; Oleques *et al.*, 2016). Schlindwein (1998) recorded visits by oil-collecting bees to *Cypella herbertii* (*Chalepogenus muelleri* and *Caenonomada brunerii*) and *H. pulchella* (*Chalepogenus* sp.) in the Serra do Sudeste region. In addition to the oil-collecting bees of the tribe Tapinotaspidini, the majority of bee species recorded in the present study were small solitary bees of the subfamilies Halictinae, Colletinae and Adreninae. Three species of social bees were recorded: *Apis mellifera* and the stingless bees *Trigona spinipes* and *Mourella caerulea*.

Our observations showed that all visits from oil-collecting bees were legitimate in *Cypella* spp., with contact between the body of bees and the surface of anthers and stigmas. In contrast, oil-collecting behaviour in *H. pulchella* and *K. brasiliensis* did not result in pollen deposition on the body of the bees or contact with reproductive structures in the flowers. Nevertheless, we did not observe pollen-collecting behaviour in the absence of oil-collecting behaviour, which indicates that oil is an important reward for attracting oil-collecting bees in flowers of *K. brasiliensis* and *H. pulchella* (Schaffler *et al.*, 2015). These results have important significance, because distinct behaviour of functional groups of pollinators can apply

Table 2. Pollination breeding systems of seven plant taxa: Akaike information criterion (AIC), deviance (G2), degrees of freedom (d.f.) and deviance significance of general linear mixed-effects model analysis

Plant taxon	Model (μ)	AIC	Deviance (G2)	d.f.	Deviance significance
Cypella herbertii subsp. *herbertii*	Result ~ Treatment (2)	130.52	7.365	2	0.025*
	Result ~ Individuals (2)	143.14			
	Result ~ Treatment + Individuals (3)	142.14			
Cypella herbertii subsp. *brevicristata*	Result ~ Treatment (2)	189.39	3.433	2	0.179
	Result ~ Individuals (2)	276.00			
	Result ~ Treatment + Individuals (3)	276.00			
Cypella amplimaculata	Result ~ Treatment (2)	109.80			
	Result ~ Individuals (2)	216.26	4.917	2	0.085
	Result ~ Treatment + Individuals (3)	214.00			
Cypella pusilla	Result ~ Treatment (2)	164.52			
	Result ~ Individuals (2)	218.50	37.94	4	< 0.001*
	Result ~ Treatment + Individuals (3)	266.38			
Kelissa brasiliensis	Result ~ Treatment (2)	90.24			
	Result ~ Individuals (2)	232.77	13.703	1	< 0.001*
	Result ~ Treatment + Individuals (3)	232.77			
Herbertia pulchella	Result ~ Treatment (2)	137.65			
	Result ~ Individuals (2)	191.15	4.618	1	0.031*
	Result ~ Treatment + Individuals (3)	191.15			
Calydorea alba	Result ~ Treatment (2)	77.070			
	Result ~ Individuals (2)	170.77	3.630	1	0.056
	Result ~ Treatment + Individuals (3)	172.00			

Numbers in parentheses indicate the number of estimated parameters.
*Significant results considering $P < 0.05$.

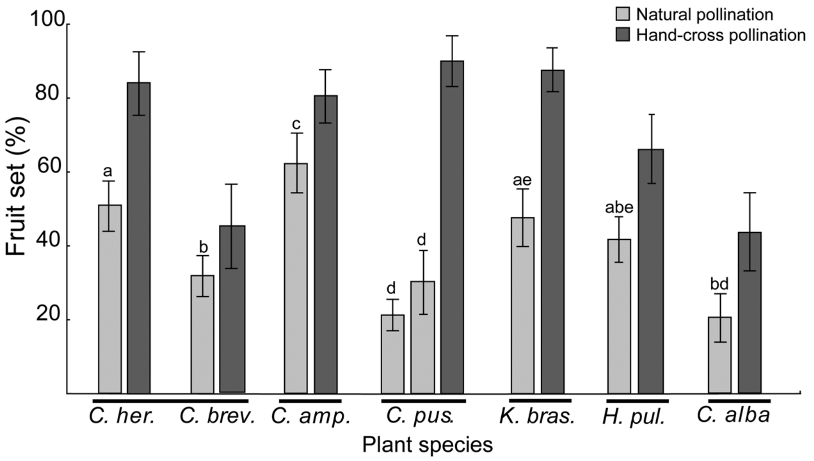

Figure 5. Fruit set (mean ± SE) of natural pollination (light grey) and hand cross-pollination (dark grey) tests. Different letters indicate differences in fruit set from natural pollination among species ($P < 0.05$). The two light grey bars for *Cypella pusilla* represent the fruit set in April and November, respectively.

distinct selective pressures on plant fitness (Ne'eman *et al.*, 2010; King *et al.* 2013; Van der Niet *et al.* 2014). In the long term, such differences in the contributions of oil-collecting and non-oil-collecting bees could result in changes in floral traits and the evolution of reward-secreting structures (Ferreiro *et al.*, 2019).

Table 3. Fruit production in seven taxa of Tigrideae after different controlled pollination treatments, and associated breeding system characteristics

Plant species	Percentage fruit set (*N*) per treatment					
	Natural pollination		Hand cross-pollination	Hand self-pollination	ISI*	Spontaneous self-pollination
Cypella herbertii subsp. *herbertii*	50.9 (55)		84.2 (19)	65.2 (23)	0.23, pSI	0.0 (13)
Cypella herbertii subsp. *brevicristata*	30.0 (100)		50.0 (20)	19.7 (20)	0.61, pSI	0.0 (28)
Cypella amplimaculata	61.3 (31)		80.9 (22)	42.4 (25)	0.48, pSI	0.0 (25)
Cypella pusilla†	April 17.4 (63)	November 29.0 (31)	90.0 (20)	27.7 (36)	0.69, pSI	0.0 (26)
Kelissa brasiliensis	47.6 (42)		87.5 (32)	0.0 (30)	1.0, SI	–
Herbertia pulchella	40.0 (70)		63.3 (30)	0.0 (28)	1.0, SI	–
Calydorea alba	20.5 (39)		43.4 (23)	0.0 (25)	1.0, SI	–

A dash indicates that an experiment was not conducted because of the absence of self-pollination.
*Index of self-incompatibility (pSI, partly self-incompatible; SI, self-incompatible).
†Natural pollination measured from both flowering periods.

Illegitimate visits can be the result of a morphological mismatch between the plant and the insect visitor (Stout, 2007; Ne'eman *et al.*, 2010; King *et al.*, 2013; Ruchisansakun *et al.*, 2016; Palacios *et al.*, 2019). Thus, an insect attracted to a flower by a specific reward, such as oil, might still fail to be a pollinator if it does not contact the anthers and/or does not contact the stigmas (Armbruster, 2017). Illegitimate visits of oil-collecting bees to oil flowers have been documented, especially in Malpighiaceae and Plantaginaceae (Sigrist & Sazima, 2004; Martins & Alves-dos-Santos, 2013). In addition, our study showed that Tapinotaspidini bees should not always be considered as pollinators during oil-collecting foraging in Iridaceae.

BREEDING SYSTEM

Specialized pollination systems could be greatly affected by lack of service by a pollinator, resulting in a decrease in their reproductive output, leading to a transition towards generalized pollination or, more commonly, a transition towards self-pollination (Moeller, 2005). Our results showed that *Cypella* spp. are partly self-incompatible and pollinator dependent, whereas *K. brasiliensis*, *H. pulchella* and *Calydorea alba* are totally self-incompatible. Self-incompatibility has been documented for *H. pulchella* and other species within *Herbertia* (Stiehl-Alves et al., 2017), whereas self-compatibility has been documented for *Cypella herbertii* (Devoto & Medan, 2004). Our study also provides the first evidence of the breeding system for *K. brasiliensis* and *Calydorea alba* and of self-compatibility for *Cypella pusilla*.

Although self-compatibility may have negative impacts on fitness as a result of inbreeding depression, it is especially important when conditions for outcrossing are unfavourable owing to an absence of mates or effective pollinators (Waser, 2006). Self-incompatibility could increase the risks of pollen limitation in specialized pollination systems and therefore intensify the risk of local extinction (Knight *et al.*, 2005). Nevertheless, the self-compatibility observed for *Cypella* spp. could be related to the high degree of specialization detected for their pollination system (Fenster & Martén-Rodríguez, 2007). In contrast, species with bimodal or more generalist pollination systems, such as *K. brasiliensis*, *H. pulchella* and *Calydorea alba*, are self-incompatible and could have lower risks of pollination limitation when compared with specialized species (Wolowski *et al.*, 2014).

Furthermore, the diversity and importance of non-oil-collecting bees as pollinators of *H. pulchella* was documented and suggests that further studies on the efficiency of pollination should be conducted in order to detect the true contribution of oil- and non-oil-collecting bees to plant reproductive output along the specialization–generalization continuum.

POLLEN LOAD

Our results for pollen load of the two functional groups of bees showed that oil-collecting bees carry mostly pollen types from Iridaceae (91.63%); however, the proportion of pollen load coming from this family was also especially significant for pollen-collecting bees (75.9%), whereas only 24.1% came from other plant

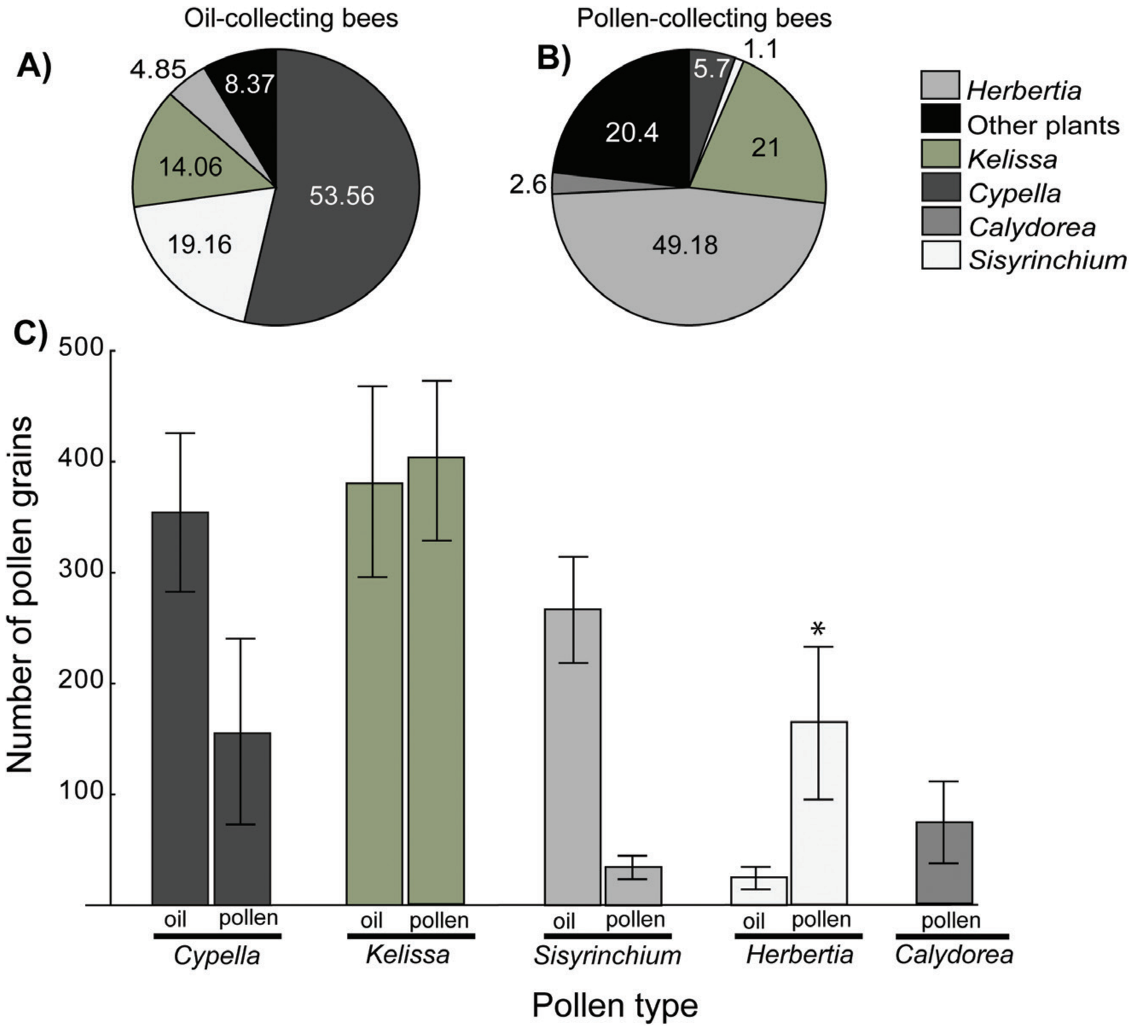

Figure 6. Results from pollen load analysis. A, percentages of pollen grains found in the oil-collecting functional group from each Iridaceae pollen type and other plant families. B, percentages of pollen grains found in the pollen-collecting functional group from Iridaceae and other plant families. C, number of pollen grains (mean ± SE) carried by oil- and pollen-collecting bees per plant genus.

families, such as Asteraceae, Solanaceae, Myrtaceae and Fabaceae. The strong attraction that oil-collecting bees have for oil-producing plant taxa could be explained by the presence of certain chemicals in the oil, mainly diacetin (Schaeffler *et al.*, 2015); however, further studies are needed to elucidate the chemical composition of the oil of Iridaceae and whether oil-collecting bees of the subfamily Apinae exhibit a similar positive response to diacetin.

Pollination systems permanently balancing on the specialization–generalization continuum and novel rewards, produced by plants to be collected by distinct functional groups of pollinators, are especially interesting because they can promote diversification through specialized plant–pollinator interactions (Armbruster & Baldwin, 1998; Waser *et al.*, 2006; Igic *et al.*, 2008; Armbruster, 2017). Our results showed that *Cypella* spp. were mainly visited by oil-collecting bees, and the pollen load results corroborated the contribution of specialized bees to the pollen transfer of the genus. Pollen transfer of *K. brasiliensis* depends on both functional groups, which visit their flowers in equal frequency, indicating a bimodal pollination system (Monty, *et al.* 2006; Shuttleworth & Johnson, 2008). The high pollen load of *K. brasiliensis* carried by oil-collecting bees results from intentional pollen collecting. Although both bee functional groups contributed complementarily to pollen transfer for *K. brasiliensis*, this bimodal pollination system includes a high level of pollen transfer by oil-collecting bees, which might have been an important factor in the evolutionary history of *K. brasiliensis* by promoting

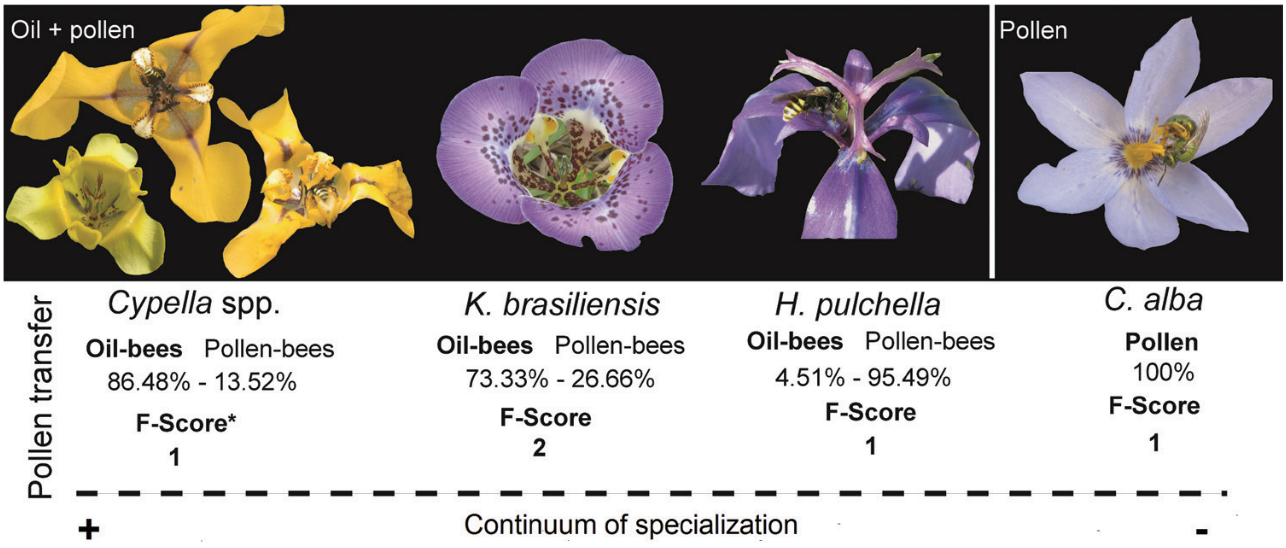

Figure 7. Diagram illustrating pollen transfer and the F-score for each genus along with the specialization–generalization continuum. Pollen flow (as a percentage) indicates the combination between visitation frequency data and pollen load from the bees' bodies. The F-score from each taxon represents the number of functional groups that contribute to > 75% of the pollen flow. Oil + pollen and Pollen indicate the type of reward available.

further floral trait displacement. Bimodal pollination systems are common, and pollination shifts are assumed to be one of the driving forces for speciation by promoting reproductive isolation in distinct plant populations (Goldblatt & Manning, 2006; Anderson *et al.*, 2009, 2016; Johnson, 2010; Ferreiro *et al.*, 2019).

Contrary to our expectation, pollen-collecting bees had a higher visitation frequency for *H. pulchella* than oil-collecting bees, assuring the pollen transfer in this species, which indicates a less specialized pollination system (Freitas & Sazima, 2003). Specialized pollination systems are most likely to develop when efficient pollinators are present (Stebbins, 1970), whereas a reduction in specialization is favoured when pollinator availability or behaviour is unpredictable (Waser *et al.*, 1996). Competition is another factor that could play an important role in the evolution of plant–pollinator interactions (Muchhala & Potts, 2007). Some of the species studied, especially *K. brasiliensis*, *Cypella amplimaculata* and *H. pulchella*, are sympatric, and all the species studies, except *Cypella pusilla*, have populations co-occuring with other oil-producing species, such as *Sisyrinchium* spp. and *Herbertia lahue*. Given that pollination is critical to plant reproduction, competition for pollinators might be expected to put strong selective pressures on coexisting species (Muchhala & Potts, 2007). When species share the same guilds of pollinators, visitation patterns can lead to competition, and competition can be reduced by specializing in different pollinators (Armbruster *et al.*, 1994). The continuum of specialization–generalization evidenced by our study could be related to distinct strategies by plants to minimize the negative effects of competition by specialized oil-bees.

CONCLUSIONS

Taken together, our results contribute to a better understanding of specialized interactions between oil-collecting bees and oil-flowers of Iridaceae. Our findings reveal a continuum in the contribution of oil-bees to the pollination system of the studied taxa. Oil-collecting bees were observed foraging in all oil-secreting plant species sampled for the present study. However, they acted as thieves in *K. brasiliensis* and *H. pulchella*, visiting flowers to collect oil without contacting the reproductive structures. Both observations of foraging behaviour and analyses of pollen load revealed that oil-bees can switch to illegitimate visitors during floral oil collection, depending on the genus studied.

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SUPPORTING INFORMATION

Appendices are available as online resources on 10.6084/m9.figshare.8188391.

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. List of all plant populations studied, with their respective collectors, herbarium voucher, localization and coordinates.
Appendix S2. Distribution of studied plant populations in the State of Rio Grande do Sul, Brazil: (1) *Calydorea alba*; (2) *Cypella herbertii* subsp. *brevicristata* + *Cypella herbertii* subsp. *herbertii* + *Kelissa brasiliensis*; (3) *Cypella pusilla*; (4) *Cypella amplimaculata*; (5) *Herbertia pulchella* + *K. brasiliensis*; (6) *Cypella amplimaculata* + *H. pulchella*.
Appendix S3. Fruit set (mean ± SE) from hand self-pollination. Different letters indicate significant differences among sampled taxa.
Appendix S4. Mainly pollen types are found on the body of bees. Pollens from Iridaceae are indicated in bold. The pollen grains are organized evolutionarily according to Angiosperm Phylogeny Group IV. Numbers in the legend are related to pollen images.
Appendix S5. List of pollen types identified in the oil-collecting functional group of bees, with their respective relative abundance and classification.
Appendix S6. List of pollen types identified in the pollen-collecting functional group of bees, with their respective relative abundance and classification.

DO POLLEN AND OIL-COLLECTING BEES DIFFER ON POLLINATION PERFORMANCE? THE CASE OF THREE OIL-PRODUCING IRIDACEAE SPECIES IN BRAZILIAN PAMPA

Do pollen and oil-collecting bees differ on pollination performance? The case of three oil-producing Iridaceae species in Brazilian Pampa

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Running head: Contribution of pollen and oil-bees on pollination of Iridaceae

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Abstract

Premise

Oil-producing flowers from distinct species could depend in different extent on pollination by oil bees and pollen bees that could also act as pollinators in some populations. The specialization-generalization continuum in pollination systems of oil-producing Iridaceae as already described. Therefore, the goal of this study was to investigate the performance of oil and pollen bees as pollinators of three oil-producing species of Iridaceae: *Cypella herbertii, Kelissa brasiliensis* and *Herbertia pulchella*.

Methods

The species varies in morphological traits, elaiophores position and visitation rates of two distinct bee functional groups. Oil bees are considered thieves when collecting oil in two of the three species studied. We combined data from visitation frequency and pollen deposition on stigmas to test the role of each functional group of bees as effective pollinators.

Results

Oil bees performed better as pollinators of *C. herbertii*; Pollen and oil bees are equally important in pollination of *K. brasiliensis* and Pollen bees performed slightly better as *H. pulchella* pollinators. In addition, oil seems to play an important role in attraction of oil bees to *K. brasiliensis* and *H. pulchella* flowers and the opportunistic behavior of oil bees made them functionally act as effective pollen-collecting bees in these two plant species.

Conclusions

These finds reinforce that specialization of oil pollination systems is a continuum and other functional groups of bees could act synergically in the pollination of oil-producing flowers.

Introduction

Flowering plants depend on animal pollinators for sexual reproduction in most species (Ollerton *et al*. 2011). Plant species can be pollinated from a single pollinator species or pollinator functional group to several species from different functional groups (Fenster *et al*. 2004). Among the floral rewards offered to pollinators by plants, floral oil is a special type of resource with some particularities about its nutritional traits, being associated with larval food provisioning and nest construction (Vogel 1974, Buchmann 1987). Oil production as a reward to pollinators has evolved multiple times along the evolution of Angiosperms in 14 unrelated plant families, including Iridaceae, Malpighiaceae, Fabaceae, Gesneriaceae and Melastomataceae (Possobom and Machado 2017, Martins *et al*. 2014, Renner and Shaefer 2010). However, the most part of oil producing plant groups has subsequently lost this trait in one or several lineages. In this way, along the Angiosperm evolution, the oil secretion as floral reward was more often lost than acquired (Renner and Shaefer 2010).

Floral oil can be considered as an ecological filter that excludes some potential pollinators in favor of specialized oil- collecting bees (Armbruster 2014, 2017). However, the unpredictability of these specialized interaction systems could play some negative impacts to these plants, including local extinction of plant species in the absence of oil-bees (Steiner and Whitehead 1996). Flowering plants with specialized pollination systems could experience high rates of pollen limitation leading to generalization in terms of pollinators in order to reduce extinction risks (Waser *et al.* 1996, Knight *et al*. 2005). In this way, plant species with some flexibility on oil-bee dependence might have their reproductive rates enhanced by other pollinators, mainly pollen-collecting bees (Steiner and Whitehead 1996, Pauw and Bond 2011, Manning and Goldblatt 2002). However, pollinators not specialized in oil-collection could exert selective pressures against oil-secretion. Indeed, several species from different groups of oil-bee pollinated plants have reduced or eliminated their dependence on oil- collecting bees locally or, in a long term, through the loss of oil-secreting structures (Anderson 1979, Cocucci 1991, Steiner and Whitehead 1996, Silvério *et al.* 2012, Chauveau *et al.* 2012, Oleques *et al.* 2020). Therefore, shifts from floral oil to other pollination systems associated with diverse floral rewards were already described (Steiner 1993, Steiner and Whitehead 1996, Steiner 1998, Goldblatt and Manning 2006, Oleques *et al.* 2020).

The number of losses in oil-production is particularly interesting in the American tribes of Iridaceae. Chauveau *et al.* (2012) have showed that the lability in the presence and absence of oil-secretion structures could be related to frequent shifts in the pollination systems. The most part of the species in the American tribes of Iridoideae present a specialized oil-bee pollination system and pollination system when pollen is the sole reward offered (Chauveau *et al.* 2012). Currently, Oleques *et al.* (2020) studying plant-pollination interactions of six Tigridieae species, evidenced differences in the level of oil-bee specialization in plants from distinct genera. It is because species belonging to Tigridieae, especially those in genera *Cypella* Herb., *Herbertia* Sweet., and *Kelissa* Ravenna, presented some differences in floral traits that are probably related to shifts in pollination strategies (Chauveau *et al.* 2012, Oleques *et al.* 2020). According to previous finding, species of *Cypella* are mainly pollinated by oil-bees and it is ensured by the adequate match between flowers and pollinators (Oleques *et al.* 2020, Baéz-Lizarazo *et al.* 2021). In contrast, the same oil-bees are considered thieves in the oil-producing species *Herbertia pulchella* Sweet. and *Kelissa brasiliensis* (Baker) Ravenna. Therefore, pollen-bees seem to play an important role as pollinators in these species (Oleques *et al.* 2020). However, to assess the contribution of distinct pollinator guilds is important to collect data from the pollinator's performance allied to plant reproduction outputs

(Ne’eman *et al.* 2010). Among the different methods to estimate the contribution of distinct pollinator species or pollinator guild, stigmatic pollen deposition is considered a direct way to measure pollination success (Ne’eman *et al.* 2010). The combination of data from visitation frequency, stigmatic pollen deposition after a single visit and pollinator behavior enhance the comprehension about the effectiveness of a given pollinator guild, allowing the comparison between distinct groups of floral visitors (Ne’enam 2010).

Pollination limitation is a very common event in plants (Wolowski *et al.* 2014, Lázaro *et al.* 2015) and it occurs due to reduction of pollinators, resulting in a decline of the amount of pollen delivered on stigmas, affecting fruit and seed sets (Nayaki and Davidar 2010). The limitation of pollinators, causing a decrease in reproductive outputs of plants, play an impact in key processes that mold the diversity of flowering plants, including pollinator-mediated selection and the evolution to elf-pollination (Ashman *et al.* 2004). Unpredictability in pollinator community composition can result in heterogeneity in floral visitation by both efficient and opportunist insect pollinators, impacting the reproductive outputs of plant species (Gómez *et al.* 2010). Pollinators from distinct species or functional groups often vary in their performance and studies linking patterns of pollination limitation with direct estimates of the pollen deposition on stigmas and visitation frequency of different pollinators is rare (Koski *et al.* 2018).

Therefore, the goal of the present study was to investigate differences in the pollination performance of oil and pollen-collecting bees of three oil-producing species from distinct plant genera in Iridaceae. The genera are grouped in the same clade within Tigridieae, Iridoideae (Chauveau *et al.* 2012). Given that *Cypella* spp. are more visited by oil-bees and they are efficient during oil-collecting behavior (Oleques *et al.* 2020), our hypothesis was that oil-bees would contribute to higher total and conspecific stigmatic pollen deposition than pollen-bees in *Cypella herbertii.* In another hand, pollen-collecting bees would be more efficient on pollen deposition in *K. brasiliensis* and *H. pulchella*, being even more important in *H. pulchella*, given the high visitation frequency of pollen-bees in this species. Concerning the levels of pollination limitation, our hypothesis was that *C. herbertii* would experience higher pollination limitation when compared to *K. brasiliensis* and *H. pulchella*, due to the more specialized pollination system and the dependence of oil-bees (Moeller *et al*. 2012, Oleques *et al*. 2020). However, we also expected differences in hand-cross pollination and natural pollination in *K. brasiliensis* and *H. pulchella* due to the antagonistic behavior of oil-collecting bees visiting these species (Oleques et al. 2020).

Material and Methods

Studied species

The plant species studied (*Cypella herbertii*, *Kelissa brasiliensis* and *Herbertia pulchella*) were chosen in order to assess the different levels of specialization in oil-bee pollination as evidenced by the previous study carried out by Oleques *et al*. (2020). According to previous finds, *C. herbertii* is mainly pollinated by oil-bees (especially *Arhysoceble picta*), while *K. brasiliensis* presented a bimodal pollination system with contribution of both oil and pollen-collecting bees and *H. pulchella* is less specialized in oil-bee pollination being pollinated by a great range of pollen-collecting bees (Oleques *et al*. 2020). These species also presented distinct morphological match with the oil-bees and oil-foraging behavior results in ineffective visits (no contact with anthers or stigmatic surface) in flowers of *K. brasiliensis* and *H. pulchella* (Oleques *et al*. 2020).

All the six populations studied were located in the state of Rio Grande do Sul, Brazil, mainly in the Serra do Sudeste region. This region is part of Southern Brazilian Campos (Overbeck *et al*. 2007) and is recognized by a high diversity of oil-producing species of Iridaceae (Eggers *et al*. 2020). Furthermore, Serra do Sudeste region is of high conservation interest due the original landscape and the endemicity of its flora (MMA, 2007).

Floral visitor observations and reproductive tests

The observations and reproductive tests were performed along the flowering season of 2016 and 2017 from September to December in two distinct populations per taxa (Appendix 1). Further observations were carried out from September to November of 2019. Data from the number of visits, type and behavior of visitors were recorded during a total of 100 hours of field observation (*C. herbertii* = 40 h; *K. brasiliensis* = 31 h and *H. pulchella* = 29 h). The visitors were divided into two functional groups: Oil-collecting bees (OB) and pollen-collecting bees (PB), and the foraging behavior was divided in three: **Pollen** - collecting behavior of pollen-collecting bees; **Oil** - oil-foraging activity from oil-collecting bees, and **Oil + pollen** - when the oil-bees collected both oil and pollen in the same visit.

To better understand the role of pollinators in the reproductive outputs of the studied species we have performed two controlled reproductive experiments: 1) Hand-cross pollination - buds were bagged one day before the anthesis. After the anthesis, pollen from distinct individuals (> 1m far) were hand deposited in the stigmatic surface of the flowers (flowers were maintained bagged until set fruit) and 2) Natural pollination - flowers naturally exposed to pollinators (Dafni 2005). To access the pollination limitation, we used pollination limitation index proposed by Duan *et al*. (2007) calculated from the following formula: **LP = 1 - Pn / Pm**, where Pn denotes the number of fruit-set from natural pollination (control) and Pm manual cross pollination. Results vary from 0 to 1, where 0 indicate the absence of pollen limitation and 1 the highest level of pollen limitation. The difference between the fruit set from hand cross pollination and natural pollination was also used as a proxy to pollination limitation.

Pollen deposition on the stigma

The number of pollen grains deposited on the stigma per single visit (legitimate visits - visitors that came into contact with anthers and stigmas) was measured, and the stigmas from the distinct plant taxa were removed after a single visit of bees from a given pollinator functional group. Data from the type of visitor and reward collected during the visit were also considered. All buds were previously bagged and the flowers were emasculated before the stigmas were removed. The flowers were not emasculated before the visits because previous observations had demonstrated that the absence of anthers do affect the pollinator behavior.

Three stigmas from each flower (approx. 10 flowers per behavior) were collected to obtain estimated data on the amount of pollen present in the stigmas after a single visit. The stigmas were placed in small plastic tubes with 1 mL of distilled water and then macerated with a glass stick. The contents were inserted into the micropipette and the Neubauer chamber was covered with a blade. All the material was observed under an optical microscope for the purpose of counting pollen grains present in the chamber quadrants according to Carvalho (1989) and Radaeski & Bauermann (2016). The procedure for inserting pollen content in the Neubauer chamber for counting was repeated three more times, totaling four counts for each sample. The pollen grain counts were used in the formula: $N = a \times 1000 / 0.1 \times 1/3$, where a corresponds to the average number of pollen grains in the four counts, the number *1000* refers to the volume of distilled water used in the procedure, the value *0.1* corresponds to the volume of the Neubauer chamber and the number *3* refers to the number of stigmas used. The final value (N) corresponds to the number of pollen grains found in one stigma of the flower.

In order to consider both visitation frequency and pollen deposition on stigmas, we have calculated the **Pollination Performance Index** (PPI) of pollen and oil-collecting bees for each plant species, following the formula: *mean of visitation frequency x relative frequency of conspecific pollen on stigmas.*

Statistical analysis

The frequencies of visits were estimated for each of the three foraging behaviors using linear mixed-effects models (GLMMs) with a Poisson distribution, with plant species, foraging behavior and the interaction between them as fixed effects. To take into account the variation in observation times, the duration of observation was included as a log 10-transformed offset. Differences in the frequency of pollen, oil and oil+pollen foraging behaviors within the same plant species and among species were assessed with Tukey's post-hoc test using the function *glht()* from the multcomp package in R (R Core Team, 2015).

To analyze binary data related to fruit set obtained from hand-cross pollination and natural pollination treatments, data were fitted to GLMMs, considering pollination treatments as fixed effects and individual plants as random effects. The fit of logistic regression models was assessed using maximum likelihood analysis (Akaike information criterion). We performed a Wilcoxon rank-sum test to assess the difference between the numbers of total and conspecific pollen grains deposited on stigmas from each plant species carried by pollen and oil-collecting bees (Rhodes *et al.* 2017).

RESULTS

Floral visitors' observations and reproductive tests

We recorded a total of 794 visits from 20 bee species: 17 pollen-collecting bees and three oil-collecting bees. The number of visits was higher in *C. herbertii* (450 visits) than *H. pulchella* (260 visits) and *K. brasiliensis* (84 visits). However, the richness of pollinators was higher in *H. pulchella* (13 pollen-bees and two oil-bees) than *K. brasiliensis* (six pollen-bees and two oil-bees) and *C. herbertii* (two pollen-bees and three oil-bees). The visitation frequency was statistically different among species (χ^2= 722.60, df= 2, P< 0.001), pollinator behavior (χ^2= 836.52, df=2, P< 0.001) and the interactions between both factors (χ^2= 738.12, df=2, P< 0.001, Fig. 1). The visitation frequency of oil-bees, collecting both oil and oil+pollen was highest for *C. herbertii*. Therefore, oil and oil+pollen collecting behavior were significantly higher than pollen-collecting behavior played by pollen-bees (pollen *vs.* oil: Z= -12.961, P< 0.01; pollen *vs.* oil-pollen: Z= -22.194, P<0.01). There was no difference in the foraging behavior of oil-bees in *K. brasiliensis* and the frequency of both oil (illegitimate visits) and oil + pollen behaviors were not statistically distinct (Z= 0.269, P= 1.00). Additionally, pollen-collecting behavior performed by pollen-bees was not distinct from oil (Z= -2.005, P= 0.494) and oil + pollen (Z= -2.804, P= 0.905) behaviors. The frequency of oil collecting-behavior (illegitimate visits) was higher than oil + pollen for *H. pulchella* (Z= -6.652, P<0.01). The pollen-collecting behavior of pollen-bees was not higher than oil + pollen behavior (Z= 2.529, P= 0.187), but smaller than antagonistic oil foraging behavior (Z= -6.288, P< 0.01, Fig. 1).

Fruit set from natural pollination differed significantly from hand-cross pollination in *C. herbertii* (χ^2= 14.086, df=1, P <0.01) and *K. brasiliensis* (χ^2= 13.703, df=1, P< 0.01), but was not different in *H. pulchella* (χ^2= 4.618, df=1, P = 0.031). The pollination limitation index of all plant species was low and although the significant difference between natural and hand-cross pollination in *C. herbertii* and *K. brasiliensis* they did not present a high value of pollination limitation (PLI< 0.60, Table I, Fig.2).

Pollen deposition on the stigma

The total pollen deposition on stigmas was better promoted by pollen than oil-collecting bees and the same pattern was observed in conspecific pollen deposition (Fig.3). Pollen-collecting bees had deposited higher total (F= 7.136, df=1, p= 0.014) and conspecific (F= 7.497, df=1, p=0.012) pollen on stigmas compared to oil-collecting bees in *C. herbertii*. The same pattern was observed in total (F=8.136, df= 1, p= 0.027) and conspecific (F=6.03, df=1, p=0.02) pollen deposition in *H. pulchella*. However, there was no significant difference between pollen and oil-bees in total (F= 0.013, df= 1, p=0.80) and conspecific pollen deposition (F= 0.564, df=1, p=0.465, Fig. 4). The pollinator performance of oil-bees was higher than pollen-bees in *C. herbertii*. In *K. brasiliensis* the value was slightly elevated to oil-bees. However, in *H. pulchella* pollen-bees have played a higher performance than oil-bees (Fig. 5).

DISCUSSION

We hypothesized differences in the pollinator performance between two functional bee groups, being oil bees more effective in pollination of *C. herbertii* while pollen bees will be more efficient in *K. brasiliensis* and *H. pulchella* (Oleques *et al.* 2020). Our results considering the pollinator performance index corroborate that oil bees presented higher performance in *C. herbertii* and pollen bees in *H. pulchella,* but non-different performance between bee functional groups were observed in *K. brasiliensis*.

The oil bee group diversity observed in this study (three oil-collecting bee species) can be considered low compared with other similar systems in other regions (Cosacov *et al*. 2008, Martins *et al.* 2013, Rabelo *et al.* 2016, Bezerra *et al.* 2009). This fact can result from geographic limitations that may constrains the spectrum of oil bee species effective in pollinating these species (Thompson 2005). Added to this, the land use intensification, common in the studied area, could be associated with changes of richness in some specialized plant-pollinator systems (Marrero *et al.* 2014).

Floral oil is a very specific resource to oil-collecting bees species and particularly to females (Vogel 1974, Buchmann 1987, Aguiar and Melo 2009). Our previous study identified two different oil bee harvest behaviors as described in Oleques *et al*. (2020): an exclusive oil-collecting and oil plus pollen collecting in the same visit. These different bee behaviors were crucial to determine and differ the functional role of this bee group in the reproductive outputs of the studied species. While *C. herbertii* presented a prevalence of oil bee visits with both behaviors, *H. pulchella* presented higher mean values of oil collecting exclusively (antagonistic behavior) and *K. brasiliensis* did not show differences between the behaviors with low visitation rates in overall. The 17 pollen collecting bee-species observed presented similar frequency of visits on flowers in the three species studied. Other specialized pollination systems with oil-flowers highlighted the role of other functional bee groups as pollinators (Sigrist and Sazima 2014) and their potential as selective pressures agents is an interesting issue. Records of pollen on stigmas, allied to visitation frequency data, allowed us a better understanding of the effective role of both bee functional groups (Ne'eman *et al*. 2010). The mean values of total pollen and conspecific pollen deposited on stigmas of the three species indicates a significant role of pollen bees to pollination of this oil-producing set of plants. However, significant effects were found in *C. herbertii* and *H. pulchella* with non-significant differences to *K. brasiliensis*.

The three plant species studied differed in how floral oil was accessible to bees. *Cypella herbertii* was a particular system presenting a very narrow access to elaiophores leading to obligate contact with reproductive organs and the oil bee body (Oleques *et al*.

2020). Thus, oil bees were able to pollinate it even collecting exclusively floral oil. In another hand, *Kelissa brasiliensis* and *Herbertia pulchella* presented a widely accessible elaiophores allowing oil collecting foraging without anthers or stigma contact. However, the close anther and stigma positions in these species could contribute to the effective contribution of pollen bees to pollination. Pollen bees in oil-producing flowers could be considered not efficient pollinators in some other systems, as occurs in *Byrsonima* species (Malpighiaceae) (Sazan *et al*. 2013, Sigrist and Sazima 2014). Therefore, to some plant species pollen bees are also considered important in ensuring reproductive outputs in populations where oil bees are not present (Manning and Goldblatt 2002, Steiner and Whitehead 1996). Total pollen grains and conspecific pollen grains on stigmas of the three Iridaceae species studied were deposited by pollen bees with higher mean values. When we calculated the proportion of conspecific pollen by total pollen in stigmas of each plant species, we observed a strong contribution of oil bees to *H. pulchella* flowers with 100% of pollen conspecific deposited in its flowers. This result could be related with oligolectic behavior of two Tapinotaspidini bee species observed that are known by collecting pollen from a narrow group of plant families or genera (Schlindwein 1998). Contrary to our expectations, pollen bees contribute to higher total and conspecific pollen deposition on stigmas in *C. herbertii* and once more, it could result from oligolectic behavior of pollen bees recorded in *C. herbertii*. It is important to reinforce that *C. herbertii* was exclusively visited by solitary and oligolectic pollen bees, while *H. pulchella* and *K. brasiliensis* were also visited by social and generalist pollen bees as *A. mellifera Trigona spinipes* and *Mourella caerulea* (Kleinert and Giannini 2012, Kaluza et al. 2018). The oligolecty contributes to higher floral fidelity and pollination efficiency in some ecological contexts (Schlindwein 2004). Oligolectic bee behavior may be common in some ecological conditions such as the dry Pampean grasslands of Southern Brazil (Schlindwein 1998) and other semi-arid conditions (Minckley and Roulston 2006).

This behavior is frequently associated with visitation of plant species with highly attractive and accessible flowers. However, the oligolectic bee behavior (oil and pollen oligolectic bees) cannot be directly associated with enhanced reproduction outputs of host plants. Temporal or geographical variation in bee groups and morphological mismatching between bee and flowers could diminish their pollination efficacy (Minckley and Roulston 2006).

The pollinator index performance of oil bees was higher than pollen bees in *C. herbertii*, even pollen bees contributing to a better conspecific pollen deposition. It is mainly because of the expressive high visitation frequency of oil bees in this species, mainly considering both effective behavior: oil and oil+pollen. Considering the pollination performance index, our results corroborate our expectations and the specialization-generalization continuum suggested by Oleques *et al*. (2020), with *Cypella herbertii* being more specialized and dependent on oil bee pollination, *K. brasiliensis* presenting a bimodal system with important contribution of both pollen and oil bees and *H. pulchella* presenting a more generalist system with a great diversity of pollen bees acting as pollinators.

Another important result was the effective visits performed by oil-bees in *K. brasiliensis* and *H. pulchella* during pollen-collecting foraging, that is, oil bees are functionally acting as pollen-collecting bees in these species (Koski *et al.* 2018). Studies on *Sisyrinchium* also evidenced that oil bees involved in the pollination of these species also collect pollen during oil foraging (Cocucci and Vogel 2001, Truylio *et al.* 2002). Observations conducted by Truylio et al. (2002) of the behavior of the specialized oil bee *Lanthanomelissa betinae* on *Sisyrinchium micranthum* Cav. flowers showed that pollen attracts primarily female bees, suggesting an essential role of pollen in oil bee pollination of Iridaceae. This information permits us to infer that floral oil plays a crucial role in the oil-bees attraction to *H. pulchella* and *K. brasiliensis* flowers, once we did not observe only pollen collecting behavior by oil-bees visits (Oleques *et al*. 2020). It could be related to the presence of diacetin in floral oil. Diacetin is considered a chemical communication channel between oil-producing flowers and oil bees, being an important mechanism related to the specificity of this specialized pollination system (Schäffler *et al.* 2012). Further studies could better describe the existence and functionality of this chemical compound in these species.

We observed lower values of natural pollination fruit set than cross pollination in the oil-producing flowers set studied (not higher than 45%). This pollination limitation (PL) widely occurs in plant species (Larson and Barrett 2000, Knight *et al.* 2005; Wolowski *et al.* 2014) and it represents a selective ecological pressure on the diversity of plant reproductive systems and predicts that plants will evolve to reduce PL. For instance, it has been argued that floral traits that enhance pollinator attraction or lessen reliance on pollinators (i.e., obligate self-pollination) may be selected to diminish the PL effects (Ashman *et al.* 2004).

Taken together, the results presented here evidenced that both pollen and oil bees, in distinct extent, are important pollinators of the studied species. This study showed that oil bees could act functionally as effective pollen-collecting bees and be opportunist when collecting specialized floral reward in *K. brasiliensis* and *H. pulchella*. The production and maintenance of a floral reward represents a high cost to plants and it is favored by selective pressure exerted by pollinators and its positive effect in plant fitness (Whitehead *et al.* 2012). Therefore, further studies on *K. brasiliensis* and *H. pulchella* populations in areas with effective oil bees, as *Centris* spp., are needed. It is because *A. picta* and *C. muelleri* observed could deplete the floral reward available to effective oil bees, acting as antagonists and impacting the reproductive outputs of the plant species (Koski *et al.* 2018). Our study, as other conducted in the same region (Schlindwein 1998, Oleques *et al.* 2020, Báez-Lizarazo *et al.* 2021) did not observe any other oil bee that could match with *K. brasiliensis* and *H. pulchella*. However, the opportunistic behavior played by the oil bees found in the populations studied, and even the contribution of pollen bees, could be negative and impact the food niche partitioning of the oil bee guild (Johnson 2010, Ponisio *et al.* 2017, Phillips *et al.* 2020).

Finally, the oil and pollen bees do differ in the pollination performance and it depends on the species. Oil bees and pollen bees are important pollinators and contribute to high pollen deposition on stigmas of *C. herbertii*, *K. brasiliensis* and *H. pulchella*. Functionally, oil bees performed better than pollen bees in the pollination of *C. herbertii*, equally to the pollination of *K. brasiliensis* and pollen bees performed slightly better in pollination of *H. pulchella*. Our results reinforce that specialization of oil pollination systems is a continuum and other functional groups of bees could act synergically in the pollination of oil-producing flowers.

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Figures

Figure 1. Visitation frequency of oil and pollen bees, including the two behaviors of oil bees (oil and oil + pollen) in the three species studied.

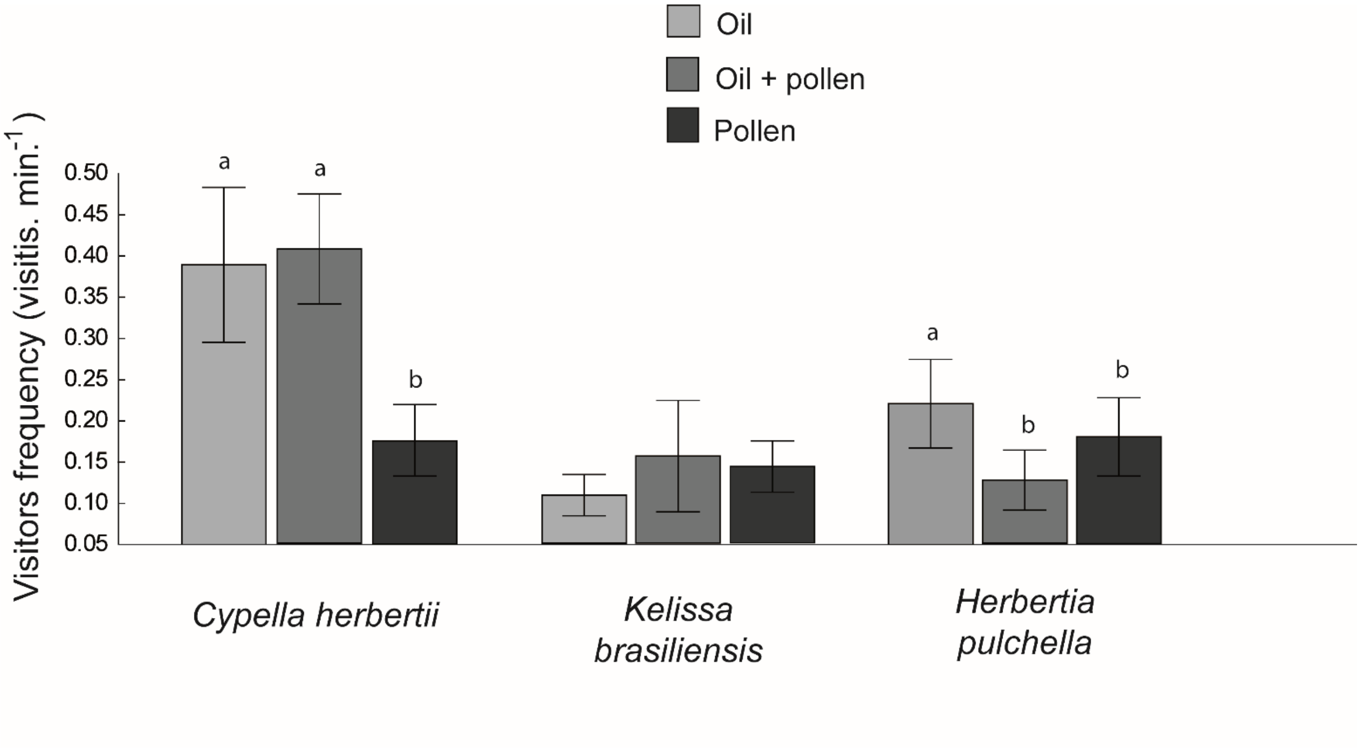

Figure 2. Fruit set from hand cross (CP) and natural pollination (NP). The asterisk and p values are indicating significant differences between treatments.

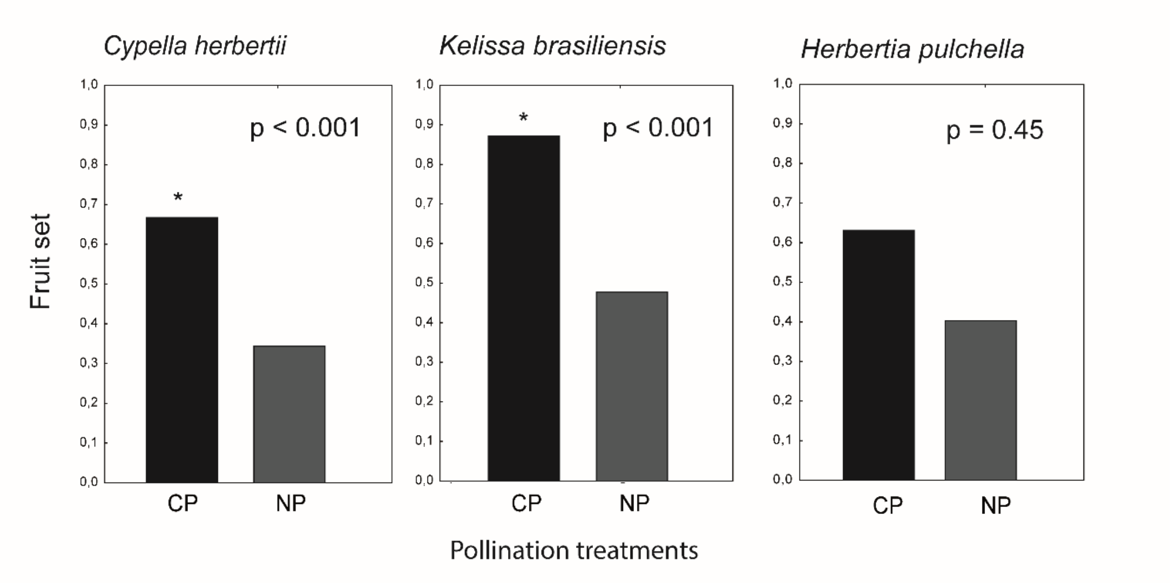

Figure 3. Total pollen grains (left) and conspecific pollen grains (right) deposited on stigmas of all species studied by pollen and oil bees. Mean ± standard error; p value is indicating the statistic significance of the tests.

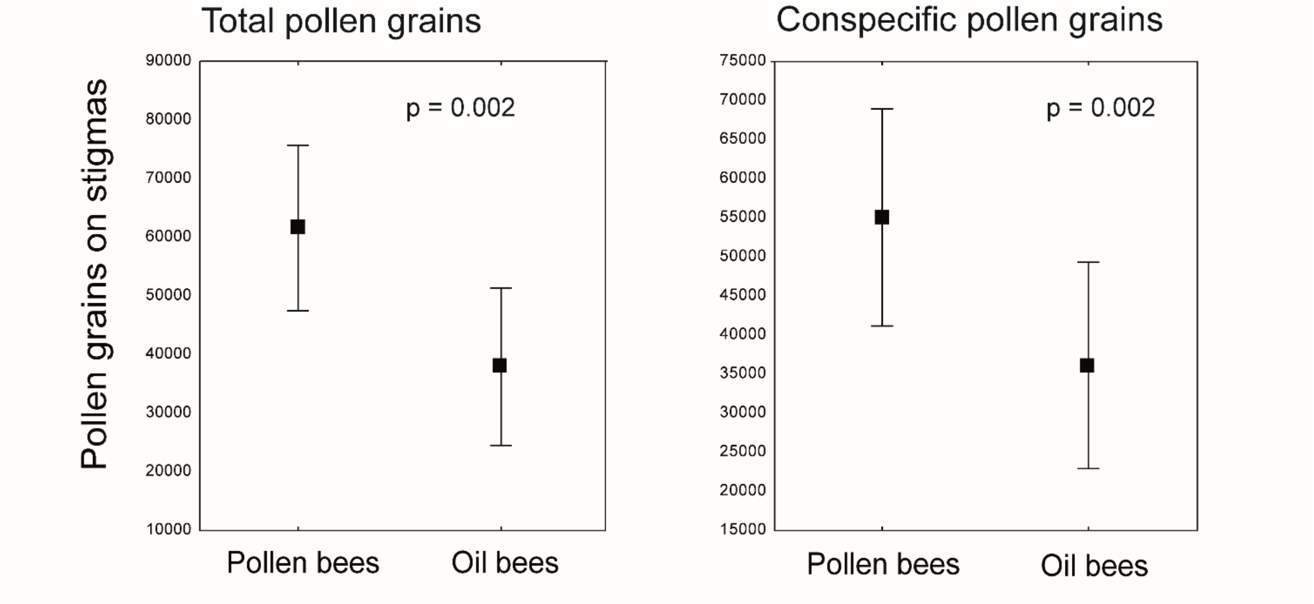

Figure 4. Pollen grains deposited on stigmas of each plant species from pollen-collecting bees (dark grey) and oil-collecting bees (light grey). From left to right: total pollen grains deposited, conspecific pollen grains and at least the relative proportion of conspecific pollen grains deposited on stigmas. * $p<0.05$; **$p<0.01$; ns: non-significant difference.

Figure 5.

CAPÍTULO III

POLLINATION FAILURE IN *TRITONIOPSIS*: A CASE OF BREAKDOWN BETWEEN OIL-BEES AND OIL-PRODUCING FLOWER INTERACTION

Pollination failure in *Tritoniopsis*: a case of breakdown between oil bees and oil-producing flower interaction

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Abstract

Among all pollination systems found in Iridaceae the predicted oil-bee pollination in the South African species *Tritoniopsis parviflora* is particularly interesting: it is the only oil-producing Iridaceae outside the Americas and compared to other Iridaceae species, it is the only system where oil and nectar are secreted in the same flower. The aims of this study were to evaluate oil and nectar secretion dynamics and the reproductive outputs of *T. parviflora* in four distinct populations; and also to identify pollinators and characterize their foraging behaviour. Our findings about floral biology and floral reward secretion dynamics suggest an inefficient attractivity of oil-collecting bees to the flowers, associated with low reproductive outputs in the four populations.. We have provided some theoretical background, based on our findings, to suggest the existence of a dependence of co-flowering oil-producing species to enhance the attractivity of *T. parviflora.* We also presented data on nectar and oil-production dynamics, suggesting a novelty in terms of segregation in reward offered by male and female phases of protandrous flowers. These results about floral anatomy, reward secretion dynamics and diacetin production in *T. parviflora* are the first evidences to support further studies on pollinator efficacy, facilitation and floral mimicry.

Key-words: floral reward – Iridaceae - oil-producing flowers - oil-collecting bees, pollination – protandry – pollination systems - specialization

Introduction

Interactions between oil-producing flowers and oil-bees are considered one of the most emblematic mutualisms among angiosperms. It is because oil-flower species usually are highly specialized in attracting a small group of oil-collecting bees compared to species which produce nectar and could be visited by a wide diversity of pollinators (Vogel 1974, Aguiar et al. 2009). Oil is considered the third most important reward collected by pollinators, after pollen and nectar (Buchmann 1987; Chauveau et al. 2012). There are approximately 2.000 oil-producing species distributed in 11 unrelated plant families which are visited by 450 specialized oil-bee species (Buchmann 1987; Martins, Melo and Renner 2015, Possobom and Machado 2017). Lately, an interesting mechanism of flower attraction in this specialized pollination system was discovered (Schäffler et al. 2015). The specificity of this pollination system can be scent-mediated through an uncommon compound among Angiosperm: the diacetin. This compound is considered a private communication channel between oil-flowers and oil-collecting bees. Shäffler et al. (2015) have shown that diacetin is widespread among oil species and detectable for oil-bees as a cue for locating oil flowers.

Iridaceae is one of the most diverse plant families who produces oil as a reward to pollinators. All oil-producing species are concentrated in New World tribes of Iridoideae (Chauveau et al. 2012), with the exception of *Tritoniopsis parviflora* (Jacq.)G.J.Lewis which belongs to Crocoideae, an exclusively African subfamily (Manning and Goldblatt 2005). The presence of elaiophores has evolved multiple times independently in the American tribes of Iridoideae, and the interaction with oil-bees possibly have played an important role in the diversification of Iridoideae on American continent (Chauveau et al. 2012). Recently, Oleques et al. (2020) evidenced different levels of specialization in oil-bee pollination systems among distinct genera of oil-producing Iridaceae in Southern Brazil.

Iridaceae presents a wide range of pollination systems, especially in the African continent where there are species pollinated by hawkmoths, butterflies, birds, beetles, flies and bees (Goldblatt and Manning 2006; van der Niet et al., 2014; Cocucci and Johnson 2017). Among all pollination systems found on African continent the predicted oil-bee pollination in *T. parviflora* is particularly interesting (Manning and Goldblatt 2002). Besides being the only oil-producing Iridaceae outside the Americas *T. parviflora* also represents the only system within Iridaceae where oil and nectar are secreted in the same flower (Manning and Goldblatt 2002). *Tritoniopsis* L.Bolus is known for the great floral variation and by the diversified pollination systems, being nectar the most important floral reward offered to distinct pollinator guilds, such as bees, butterflies, birds and hawkmoths (Manning and Goldblatt 2005).

The first record of oil-producing flowers and possible oil-bee pollination in *T. parviflora* was reported by Manning and Goldblatt (2002). In this paper the authors have described important aspects concerning floral biology of the species as hercogamy, protandry, scent chemical composition, amount of nectar produced and the observation of oil-bee visits. However, all the finds were based on a few hours of observation conducted in only one population situated in Kogelberg Biosphere Reserve, in Kleinmond, South Africa. In addition, the oil-secretion was tested with crystals of Sudan IV and no anatomical analysis was conducted. According to Manning and Goldblatt (2002) several flowers of *T. parviflora* were visited by the oil-bee *Rediviva gigas* and the bees cached had *T. parviflora* pollen attached in the anterior part of the dorsum and the vertex of the head. They also have found several pollinias of an oil-producing orchid attached to the bees. *Tritoniopsis parviflora* shares some characteristics as strong scented yellowish/maroon flowers with a guild of oil-producing orchids from the genera *Pterygodium* Sw. and *Ceratandra* Eckl. & F.A.Bauer (Pauw 2006, Pauw & Bond 2011). One of the hypotheses assumed by Manning and Goldblatt (2002) to explain the production of both oil and nectar by *T. parviflora* is that nectar would be an important reward to attract nectar-feeding bees in regions where the occurrence of *R. gigas* is unpredictable.

The most part of the oil-producing flowers lack nectar as a reward to pollinators and there are two hypotheses behind this: the ancestors of oil-flowers were nectar-less or oil was strongly selected to be the only reward offered (Ferreiro et al. 2017). The coexistence of both rewards could represent an evolutionary scenario when one of them is under selective pressure to become the only reward available or nectar is essential to ensure the reproduction in regions where the oil-bees are unpredictable (Ferreiro et al. 2015, 2017). The unpredictability of oil-bees is well described in the literature and the local extinction of these specialized bees results in a decrease or even failure in plant reproduction (Cocucci 1991, Steiner 1993, Steiner and Whitehead 1996). Therefore, the reduction or elimination of oil-bees dependency is expected and could result in the loss of oil-producing structures followed by a shift to other pollination systems with pollen as the sole reward (Steiner and Whitehead 1996, Chauveau et al. 2012, Renner and Schaefer 2010).

The aim of this study was as follows: (1) to identify pollinators and characterize their foraging behaviour; (2) to characterize the oil and nectar secretion dynamics along the lifespan of *T. parviflora* flowers and (3) to describe the general anatomical floral traits.

Our expectations concerning *T. parvilflora* were: 1) To find differences in oil and nectar-feeding bees visitation rates among the four populations studied, being the reproductive outputs higher in populations mainly visited by oil-bees than compared to populations visited by nectar-feeding bees; 2) Nectar production would be higher in populations visited by nectar-feeding bees; 3) Oil-secretion, assessed by the amount of diacetin, would increase in populations visited by oil-bees; 4) The oil-secreting structures would be well developed septal elaiophores and oil the main reward secreted along the flowers lifespan.

Material and Methods

Plant species and studied area

Tritoniopsis parviflora is a seasonal geophyte found in the western areas of the Cape Floral Region in South Africa. It is usually distributed in mountain fynbos on sandstone slopes and flowering abundantly in burnt areas just after the season following the fire (Manning and Goldblatt 2002). The distribution of *T. parviflora*, as the most part of species in the genus, is restricted to the summer-dry Cape Floristic Region (Manning and Goldblatt 2005). The flowers are distributed in a dense spike and are strongly bilabiate (ca. 20mm long). Most species of *Tritoniopsis* are characterized by odorless flowers (Manning and Goldblatt 2005). However, *T. parviflora* flowers produce a strong sweet/acrid scent resembling the same scent produced by orchids from the same guild of oil-producing flowers in the genera *Disperis* and *Pterygodium*. The flowering peak is during the summer months from November to January, overlapping with the late-flying oil bee *Rediviva gigas* (Whitehead and Steiner 1993).

Field observations were conducted in four distinct populations of *T. parviflora*. The populations were distributed along the Western Cape province in South Africa in order to cover the range of *R. gigas* distribution and post fire areas (Appendix I), being the first the same local when Goldblatt and Manning (2002) have found *R. gigas* visiting *T. parviflora*. The number of individuals in each population was recorded and we also searched for other oil secreting plants in the community. To evaluate the existence of other co-flowering oil secreting plants a transect of 100m was tracked.

Western Cape province is part of the Greater Cape Floristic Region of Southern Africa. It is considered one of the most florally diverse regions of the world (van der Niet et al. 2014). Studies suggest that abiotic factors are the most important drivers of speciation in the Cape environment. Moreover, Cape Floristic Region is known by the high diversity of specialized plant-pollinator interactions, including oil-collecting bees, Table Mountain Pride butterfly, nectarivorous birds and long-tongued flies pollination systems (Alexandersson and Johnson 2002; Johnson, Harris, and Proche 2009; Johnson and Wester 2017). This high diversity of specialized pollination systems may possibly act together with habitat changes to explain the high species richness of the Cape Region (Alexandersson and Johnson 2002; Van der Niet et al. 2014).

Floral visitors

Floral visitors were monitored on sunny days, during intervals of 15-30 min from 8h00 am to 17h00 pm. Multiple randomly chosen flowers were surveyed in each population, and the total number of visits, in addition to the type and behaviour of the visitor, were consistently recorded. A total of 94 hours of observation were conducted by two observers. Visitation frequency was calculated as the number of visits per minute of observation, and flower visitors were classified as legitimate and illegitimate according to the behaviour and reward collected (pollen, nectar and oil).

Reproductive outputs and floral life span

In order to indirectly assess the impact of pollinators in the reproductive outputs of *T. parviflora* we conducted natural pollination treatments in all populations studied. A total of 267 flowers from 61 different individuals were marked (Appendix I). We also collected a total of 37 fruits to measure the seed-set, being nine fruits from Greyton, seven from Michell's Pass, eight from Kogelberg and 13 from Palmiet.

To better understand the nectar and oil secretion dynamic we divided the flower lifespan in five stages namely: **Stage I** (flower bud, anthers closed and stigma not receptive), **Stage II** (flower opened, one of the anthers with small amount of pollen, stigma not receptive, lower tepals start flush maroon), **Stage III** (anthers with pollen and stigma not receptive, lower tepals completely maroon), **Stage IV** (the style descends and the stigmatic arms partially diverge and are then assumed to be receptive, anthers and lower tepals are dry and senescent). The stages were based on Manning and Goldblatt (2002) and field observations.

Nectar volume measurements were made from previously bagged flowers on stage II, III and IV. Nectar was withdrawn from the base of the floral tube flowers with 1μl capillary tubes.

Diacetin tests

Floral scents for chemical analyses were collected from 60 flowers in 15 individuals distributed in all populations (at least three individuals per population). Flowers were removed from the plants using clean forceps and extracted for one minute in 2–3 ml pentane. Flowers from the same individual were put together and considered as a single sample.

To identify the presence of diacetin, 1 μl of the flower extracts was analyzed on a Varian CP-3800 gas chromatograph (Varian, Palo Alto, California, USA) with a 30×0.25-mm internal diameter (film thickness 0.25 μm) Alltech EC-WAX column, coupled to a Bruker 300-MS quadrupole mass spectrometer in electron-impact ionization mode and a 1079 injector. Compound identification was done by searching mass spectra and the Kovats

Retention Index of monoacetin, diacetin and triacetin using the Bruker Workstation software Version 7.0 in combination with the NIST Mass Spectral Program for the NIST Spectral Library Version 2.0. Once diacetin peaks were identified the quantification was based on integrating the area under peaks in chromatograms.

Anatomical analyses

For anatomical investigations of secretory structures and to identify the secretion in flowers from different stages, fresh flowers were field fixed in two fixatives: (1) Formaldehyde, acetic acid and ethanol (FAA) (formaldehyde: acetic acid: 50% ethanol, 1:1:18 by volume) (Johansen, 1940) and (2) Glutaraldehyde (2.5%) and formaldehyde (4.5%) in 0.1 M phosphate buffer, pH 7.2 (Karnovsky 1965). We fixed four flowers per flower stage in each fixative: pre-anthesis buds, Stage I, II, III and IV. The material was maintained in the fixing solution for the further anatomical and histochemical analyses. For the investigations of secretory structures, inner and outer tepals and gynoecium were subsequently examined using light microscopy (LM). In addition, scanning electron microscopy (SEM) was used for tepals.

For structural observations, samples were washed three times in 0.1 M sodium phosphate buffer, dehydrated in ethanol series, and embedded in 2-hydroxyethyl methacrylate resin Technovit 7100 (Heraeus Kulzer, São Paulo, Brazil). Transverse and longitudinal sections (5 µm) obtained with a Leica RM 2245 microtome were stained for structural observations with 0.05 % toluidine blue O pH 7.2 in phosphate buffer (O'Brien and McCully, 1981). Semi-thin sections were submitted to histological tests to the following metabolic groups: Lugol solution to detect starch (Johansen, 1940), periodic acid-Schiff (PAS Reaction) for total polysaccharides (O’Brien and McCully 1981), Ponceau Xylidine for total proteins (Vidal 1970), Ruthenium red for pectins and mucilages (Johansen 1940; Mariani and Rascio 1977).

Additionally, free hand-sections of tepals were washed three times in 0.1 M sodium phosphate buffer for 20 min and submitted to test for detections of total lipids with Sudan IV (Jensen 1962), and Sudan Red 7B (Brundrett et al. 1991) and appropriate negative controls were run simultaneously with methanol/chloroform/H2O/HCl (66: 33: 4:1). Observations were carried out using a bright field microscope Leica DMR-HC equipped with a digital camera AxioCam HRc Zeiss and software ZEN Light 2012. The images were recorded in the database of the plant anatomy laboratory at University of Rio Grande do Sul (LAVEG – UFRGS).

Scanning Electron Microscopy (SEM) – For SEM, fixed inner and outer tepals were dehydrated with 2,2 dimethoxypropane (Thorpe and Harvey, 1979) for 30 min (2x), and stored for 24 hrs. Subsequently, the tepals were subjected to the drying process by the critical-point drying using liquid CO_2 method on the BAL-TEC CPD 030 equipment. After, the tepals were then sputter-coated with gold and examined using a JEOL JSM 6060 scanning electron microscope, at an accelerating voltage of 10 kV in CME – UFRGS (Microscopy and Microanalysis Center).

Statistical analysis

To analyze binary data related to fruit set (natural pollination) obtained from the different populations, data were fitted to using general linear mixed-effects models (GLMMs), considering populations as fixed effects and individual plants as random effects. The fit of logistic regression models was assessed using maximum likelihood analysis (Akaike information criterion). Fruit and seed set from plants of distinct populations was respectively compared using GLMMs with a binomial and Poisson distribution. Differences in the presence and absence of diacetin among populations were assessed using GLMMs with a binomial distribution. Finally, a deviance analysis was performed within each GLMMs analysis to test whether model deviance was greater than expected by chance and whether each model as a whole was better than the null model (Bolker et al., 2008).

Difference in nectar production between flowers from stage III and IV was analyzed by Mann-Whitney Test. To evaluate differences in nectar production among populations we performed a Kruskal-Wallis rank sum test. All statistics were performed in R (R Core Team 2015).

RESULTS

Floral visitors

We recorded 49 flower visits (19 illegitimate and 20 legitimate) from five bee species during a total of 94 hours of observation. Legitimate visits were considered when the bees touched anthers and/or stigmatic surfaces of the flowers during the foraging behavior. The bees were caught during nectar and/or pollen foraging behavior, being recorded 20 nectar-feeding visits, nine pollen-collecting visits and nine visits by bees foraging both nectar and pollen. No oil-bees were observed in the four populations studied. The mean of visitation frequency was 0.12 and 0.08 (visits/min) in Palmiet and Kogelberg, respectively. No visits were observed in plants from Greyton and Michell’s Pass populations.

Floral lifespan and reproductive outputs

In general fruit set was low (about 16%), being higher in Palmiet, when 21% of the flowers marked set fruits, and lower in Greyton when only 11% of flowers marked set fruits. However, the seed set significantly differed among populations; it was higher in plants from Palmiet ($\chi^2 = 50.116$ d.f = 3, $P < 0.001$, Table I, Fig. 1).

Floral lifespan was monitored and we observed the stigmatic receptivity dynamic, anthers opening with pollen availability and floral reward offered (oil and nectar). Stage I was characterized by immature reproductive organs and no nectar or oil secretion. In the Stage II pollen exposure started and we observed the first signals of lipid-secreting but only in the stage III we observed active oil secretion. In this stage pollen was totally exposed (dehiscent anthers) and we recorded small amounts of nectar secreted (Fig. 2). However, 50% of flowers from stage III were nectarless. The volume of nectar secreted was significantly higher in flowers in the stage IV, compared to flowers in stage III (U= 80.5, Z = 5.71, p <0.001; Fig. 2). Considering only flowers in stage IV, we observed significant variation in nectar volume secreted among populations with higher mean values in Kogelberg population ($\underline{X} = 0.57\, ul \pm 0.24$, $\chi^2 = 15.82$, d.f = 3, p = 0.001) when compared to flowers from Palmiet ($\underline{X} = 0.47\, ul \pm 0.11$), Michell's Pass ($\underline{X} = 0.33\, ul \pm 0.09$) and Greyton ($\underline{X} = 0.27\, ul \pm 0.08$). Fruit set from natural pollination did not differ significantly among plant populations ($\chi^2 = 4.204$. d.f = 3, P = 0.240; Fig 1).

Diacetin tests

The trace amount of diacetin found in each sample was not enough to quantify and compare populations. Thus, we only consider the presence and absence of the compound in the samples analyzed. Trace amounts of diacetin were found in six of the 15 samples collected: two samples from Kogelberg, three from Palmiet and one from Greyton with no significant difference among them ($\chi^2 = 2.092$ d.f = 2, P= 0.351). No diacetin traces were found in samples from Michell's Pass. No traces of monoacetin and triacetin were found in all samples collected.

Secretory structures

Morphological and histological analyses revealed secretory structures located in tepals and ovaries of *T. parviflora*. On the adaxial surface of the three lower tepals was possible to observe a region of papillate epidermal cells (Fig 3 A). The papillate cells have a striated cuticle without visible ruptures (Fig 3 C), and the accumulation of secretion below the cuticle ("blister") has rarely been recorded. In stage III, the secretion observed in the papillae stained positively for the total lipid test with Sudan IV (Fig 3 D) and Sudan Red 7B (Fig 3 E-F). In the lower tepal cross section it was possible to observe a layer of papillary cells in the median region (Fig. 3 G). Under the papillate epidermis, the mesophyll has 5 to 8 cell layers (Fig. 3 J) with large intercellular spaces and numerous idioblasts. In some regions of mesophyll, during pre-anthesis and Phase I stages, cells accumulate starch and polysaccharides (Fig. 3 H-I) and intercellular spaces mucilage/pectic substances (Fig 3 J). On the abaxial face, the epidermis cells have an elongated shape in the anticlinal direction and thickened cuticle on the external periclinal wall.

Results indicate that the lipid-secreting activity occurs between stages II and III of the anthesis (Fig. 3 K-L), but decreases as the flower approaches senescence, in stages IV. In the final stage of anthesis, apoptosis occurs in the cells of tepal, so that only the cell walls are observed, including the lignified wall of the xylem vessel elements (Fig. 3 M).

Anatomical analyses of *T. parviflora* allow the identification of a gynopleural (or septal) nectary consisting of three clefts located in the septal region of the ovary, between adjacent carpels (Fig. 3 N). The nectary cavity is very narrow and has a single-layered epithelium, characterized by cells compactly arranged, with dense cytoplasm and large nuclei (Fig. 3 O-P). Under the epithelium of each secretory cavity there is a compound subsidiary tissue 4 - 5 cell layers that are smaller than the cells of the other parts of the ovarian parenchyma. Vascular bundles, with phloem and xylem, and idioblastic cells were observed surrounding this tissue.

DISCUSSION

Contrary to our expectations oil-bees were not observed in any *T. parviflora* populations and the reproductive outputs were low in all of them, mainly in those where no visits were recorded. The frequency of illegitimate visits was quite impressive as well the failure in attracting oil bees and the low visitation frequency of pollen and nectar-feeding bees. The unpredictability and the very low levels of diacetin detected was also unexpected, and probably this trait can be associated with failure in attracting oil-bees. The anatomical analysis confirmed the presence of oil-secretion structures, but the secretion was rarely found. Oil and nectar had distinct secretion dynamics and it might be the first evidence of partitioning reward along the flower lifespan of a protandrous species.

Taking together, our findings about the floral biology and the floral reward secretion dynamics suggest an inefficient attractivity of oil-bees to the flowers on the populations studied, associated with low reproductive outputs along *Tritoniopsis parviflora* distribution. We have provided some theoretical background, based on our findings, to suggest some hypothesis behind the *T. parviflora system*: First, the existence of a dependence of co-flowering oil-producing species, as Orchids,to enhance the attractivity of *T. parviflora.*

Tritoniopsis parviflora was previously described as having protandrous and herkogamous flowers (Manning and Goldblatt 2002). Protandry is an important adaptative strategy in hermaphrodite plants and it is related to the temporal separation of male and female phases in order to reduce self-pollination within and among flowers in the same inflorescence (Lloyd 1986, Jersáková and Johnson 2007). There are several reports on differences in nectar production between sexual stages in protandrous plant species (Devlin and Stephenson 1987, Aizen and Basilio 1998, Varga *et al.* 2013, Stpiczynska *et al.* 2015). However, this is the first report of different types of reward being secreted in distinct sexual phases. Our results suggest different reward secretion dynamics in distinct stages along the flower lifespan. *Tritoniopsis parviflora* secretes nectar during the female phase, mainly in the stage IV, and the anatomical analysis reveals that before that the ovary and floral tube are undeveloped. The lipid-secretion activity and pollen exposure start at the same stage, during phase II.

Bee pollination was confirmed in our study and *Amegilla* sp. was the most frequent floral visitor, mainly in the Palmiet population. However, we recorded an extremely low visitation frequency in *T. parviflora* flowers and no oil-bee visits in all populations studied. Therefore, few bees had played legitimate visits during the foraging behaviour and a great number of visits results in bees collecting pollen and nectar without contact anthers or the stigmatic surface.

The low visitation frequency and reproductive outputs with some evidence of pollinator limitation have already been suggested as an effect to post fire flowering species from Orchidaceae, Amaryllidaceae and Iridaceae plant families in South Africa (Johnson and Bond 1997). This low frequency of oil-bee visits is consistent with the Manning and Goldblatt (2002) records that observed a couple of visits of *Rediviva gigas* in *T. parviflora* and the extremely low frequency of *R. peringuey* in some oil-bee pollinated Orchidaceae in the Cape region (Pauw 2006). Oil-producing species from the genus *Ixianthes* (Stilbaceae) pollinated by *R. gigas*, showed extremely low reproductive rates in the absence of *R. gigas* visits and some populations had experienced no visits of the specialized pollinator along years (Steiner 1993, Steiner and Whitehead 1996). The oil-bee *R. gigas* is known by the late-flying behaviour, compared to other species in the same genus, and as the main pollinator of the oil-flowers from the genus *Ixianthes*.

According to Steiner and Whitehead (1996) the relationship between *R. gigas* and *Ixianthes* plus Scrophulariaceae species are older than the evolution of the oil-producing orchids (Renner and Shaefer 2010, Kahnt *et al.* 2019). Thus, the guild of oil-producing orchids probably evolved in order to take advantage of the pre-existing interaction between *Ixianthes* and *R. gigas.* The same pattern was recorded in the interactions of orchids and other oil-bees from the genus *Rediviva*. Thus, the interactions between orchids and *Rediviva* bees has probably evolved opportunistically (Johnson and Steiner 1994, Pauw 2006).

Our finds suggest that besides the absence of oil-bee pollination in the populations studied, nectar and pollen collecting bees' visits were low, resulting in equally low reproductive rates. However, nectar is probably important to ensure some fruit and seed set, even if at minimal levels, as we observed in all *T. parviflora* populations.

The unpredictable and low amount of diacetin produced by *T. parviflora* flowers can be considered as a key result in our study. Diacetin is an uncommon compound, found exclusively in oil-producing flowers around the world. It is effectively detected by different oil-bee species indicating a coadaptative adjustment in chemical signaling between plants and pollinators (Schäffler *et al.* 2015, Castañeda-Zárate *et al.* 2020). Taken together, the trace amount of diacetin, the rare accumulation of oil secretion and the absence of oil-bees could be an evidence of oil-pollination system failure in not co-flowering populations from the same guild or a local decline in oil-bees populations. Oil secretion is a novelty in African Iridaceae. The genus *Tritoniopsis* presents high diversification in pollination systems with nectar and pollen flowers (Manning and Goldblatt 2005). In this way, the maintenance of nectar secretion, common in the genus, added to an innovative floral reward could be the result of some strategies to ensure the reproductive rates in a very diverse region of southern Africa with some implications of interspecific competition (Van der Niet *et al.* 2014).

The species presented an epithelial elaiophore, being the lipid secretion protected by a cuticle, contrasting with the trichomatous elaiophores found in the most part of American species of oil-producing Iridaceae (Chauveau *et al*. 2012). Epithelial elaiophores presented the lipid secretion accumulated and protected by the cuticle. In some species the pollinators have to promote the rupture of the cuticle to access the lipid secretion. However, trichomatous elaiophores presented a continuous and unprotected secretion (Tölke *et al*. 2019). Although the type of elaiophore could represent an adaptation to distinct bees, *R. gigas* pollinate plant species with trichomes and glandular elaiophores (Steiner 1998, Pauw 2006).

Our results highlighted the existence of lipid secretion that probably evolved by selective pressures alongside the guild of oil-producing orchids, in order to take advantage of the pre-existing relationship between this pre-existent guild and *R. gigas.* Geographical area of *T. parviflora* occurrence is characterized by frequent fire followed by flowering of several plant species, including other species pollinated by *R. gigas* as the orchids *Ceratandra atrata* (L.) T. Durand & Schinz, *C. bicolor* Sond.ex Bolus, *C. harveyana* Lindl., *Evotella rubiginosa* (Sond.ex Bolus) Kurzweil & H.P. Linder, *Pterygodium acutifolium* Lindl. and the *Ixianthes retzioides* E. Mey. ex Benth. (Steiner 1993, Manning and Goldblatt 2002, Kuhlmann and Hollens 2014).

The co-occurrence of a guild of plants could facilitate the attractivity of the pollinators and enhance visitation frequency and consequently the reproductive outputs (Rathke 1983, Ogilvie and Thomson 2016). However, no other co-occurring oil-flowers were found in the populations studied areas. It could result in the absence of oil-bees in the *T. parviflora* populations and the low reproductive rates found. The studied species shares some floral traits with other oil-secreting orchids and probably the oil has evolved in *T. parviflora* as a way to take advantage of pre-existing interactions between orchids and *R. gigas.* Manning and Goldblatt have found pollinias of *Pterygodium* attached in the *R. gigas* individuals catched during the visits to *T. parvilflora.* In this way, the interactions between *T. parviflora* and *R. gigas* could be dependent on co-flowering orchids, or other oil-producing flowers as Scrophulariaceae species, in order to enhance the attractictity and the efficacy of the opportunistic nature of this interaction. Cavalheiro *et al.* (2014) have shown that the potential for one plant species to influence another indirectly via shared pollinators was higher for plants whose floral rewards were more abundant. Variations in the pollinator assemblage can be caused by spatial (Herrera 2005) or temporal (Price *et al*. 2005) fluctuations in the population densities of pollinator species, but it could also be caused by variation in the plant community composition. Therefore, co-flowering plant species, offering the same reward to the same guild of pollinators, may facilitate each other in terms of pollinator attraction (Rathcke 1983, Waser 1983, Callaway 1995) and therefore they can affect each other's pollinator visits and reproductive outputs. Some studies have investigated how pollinator-sharing influences the patterns of pollinator foraging behaviour (Stout et al. 1998, Johnson *et al*. 2003), and how this affects visitation rates and plant reproduction (Feinsinger *et al.* 1991, Brown *et al.* 2002, Ghazoul 2006). Beyond that, the co-flowering species can also influence the diversity and composition of visits that particular plant species receives, because the attractiveness of a plant species for a particular pollinator, especially in specialized reward offered as oil, might not only depend on the traits of the individual plant species itself, but also on the presence, attractiveness and abundance of co-flowering plants (Grindeland *et al*. 2005, Kudo and Harder 2005, Hercsh and Roy 2007).

The absence or low visitation rates of oil-bees could act as selective pressure to the maintenance of nectar secretion in order to ensure that the flowers will be attractive to other bees than *R. gigas* and then ensure some fruit and seed sets (Manning and Goldblatt 2002). Shifts in pollination by oil-bees to other pollination systems have already been described (Steiner 1993, Steiner and Whitehead 1996). In some cases, there is a reduction in the levels of dependency followed by the loss of oil secretion structures, being the reversion to the absence of oil secretion recorded in several plant families (Renner and Shaefer 2010).

Taken together, the trace amount of diacetin, the absence of oil-bee visits, the small amount of lipid secretion and low reproductive rates could be an evidence of two possible pathways: 1) Incomplete shift from a specialized pollination system to another due to reduction of oil-secretion activity as a result of local unpredictability of specialized oil-bees, leading to a reduction or elimination of oil-pollination dependency in favor to pollen and nectar- collecting bees (see Steiner 1998) and 2) The residual lipid secretion found is an opportunistic strategy to take advantage of co-flowering oil-producing species, mainly orchids, and the absence of them in the populations studied lead to the non-attractivity of oil-bees and consequently to low reproductive rates.

To test both hypotheses presented here, more data need to be collected from population along all the *T. parviflora* range and in populations flowering in sympatry with plant species from the same guild.

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Tables

Table 1 - Reproductive outputs, observation hours and visitation frequency from each population studied.

Populations	Fruit-set	Seed-set	Observation hours	Visitation frequency[1]
Greyton	0.11 ± 0.03	4.33 ± 0.98	27h	0
Mitchell's Pass	0.12 ± 0.04	6.00 ± 1.06	32h	0
Kogelberg	0.19 ± 0.05	7.87 ± 1.31	18h	0.08 ± 0.01
Palmiet	0.21 ± 0.04	12.53 ± 1.33	17h	0.12 ± 0.01

Mean ± Standard Error; [1] Number of flowers per observation period (legitimate visits)

Table 2 - Akaike Information Criterion (AIC), deviance (G2), dregrees of freedom (df) and deviance significance of GLM model analysis of fruit and seed-set.

Data	Models (μ)	AIC	Deviance (G2)	df	Deviance significance
Fruit-set	Result ~ Population (2)	236.18	4.204	3	0.24
	Result ~ Individual (2)	288.26			
	Result~Individual + Population (3)	288.26			
Seed-set	Result ~ Population (2)	202.74	50.116	3	p<0.000*
	Result ~ Individual (2)	229.08			
	Result~Individual + Population (3)	204.30			

Figures

Figure 1

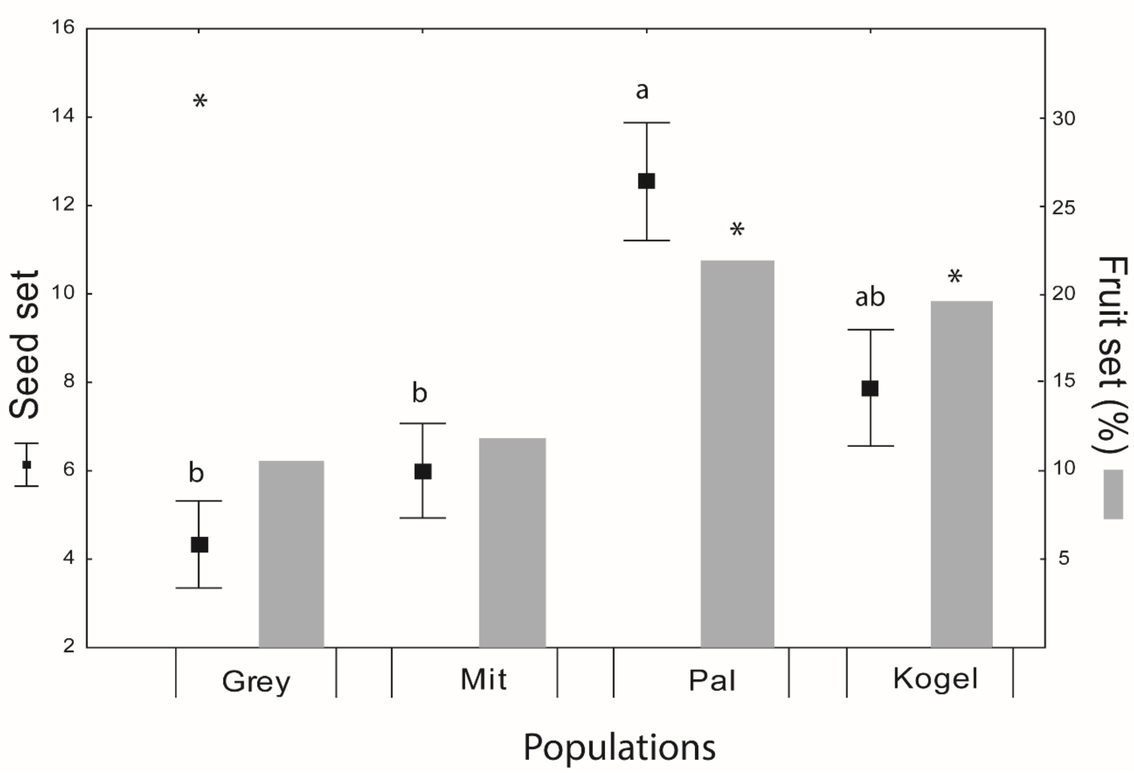


Figure 2

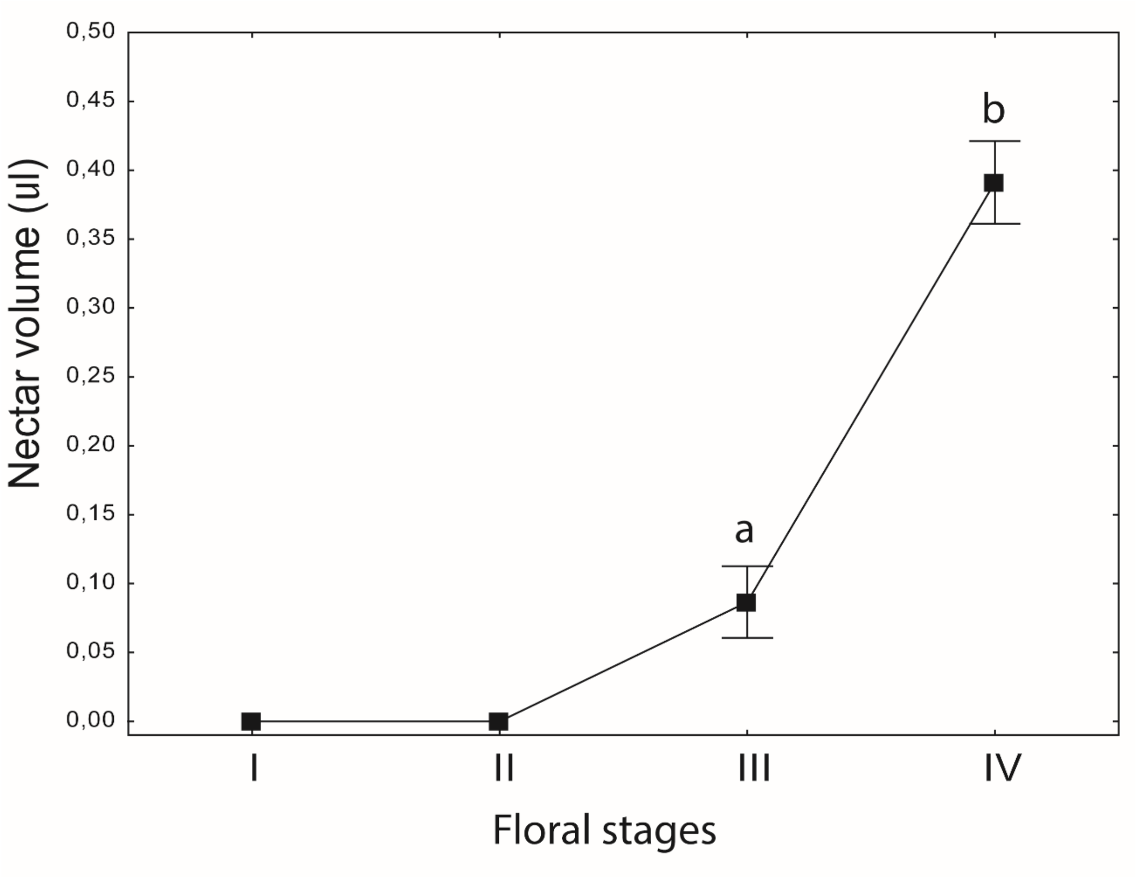
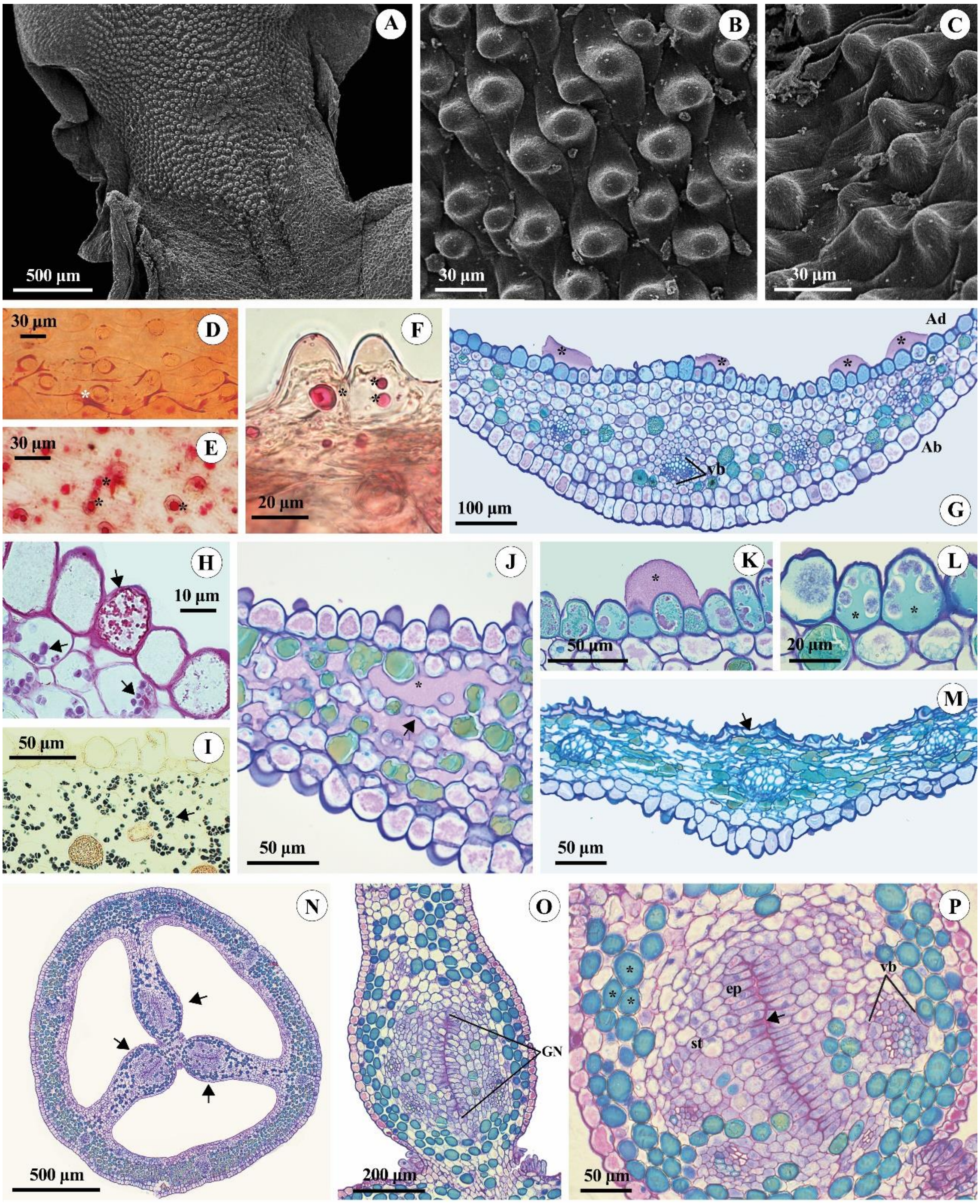

0,50
0,45
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
Nectar volume (ul)
a
b
I
II
III
IV
Floral stages

Figure 3


A
500 µm
B
30 µm
C
30 µm
D
30 µm
E
30 µm
F
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G
Ad
Ab
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I
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J
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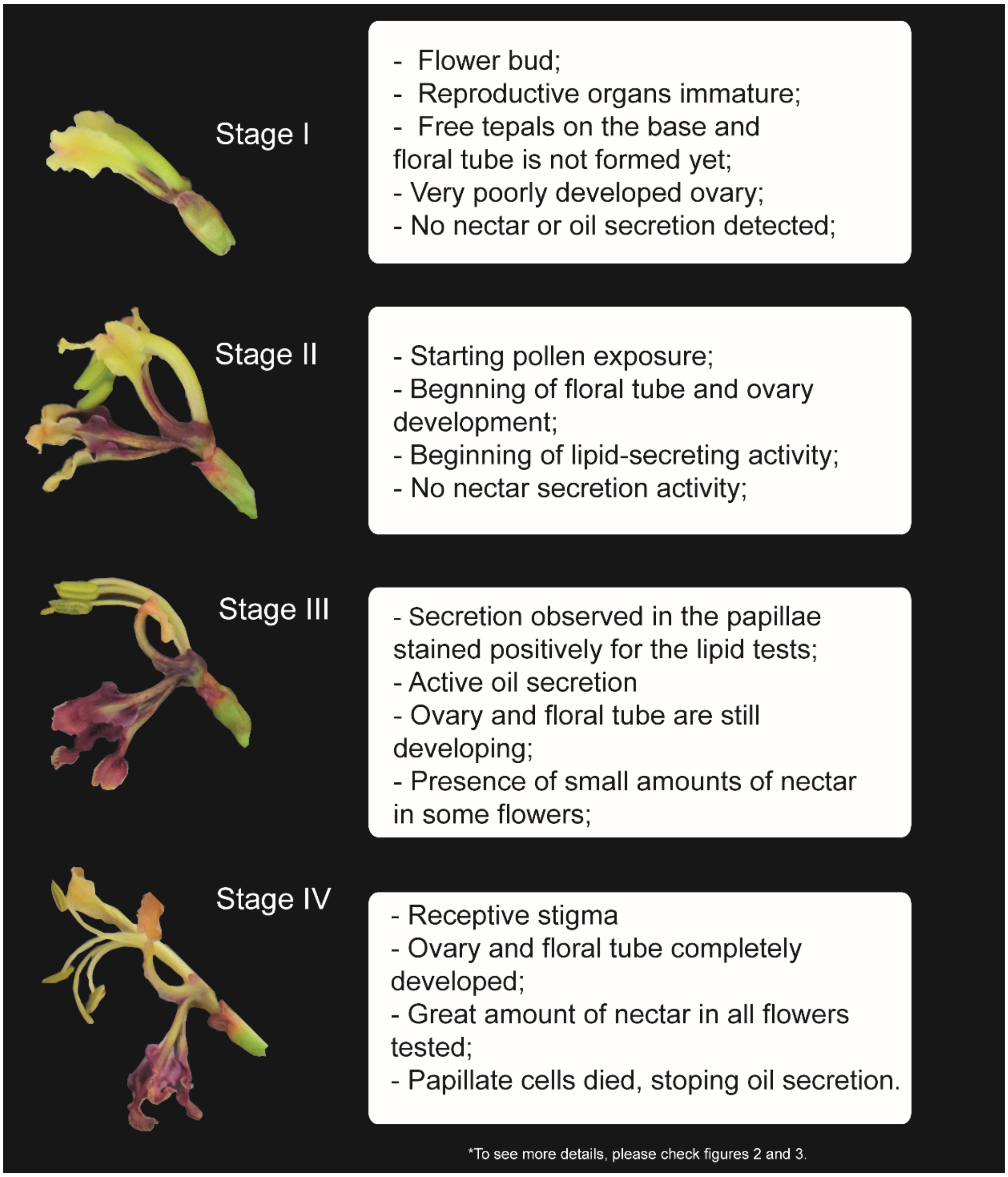
Figure 4



Stage I

- Flower bud;
- Reproductive organs immature;
- Free tepals on the base and floral tube is not formed yet;
- Very poorly developed ovary;
- No nectar or oil secretion detected;

Stage II

- Starting pollen exposure;
- Begnning of floral tube and ovary development;
- Beginning of lipid-secreting activity;
- No nectar secretion activity;

Stage III

- Secretion observed in the papillae stained positively for the lipid tests;
- Active oil secretion
- Ovary and floral tube are still developing;
- Presence of small amounts of nectar in some flowers;

Stage IV

- Receptive stigma
- Ovary and floral tube completely developed;
- Great amount of nectar in all flowers tested;
- Papillate cells died, stoping oil secretion.

*To see more details, please check figures 2 and 3.

Figure Captions

Figure 1 – Seed and fruit sets of *T. parviflora* populations. Distinct letters indicate significant difference in seed-production among populations. Asterisks are indicating differences in fruit production**.**

Figure 2 – Nectar volume along floral lifespan, represented by phases I, II, III and IV. Distinct letters are indicating significant differences between nectar production of flowers from phase III and IV.

Figure 3 - Morphology and anatomy of the tepals of *Tritoniopsis parviflora* in SEM (A-C) and light microscopy analysis (D-M), and ovary (N-P). A) Lower tepal surface showing the papillate region; B and C) details of the papillate cells with a striated cuticle; D-E) Surface tepals in light microscopy highlighting the drops secretion in papillae (*) stained positively for the total lipid test with Sudan IV (D) and Sudan Red 7B (E), and details of papillate cells with drops of lipidic (F). G) Cross sections of the lower tepal stained with toluidine blue O, demonstrating the adaxial (Ad) and abaxial (Ab) surface, vascular bundle (vb) and the secretion (*). H-I) Stage I, cells accumulate polysaccharides (H) and starch (I), indicating for black arrows. J) Cross sections of the lower tepal stained with toluidine blue O showing the intercellular spaces with mucilage/pectic substances (*), and arms of paraenchyma cells. Details of papillate cells (K – L), indicating the secretion (*) in K, and L the metabolizing cells (*). M) Stage IV, apoptosis of tepals cells (arrow) (Fig. X M). N–O) N-P) Cross section of ovary stained with toluidine blue O, with black arrows indicating gynopleural (or septal) nectary consisting of three clefts located in the septal region. O) Details of gynopleural (or septal) nectary (GN), and (P) a black

arrow indicating a nectary cavity, (ep) single-layered epithelium, (st) subsidiary tissue, (vb) vascular bundles and (*) idioblastic cells.

Figure 4 – Illustrative scheme summarizing the main results of floral anatomy and reward secretion dynamics along the flowers lifespan.

Appendices

Appendix 1

Populations studied and details about localization, number of flowering individuals and marked flowers.

Population	Coordinates	Nº of individuals in the population	Nº of flowers marked to set fruit (individuals)
Greyton	34°02’23.0”S 019°35’54.2”E	64	81 (14)
Michell’s Pass	33°23’42.3”S 019°17’05.2”E	52	65 (11)
Kolgelberg	34°19’07.0”S 018°58’07.0”E	14	47 (12)
Palmiet	34°20’22.5”S 019°00’09.9”E	20	74 (14)

CONSIDERAÇÕES FINAIS

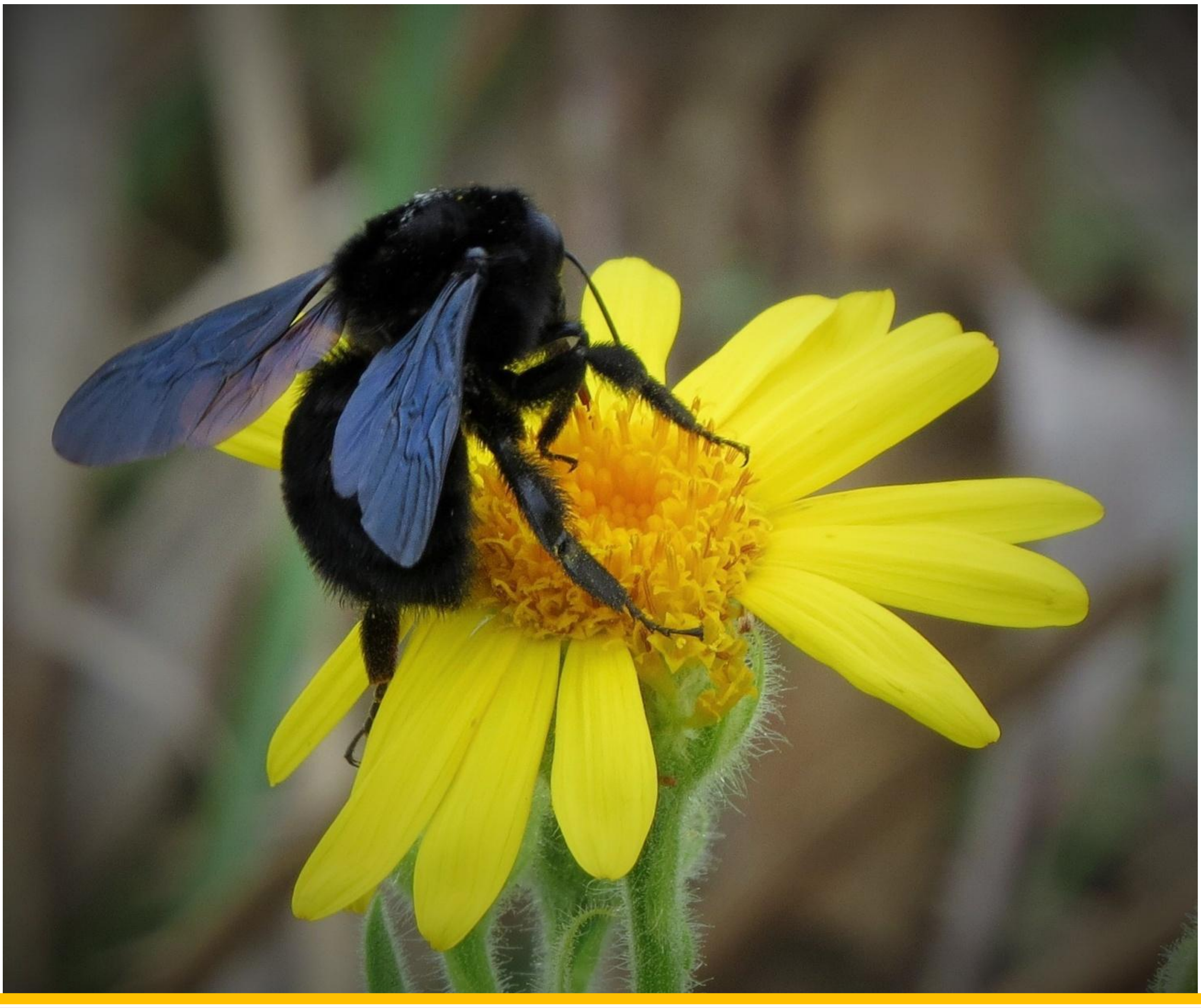
Considerações finais

Os dados levantados e apresentados nesta tese demonstram, primeiramente, a importância das abelhas como polinizadores das espécies de plantas que ocorrem nos campos do bioma Pampa e Mata Atlântica. Muito embora exista uma carência de estudos que visem investigar aspectos reprodutivos e da polinização de espécies visitadas por outros grupos funcionais como mariposas, moscas e besouros. As espécies campestres demonstraram ser pouco restritivas em termos de morfologia floral e de forma geral pouco especializadas em relação ao sistema de polinização. No entanto, dentre as espécies visitadas apenas por um grupo funcional de polinizadores, a grande maioria apresenta as abelhas como principais agentes polinizadores. A revisão bibliográfica apresentada nesta tese, ressalta a importância de estudos que visem investigar a fundo a biologia reprodutiva e ecologia da polinização de espécies campestres, principalmente se considerarmos a polinização como essencial para manutenção das comunidades vegetais e com amplo potencial para conservação de espécies e ecossistemas. A compreensão dos requerimentos das espécies vegetais, em termos de agentes polinizadores, tem um papel essencial no estudo da dinâmica das comunidades, na evolução e diversificação de grupos e na conservação de plantas, animais polinizadores, interações e habitats.

Em relação às interações entre abelhas coletoras de óleo e espécies de Iridaceae, este estudo auxiliou no preenchimento de uma grande lacuna em relação à polinização de Iridaceae Sul-Americanas. Dados inéditos sobre a biologia reprodutiva e sistemas de polinização de Iridaceae foram apresentados nos manuscritos redigidos, incluindo dados da única espécie de Iridaceae produtora de óleo floral fora das Americas: *Tritoniopsis parviflora*. Este estudo propiciou uma visão mais ampla sobre as interações entre abelhas coletoras de óleo e espécies de Tigridieae, demonstrando diferenças no comportamento e na performance de distintos grupos de abelhas e também diferença na diversidade de polinizadores das diferentes espécies de plantas estudadas. O sistema de polinização de espécies produtoras de óleos florais é reconhecido como especializado, por envolver um recurso que é coletado por um grupo específico de abelhas, que apresentam especificidades morfológicas e comportamentais para coleta de óleos. No entanto, as espécies de Tigridieae de diferentes gêneros, nas populações estudadas, apresentaram diferentes níveis de dependência e especialização em seus sistemas de polinização. Estes resultados reforçam que aspectos comportamentais, a disponibilidade de polinizadores, bem como as diferenças na performance dos mesmos, pode tornar algumas espécies mais flexíveis e generalistas em relação ao sistema de polinização. Neste sentido, espécies do gênero *Cypella* spp. foram elencadas como mais especializadas na polinização por abelhas de óleo, enquanto *K. brasiliensis* demonstrou um sistema misto, com contribuição tanto de abelhas de óleo como de pólen, e por último, *H. pulchella* apresentou um sistema menos especialista, com contribuição de várias espécies de abelhas de pólen e *Calydorea alba* (única espécie não produtora de óleo floral) foi exclusivamente visitada por abelhas de óleo. Dentre os principais resultados, pode-se destacar também as baixas taxas reprodutivas nos dois picos de floração da espécie *Cypella pusilla*.

As diferenças comportamentais das abelhas coletoras de óleo e pólen foram também um aspecto importante levantado no decorrer deste estudo. Isso porque as abelhas de óleo observadas atuam como oportunistas, forrageando óleo floral sem contactar anteras e estigmas, nas espécies *K. brasiliensis* e *H. pulchella*. Este comportamento pode ter um impacto negativo para as espécies de plantas em um cenário onde espécies coletoras de óleo compatíveis com a morfologia floral de *K. brasiliensis* e *H. pulchella* possam competir por este recurso que está sendo retirado por abelhas oportunistas. Embora as interações entre abelhas de óleo e as espécies estudadas sejam de extrema importância, as abelhas de pólen também se mostraram essenciais como polinizadoras, garantindo boas taxas de deposição polínica e visitação, principalmente em *H. pulchella*.

Este estudo traz a importância de perspectivas futuras que visem compreender o papel das interações planta polinizadores na diversificação de Iridaceae e como estes dados sobre os sistemas reprodutivos e da polinização podem estar relacionados com aspectos citogenéticos, morfológicos e da diversidade genética de diferentes populações das espécies já estudadas. Sendo assim, além de contribuir para o conhecimento dos sistemas de polinização de Iridaceae, esta pesquisa lançou luz sobre novas perguntas e novas perspectivas de estudo sobre a família e seus aspectos reprodutivos e evolutivos.



ELUCIDATING PLANT-POLLINATOR INTERACTIONS IN SOUTH BRAZILIAN GRASSLANDS: WHAT DO WE KNOW AND WHERE ARE WE GOING?

Elucidating plant-pollinator interactions in South Brazilian grasslands: What do we know and where are we going?

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Running head: Plant-pollinator interactions in grasslands.

Abstract

Grassland ecosystems present plant-pollinator interactions patterns due to habitat heterogeneity, plant composition and disturbances. Most studies about plant-pollinator interactions in the Neotropics were conducted in forest, savanna-like, or Andean vegetation. However, the current increase in the number of studies about interactions from grasslands vegetation can enable a better understanding of the ecology of these landscapes. In this systematic review, we summarised information from 24 published papers about plant-pollinator interactions in South Brazilian grasslands. We highlighted important aspects of the plant-pollinator interaction patterns, indicating its particularities compared to other grassland communities in South America. Bees are important pollinators of many plant species in these grasslands and most species are visited by more than one group of pollinators. Among the plant species visited by one pollinator group, most were visited by bees. However, many types of pollinators, plant species, habitats, and regions have, thus far, received little attention. Pollination by groups other than bees, such as nocturnal pollinators, flies, beetles, and birds, is particularly understudied. The nature of the information provided in this review is an important source of data that could be used to further pollination niche studies to understand the diversification and maintenance of South Brazilian grasslands flora.

Introduction

Plant-pollinator interactions play a fundamental role in biodiversity integrity (Potts *et al.* 2010) and are fundamental to plant population dynamics as they ensure population recruitment by fruit and seed set. In a plant community context, plant-pollinator interactions constitute one of the most important biotic factors, structuring community assemblage in temporal and spatial scales (Sargent and Ackerly 2008). In addition to the functional importance of such interactions, they lead to a set of different pollination niches resulting from selective pressures played by distinct pollinators along the evolutionary history of plants (Johnson 2010).

Plant-pollinator interactions are under an increasing threat from human activity in grasslands (Corbet 2006; Weiner et al. 2014). The constant conversion of native landscapes could be putting a set of diverse organisms associated with still unknown ecological interactions at risk (Valiente-Banuet *et al.* 2015; French *et al.* 2017). Data about this mutualistic interaction from different plant communities and physiognomies could help us to understand the ecological-evolutionary processes that determine the occurrence patterns of a set of plant species (Wołowski *et al.* 2017) and to evaluate potential species extinction through plant-pollinator interactions (Memmot *et al*. 2007). Information about plant-pollinator interactions allied to reproductive system information can help in the proposition of optimal management strategies and conservation by the identification of pollen or pollinator limitation, specialised groups of plants (e.g., oil-producing flowers pollinated by specialised oil-collecting bees), vulnerable species in terms of reproductive outputs, and core species that can be used to attract a great richness of insects (Kearns *et al*. 1998). Additionally, the understanding of plant-pollinator interactions can provide information about the vulnerability of habitats, assessing the risks of local extinction of plants, animals, and the interactions among them (Simmons *et al*. 2020).

Although we have information about plant diversity and its heterogeneity along South Brazilian grasslands (Overbeck *et al*. 2007), there is a gap of studies concerning insect diversity in both the Atlantic Forest and Pampa phytogeographic domains (Rafael 2009; Bencke 2009) and their mutualistic plant interactions, mainly in Pampa (BPBES/REBIPP 2019). Most plant interaction studies in this region are associated with plant species of economic interest (Witter *et al.* 2014; Garibaldi *et al.* 2016; Nunes-Silva *et al.* 2016; BPBES/REBIPP 2019), besides some studies on bee foraging behaviour (taxonomy and plant source of pollen) (Schlindwein and Wittman 1995; Alves dos Santos 1997; Schlindwein 1998; Blochtein 2014). To understand the importance of pollinators as selective agents on floral traits, we must assess who they are and the level of specialisation or generalisation of these interactions in the community (Fenster et al. 2004). The degree of specialisation and generalisation of plant pollination systems could be an important tool to understand the ecology of pollinator services and aspects of reproductive isolation, speciation, extinction, and assembly of communities. The specialisation degree of the interactions also has several implications for community ecology and the resilience of pollinator services in the face of climate changes, land use, and all types of environmental disturbance (Armbruster 2017). In the past, most studies about plant-pollinator interactions in the Neotropics were conducted in forest, savanna-like, or Andean vegetation. However, the recent increase in the number of studies about plant-pollinator interactions from grassland vegetation could enable a better understanding of the ecology of these landscapes (Freitas *et al*. 2007)

The South Brazilian grasslands englobe two different phytogeographic domains (Coutinho 2006; Batalha 2006); the most southern portion belongs to the Pampa domain, while the northern portion is part of the Atlantic Rain Forest domain (hereafter, called ARF) (Overbeck *et al.* 2007). The Brazilian Pampa accounts for 63% of the Rio Grande do Sul State area, where grasslands with scattered shrubs and tree formations represent the dominant vegetation in the landscape (Carvalho *et al.* 2015). The Atlantic Rain Forest comprises the grasslands of the Brazilian Plateau and is characterised by a mosaic of grasslands and forests in the northern half of the state of Rio Grande do Sul, Santa Catarina, and some areas of Paraná state. Including Pampa and ARF, an estimated 3,000 plant species exist in the South Brazilian grasslands (Boldrini 1997; Overbeck *et al.* 2007). Poaceae, Asteraceae, Cyperaceae, Fabaceae, Apiaceae, Oxalidaceae, Verbenaceae, and Iridaceae are the plant families with the highest richness in the Pampa (Overbeck *et al*. 2006, 2007; Andrade *et al*. 2018). Asteraceae, Apiaceae, and Verbenaceae are considered important floral reward sources to a wide range of pollinators (Pinheiro *et al.* 2008; Oleques *et al.* 2019). The unspecialised flowers of these plant groups and high population abundance, mainly Asteraceae, enhance the pollinator richness making these species crucial in pollination niche structuring in this region (Torres and Galetto 2002, 2011).

In this paper, we provided a systematic review and a general perspective of plant-pollinator interactions in South Brazilian grasslands. Our goal was to describe the community-level interactions between plants and distinct pollinator groups of South Brazilian grasslands to understand the main pollination systems and level of specialisation of this flora region compared to other South American grasslands. We also aimed to collaborate on a synthesis of knowledge and highlight the gaps and potentialities of plant-pollinator interactions studies in South Brazilian grasslands.

Our first expectation was that the high richness of unspecialised flowers, such as Asteraceae flowers, would contribute to more generalised pollination systems with species being visited by two or more pollinator groups (Pinheiro *et al.* 2008, Oleques *et. al.* 2019). Regarding pollinator groups, our expectation was that bees would play an important role as pollinators of a great number of species, considering the diversity of bees in this region and the floral traits of the main plant families (Schlindwein 1998). Considering the richness of species from Asteraceae, Verbenaceae, Apiaceae. and Iridaceae plant families in South Brazilian Grasslands, our expectation about floral traits was that the majority of species would present open flowers (dish flowers), easily accessible by insects, with a short or absent floral tube (Herrera 1996).

Methods

We compiled studies on plant-pollinator interactions with the help of extensive literature available on Google Scholar, ISI Web of Science, Scopus, Scielo, and the World Wide Web using the following search terms: “pollination AND Brazilian grasslands”, “pollination AND Southern Brazil”, “pollinators AND Southern Brazil, “flower visitors AND Southern Brazil, “pollination AND Pampa”, “pollination AND South Brazilian Campos” (all terms were searched in both Portuguese and English). In addition, we searched for grassland data sets in the Interaction Web database (NCEAS) and used some information in unpublished manuscripts (Avila Jr and Schlindwein 2020; Lopes 2017). We have chosen specific papers with information about plant-pollinator interactions or plant-floral-visitor interactions (with plant and animal taxonomic information) from Rio Grande do Sul, Santa Catarina and Paraná states without a temporal frame. We only considered studies based on field observations conducted in South Brazilian grasslands, which includes grassland vegetation of both Pampa and Atlantic Rain Forest domains. In our first search, we found five community-level papers. However, to avoid a bias in this review, we excluded two community papers: one of them the interactions of plant-pollinators were collected exclusively by the pollen loads of bees (Schlindwein 1998). The other was a study that covered both grassland and forest vegetation without differentiation in the results presentation (Mouga *et al.* 2012). Therefore, a total of 26 papers were reached and two were excluded by filters, resulting in 24 data sources (three studies at the community level and 21 case studies with one or a few plant species).

We categorised pollinators into eight distinct groups: bees, wasps, ants (Hymenoptera), flies (Diptera), beetles (Coleoptera), butterflies, hawkmoths (Lepidoptera), and hummingbirds (Apodiformes). Although species of ants are not usually considered pollinators, ants were treated as an independent category, herein, because they are frequently observed on flowers in many communities (García *et al.* 1996). To improve the discussion on bee-pollinated plants, we sub-categorified bees as carpenter bees (*Xylocopa* and *Ceratina*), bumblebees (*Bombus*), oil-bees (*Centris, Epicharis, Arhyssoceble, Chaleopogenus, Lanthanomelissa*), stingless bees (*Plebeia, Mourella, Trigona, Melipona, Tetragonisca*), and other bees, according to the subfamilies Colletinae, Megachiliinae, and Halictinae.

To classify the main pollination system of each plant family, we categorised specialists in a particular group using the species with more than 85% of visitors from that group. Species of plants with no group, encompassing more than 85% of visitors, were classified as insect/vertebrate generalists (Ollerton *et al.* 2006). Plant classification followed APG IV (2016) and Flora do Brasil

2020 (http://floradobrasil.jbrj.gov.br), and we categorised floral type according to Faegri and van der Pijl (1979) and used by Freitas & Sazima (2006) in a community-level study.

Results

Plant-pollinator interactions studies

Our search resulted in three plant community-level studies in the grasslands of the Pampa domain of Rio Grande do Sul (Pinheiro *et al*. 2008; Oleques *et al*. 2019; Deal-Neves *et al.* 2020). In addition, were found another 21 case pollination studies published in grasslands of both phytogeographical domains, including two unpublished manuscripts (two dissertations and one *in prep* manuscript of R. Avila Jr) (Table I). The studies consider to the systematic review were published from 2001 to 2020, being the most part of them published between 2017 and 2020. The latitudinal range varied from 31°48’36.96” S, 52° 24’53.13” W (Pelotas, Rio Grande do Sul State) to 24°33’16.79” S, 50°13’58.26” W (Tibagí, Paraná State). This data set allowed us to collect information of 203 plant species from 124 genera and 40 families, approximately 16% of the flora from Southern Brazilian grasslands (Boldrini 1997) (Table I, Fig. 1).

Floral traits

Most plant species recorded in this review presented a readily accessible dish flower type (30.0%) and brush flowers (24.6%). Narrow tube flowers were present in 15.7% of the species sampled. However, we found plant species presenting large tubes of approximately 10 cm, belonging to Solanaceae *Nicotiana alata* Link & Otto and *Petunia* spp. or in *Oenothera affinis* Cambess. Those with small and inconspicuous flowers were found in Anacardiaceae and Apiaceae species (Table I, Fig. 2). Both nectar and pollen were the most common floral rewards observed in 53.6% of our sampled species, and we recorded five Orchidaceae species (3.3%) and one case of sexual mimicry with female flowers without floral rewards (*Begonia cucullata* Willd.). However, in the Southern Grasslands, 12.3% of the plant species offered just pollen as a floral reward, and pollen and oil were present in 7.3% of this set of plants.

Pollination systems

Of the plant species, 56.7% were visited by more than one group of pollinators (Fig. 3). We observed the prevalence of Asteraceae species (47 species) with this wide spectrum of flower visitors. Among them, 30.4 % presented an extremely generalist pollination system with four or more flower visitor groups. Asteraceae, Verbenaceae, Apiaceae, and Myrtaceae can be cited as examples of this pollination system. However, there was a significant difference in this generalisation between plant families (Fig. 4). Asteraceae and Fabaceae presented a wide generalised pattern, while Orchidaceae and Solanaceae presented different pollinator groups at the family level but with a high degree of specialisation at the species level.

South Brazilian grasslands presented a great number of species visited by more than three groups of pollinators compared to other grassland plant-communities in South America. Most species from Venezuela and Mendoza (AR) grassland communities were pollinated by one or two groups, with few species visited by four groups. In contrast, South Brazilian grasslands and Bocaina grasslands (BR) had a similar frequency of extremally generalist species, pollinated by four distinct groups of pollinators (Fig. 5).

Pollinator groups

Bees were the most important and diversified pollinator groups in the Southern grasslands of Brazil. *Apis mellifera* was recorded in 59 plant species from different plant families, while native bees were observed as potential pollinators of 130 Southern Brazilian grassland species. Five subfamilies were found in our survey (Apinae - bumblebees: 4 spp.; carpenter bees: 11 spp.; oil-collecting bees: 11 spp.; stingless bees: 15 spp.; and others: 6 spp.; Andreninae: 13 spp.; Colletinae: 20 spp.; Megachiliinae: 15 spp.; and Halictinae: 50 spp.).

Few species were exclusively pollinated by groups other than bees. Plant species pollinated exclusively by beetles accounted for 10.7%, while 7.1% were pollinated by wasps, 6% by flies, 6% by hawkmoths, 4.8% by butterflies, and 3.6% by hummingbirds (Fig. 3). The plant species pollinated exclusively by hawkmoths belonged to the families Solanaceae (two species), Orchidaceae (three species) and Cactaceae (one species). Plant species pollinated by wasps belonged to four different plant families, namely Apiaceae, Apocynaceae, Asteraceae, and Fabaceae. Four plant species were pollinated exclusively by butterflies, with one species belonging to Asteraceae, one to Fabaceae, and two species to Orchidaceae, and hummingbirds were the sole flower visitors of only three plant species.

Discussion

Floral traits

The prevalence of easily accessible flowers in this review had a similar pattern in many other plant communities from Brazilian high-altitude grasslands (Freitas and Sazima 2006) to non-grassy ecosystems, such as the Caatinga (Machado and Lopes 2004) in Brazil, and from herbaceous Mediterranean communities (Bosch *et al.* 1997) to Alpine communities (Makrodimos *et al*. 2008) in Europe. Both nectar and pollen were the most common floral rewards, observed in 53.6% of our sampled species, corroborating this common trait in most flowering plants (Galetto 2007). The same pattern was observed in Southeastern grasslands (Bocaina grasslands) (Freitas and Sazima 2006). However, no reference, regardless of flower species, was made by Freitas and Sazima (2006), while in the Southern Grasslands, we found five Orchidaceae species (3.3% of the plant set) besides the female flowers of *Begonia cucullata* Willd., representing sexual mimicry (Avila Jr. *et al*. 2017). Regarding oil-flower species, we only found a similar frequency of oil-flowers in the Caatinga domain (Machado and Lopes 1999).

Pollination systems

Some plant families were evaluated regarding the prevalence of pollination systems. Iridaceae and Cactaceae, except *Cereus hildmannianus*, were strongly associated with bee pollination. Iridaceae in South Brazilian grasslands comprehend several oil-producing flowers, such as the genera *Herbertia* Sweet, *Cypella* Herb, *Sisyrinchium* L. and *Kelissa* Ravenna, which are associated with oil and pollen collecting bees (Oleques *et al.* 2020). (Oleques *et al.* 2020). This prevalence of the bee-pollination system contrasted with Iridaceae assemblages in South Africa, for example, where besides bees there are plants pollinated by sunbirds, longue-tongue flies

(Goldblatt and Manning 2006). This plant group could, therefore, be an interesting model to verify the different efficiencies of both bee groups in the reproductive outputs.

Another predominantly bee-pollinated plant group was the family Cactaceae. The state of Rio Grande do Sul is one of the most important diversity centres of cacti in South America (Silva *et al*. 2011). The genus *Parodia* (besides *Gymnocalycium*, *Frailea* and *Opuntia*) presents strong convergence in floral traits, such as yellowish corolla and numerous stamens with high amounts of pollen as floral resources to several bee species. Its' wide corolla ensures that bees of different sizes collect pollen (from the small *Dialictus* to the large *Cephalocolletes bipunctata*, observed in *Parodia crassigibba*, for example). An interesting phenomenon in this cacti group is stamen movement (thigmonastic stamen), which ensures pollen deposition by these bees (Schlindwein and Wiettman 1997). In this plant group, we may observe a wide spectrum of bee pollinators to extremely bee-specialised pollination systems (Cerceau *et al*. 2019). Furthermore, in this genus, there are some interesting cases of bee copulation behaviour during *Opuntia* flower visits, where male bees forage for females visiting flowers (Oliveira and Schlindwein 2010). Plantaginaceae is another oil-producing flower group that, together with Iridaceae and Malpighiaceae, is exclusively pollinated by bees. Although some studies recorded other floral visitors (beetles and flies in *Scoparia dulcis*), the prevalence of bee species acting as potential pollinators was highlighted. This fact probably results from the kind of reward offered to pollinators.

Mixed pollination systems, with more than one single pollinator group, characterised most plant species in the Southern grasslands. The prevalence of Asteraceae species could contribute to this pattern with this wide spectrum of flower visitors, which agrees with previous studies in South America that report the high importance of this plant group as a key resource for different guilds of pollinators (Torres and Galetto 2009; Antonini and Martins 2003; Freitas and Sazima 2006, Pinheiro *et al.* 2008; Martins *et al.* 2013; Oleques *et al.* 2017). Those species generally have numerous flowers per inflorescence and present floral traits that make them accessible and attractive to a broad range of flower visitors, such as small floral tube size and secondary pollen presentation (Torres and Galetto 2002; Antonini and Martins 2003; Lunau *et al.* 2018). Moreover, Asteraceae is the most diverse plant group in the Southern Brazilian grasslands (excluding Poaceae) (Boldrini 1997; Overbeck 2007; Andrade *et al*. 2019) and could be considered crucial to the maintenance of many plant-pollinator interactions in grassland communities (Pinheiro *et al.* 2008; Oleques *et al.* 2019).

The different aspects of generalisation among plant families was interesting. While most plant families presented wide generalisation spread in its species (i.e., the most of species presenting diverse pollinator groups), some other generalist plant groups presented a generalisation pattern but with some specialisation degree in pollination niches at the species level (Solanaceae and Orchidaceae, for example). This could be an interesting aspect of diversification associated with pollinator pressure along the evolutionary history of these plant groups. These species are good models to test the effective role of pollination strategies promoting diversification in grassland ecosystems. It is because these species could represent effective cases of selective pressures of some pollinator groups (Gómez *et al.* 2015).

Freitas and Sazima (2006), studying plant-pollinator interactions in Bocaina highland grasslands (Southeastern Brazil), found a similar pattern with a high prevalence of plants with two or more groups visiting flowers. However, these typical high-altitude grasslands presented higher equitability among pollination system frequencies (Freitas and Sazima 2006) compared to our findings. Contrasting this pattern, in grasslands of extreme northern South America (Venezuela), Ramirez (2004) found a very high percentage of plants with only from one group of floral visitors. Although with an overall lower number of specialised plant species than plants with generalist systems, the proportion between specialised-generalised pollination systems was quite similar in Venezuela, with a low number of extremely generalist plants (Ramirez 2004). The same proportion was observed in grasslands of Mendoza (Argentina), with an overall prevalence of pollination systems with more than two pollinator groups but with a low prevalence of extreme generalists (Vázquez 2007). Generalisation in pollination systems could be favoured by various ecological factors, such as unpredictability of the most important pollinator promoted by spatiotemporal variability in the pollinator assemblage, similarities among pollinators as selective agents, and geographical variations in the pollinator fauna along latitudinal gradients (Ollerton *et al.* 2006). Tropical areas generally present a larger number of potential pollinator groups that could promote higher specialisation, contrasting with generalisation systems in subtropical and temperate regions. However, this is not a pattern concerning plant-pollinator interactions (Ollerton and Cranmer 2002; Bascompte 2009), and plant-pollinator interactions in grasslands can reinforce this aspect. Grasslands from both extreme northern and Southern South America presented a similar frequency of plant species, with pollinators belonging to one pollinator group, however, with the prevalence of plants pollinated by two or more groups.

Pollinator groups

Our findings point out the importance of bees as pollinators of species in the Southern grasslands of Brazil. Bees are considered the most important and dominant pollinators in most plant communities (Proctor *et. al.* 1996). They are related to plant species with different floral traits, and their behaviour could vary according to their nutritional requirements, type of reward collected, and habitat (Stallman 2011; Gianinni *et al.* 2012). Native stingless bees play an important role as pollinators of species occurring in the state of Rio Grande do Sul, and several species are considered oligolectic (Schlindwein 1998). Furthermore, stingless bees are known for their positive influence on the pollination of canola crop yields in Southern Brazil (Halinsk *et al.* 2018). The most diverse bee subfamily was Halictinae, being related to 66 plant species from 26 plant families. Although *A. mellifera* has been recorded as a visitor in several plant species, our finds elucidate the great diversity of native bees and their importance as pollinators of grassland plant species. All plants pollinated exclusively by hawkmoths present floral traits compatible with sphingophily, such as nocturnal anthesis, white/greenish colour, and nectar secreted in a tube or spur (Herrera 1996). The low prevalence of sphingophilous plant studies reflects the rarity of this pollination system in the Southern grasslands.

The plant species pollinated by wasps presented morphologically generalised flowers; however, the unspecialised floral morphology could indicate the existence of other filters to exclude other pollinators, such as chemical characteristics of scent and nectar (Johnson and

Steiner 2000). Specialised interactions between plants and wasps are typically associated with sexually deceptive or food-based mimicry systems. Besides being uncommon, there are examples of rewarding plants, including pollination by vespids in *Oxypetalum* spp. and *Blepharodon nitidus* (Vell.) J.F.Macbr. (both milkweeds) in South America (Vieira and Shepherd 1999).

Few species were exclusively pollinated by butterflies because specialisation in pollination by butterflies is rarely found in plants (Johnson and Bond 1994). Among all species visited exclusively by butterflies, only *Epidendrum fulgens* Brongn. presented floral traits considered adapted to butterfly pollination. In the last few years, there was an increase in studies of butterfly diversity in the South Brazilian grasslands. However, data about plant species pollinated by butterflies are still scarce and new studies on pollination ecology of species visited by this group are promising (Paz *et al.* 2013; Carvalho *et al.* 2015).

Species pollinated by hummingbirds share conspicuous reddish flowers, an important floral trait of bird pollination systems. Except for *Cattleya coccinea* Lindl. (rewardless orchid), *Nicotiana forgetiana* Hemsl., and *Petunia exserta* Stehmann, secret nectar is stored in a long corolla tube. Pollination by hummingbirds is a highly frequent pollination system in other plant communities studied in Brazil. However, most of the studied species are concentrated in forests of South-eastern Brazil (Buzato *et al.* 2000; Canela and Sazima 2005; Rocca and Sazima 2008; Buggoni and Sazima 2012; Lunau *et al.* 2011).

Conclusions

In conclusion, our review points out the lack of studies of plant-pollinator interactions in South Brazilian grasslands, mainly concerning some attributes of plants, such as nocturnal long-tube flowers pollinated by hawkmoths. In addition, there are no studies about the importance or efficiency of flies and beetles as pollinators of this particular vegetation. This is especially concerning in the Pampa domain because of the small extent of effectively protected areas and accelerated conversion of natural areas into extensive soybean monocultures in the last decade.

Furthermore, an insufficient number of studies about mutualistic interactions and reproductive biology could put several species of plants and pollinators at risk. The prevalence of plants visited by more than three groups of pollinators highlight the generalist aspects of the interactions, which could be the result of the great diversity of plants with generalist flower traits, such as Asteraceae species. Based on our findings, bees are the most important group of pollinators related with both generalist and specialist plant groups, such as Asteraceae and Iridaceae. The nature of the information provided in this review is an important source of data that could be used to further pollination niche studies to understand the diversification and maintenance of South Brazilian grassland flora.

Many types of pollinators, plants, habitats, and regions have received little attention thus far. Pollination by groups other than bees, such as nocturnal pollinators, flies, beetles, and birds, is particularly understudied. Community-level studies on many ecosystems across the entirety of South Brazil are, therefore, needed to understand general trends in plant-pollinator interactions in South Brazilian grasslands and increase the level of acknowledgement of Neotropical grassland vegetation. It is essential to facilitate the development of adequate conservation strategies associated with the preservation of crucial ecosystem services provided by grasslands.

Acknolegments

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Figure Captions

Figure 1. Some interactions registered in Southern Brazilian Grasslands. A, *Campsomeris* sp. (wasp) visiting Senecio sp.; B, a beetle visiting flowers of *Pffafia tuberosa*; C, Apis mellifera visiting flowers of Richardia grandiflora; D, beetles (Curculionidae) visiting *Eryingium horridum*; E, a fly (Tachinidae) visiting *Aspillia montevidensis* and F, the oil-collecting bee *Arhysoceble picta* foraging oil in *Kelissa brasiliensis*.

Figure 2. Number of plant species per flower type. Note that Asteraceae and Verbenaceae species are counted here as brush and dish, according to the morphology of the inflorescence.

Figure 3. Number of species visited by more than one and by a single functional pollinator group (left). The numbers inside the bars (distinct shades of grey) are indicating the number of plant species pollinated by two, three and more than three groups. On the right, the percentage of plant species visited only by bees, beetles, hawkmoths, butterflies, hummingbirds, flies and wasps.

Figure 4. Frequency of different pollination systems within plant families in South Brazilian Grasslands (plant families with more than three species presented in this review). The pollinator groups described in the side of the graphs refers to specialized system cases observed in each plant family.

Figure 5. Relative frequency of plant species visited by one, two, three and four pollinator groups (functional groups) in distinct grassland communities of South America. The yellow area in the map is indicating South Brazilian Grassland from Rio Grande do Sul, Santa Catarina and Paraná states in Brazil.

Fig. 1

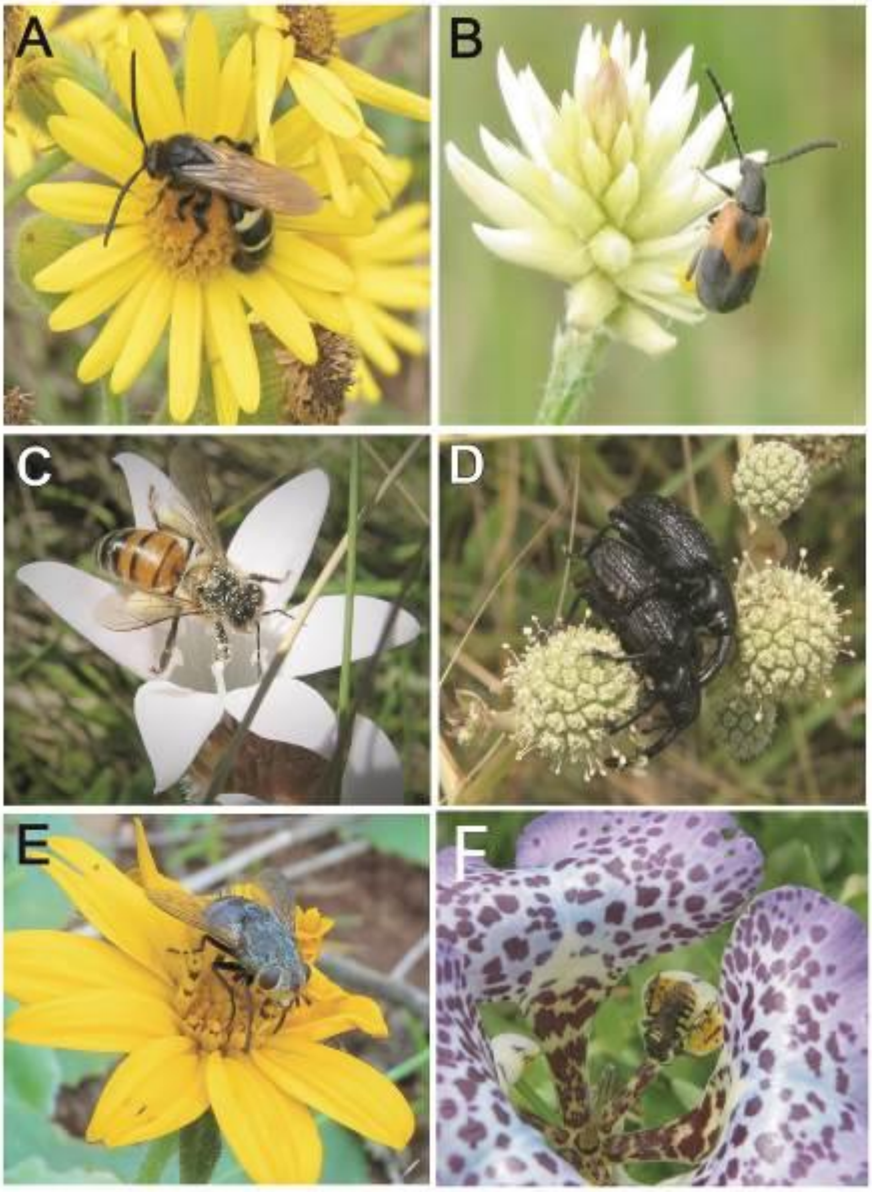


Fig. 2

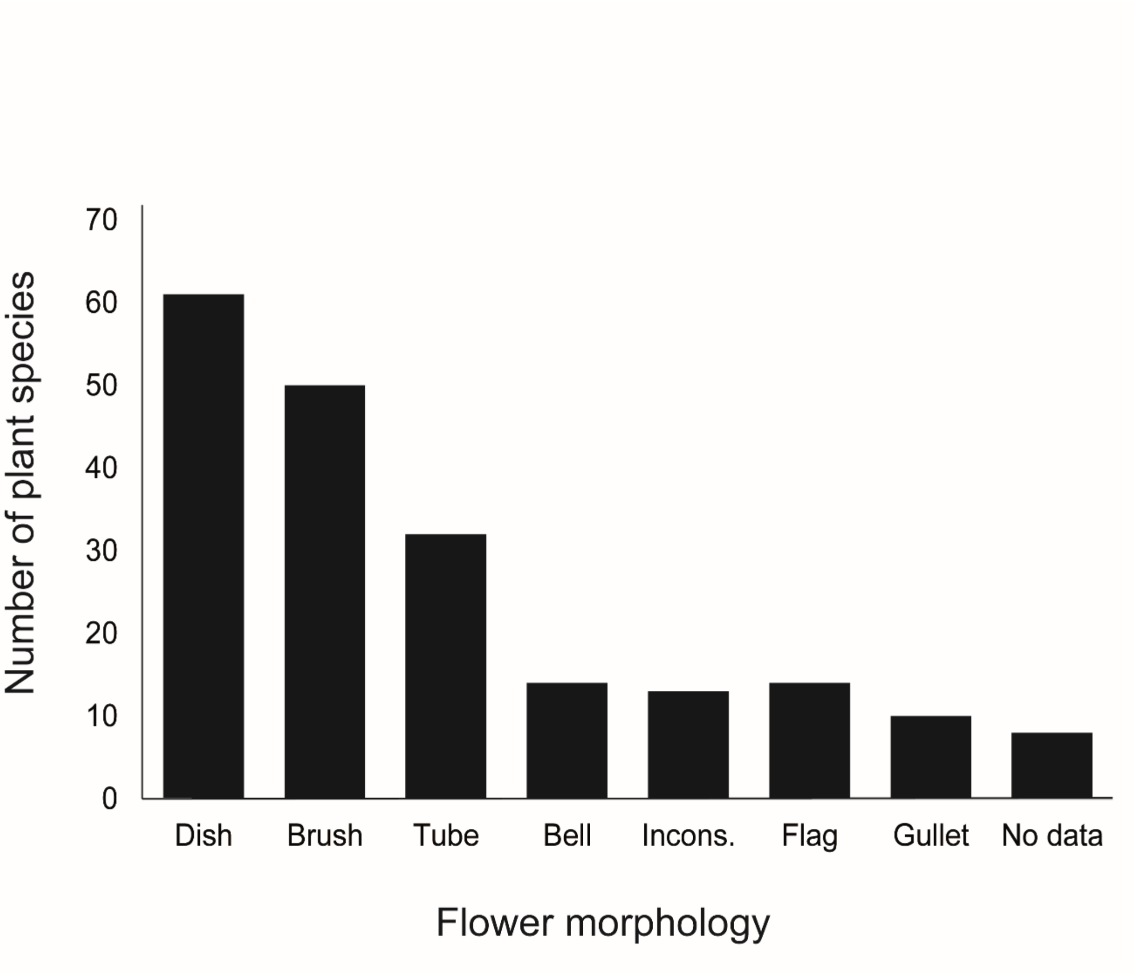

Number of plant species
70
60
50
40
30
20
10
0
Dish
Brush
Tube
Bell
Incons.
Flag
Gullet
No data
Flower morphology

Fig. 3

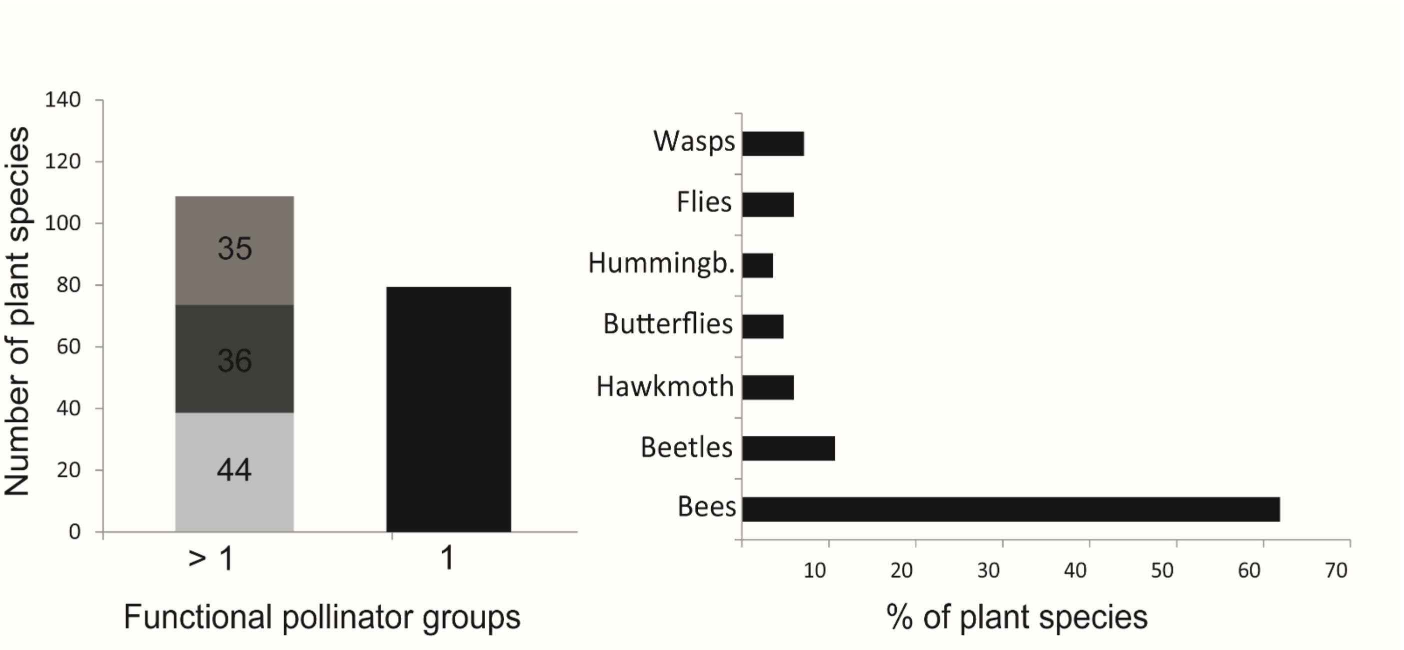

140
120
100
80
60
40
20
0
Number of plant species
35
36
44
> 1
1
Functional pollinator groups
Wasps
Flies
Hummingb.
Butterflies
Hawkmoth
Beetles
Bees
10
20
30
40
50
60
70
% of plant species

Fig. 4

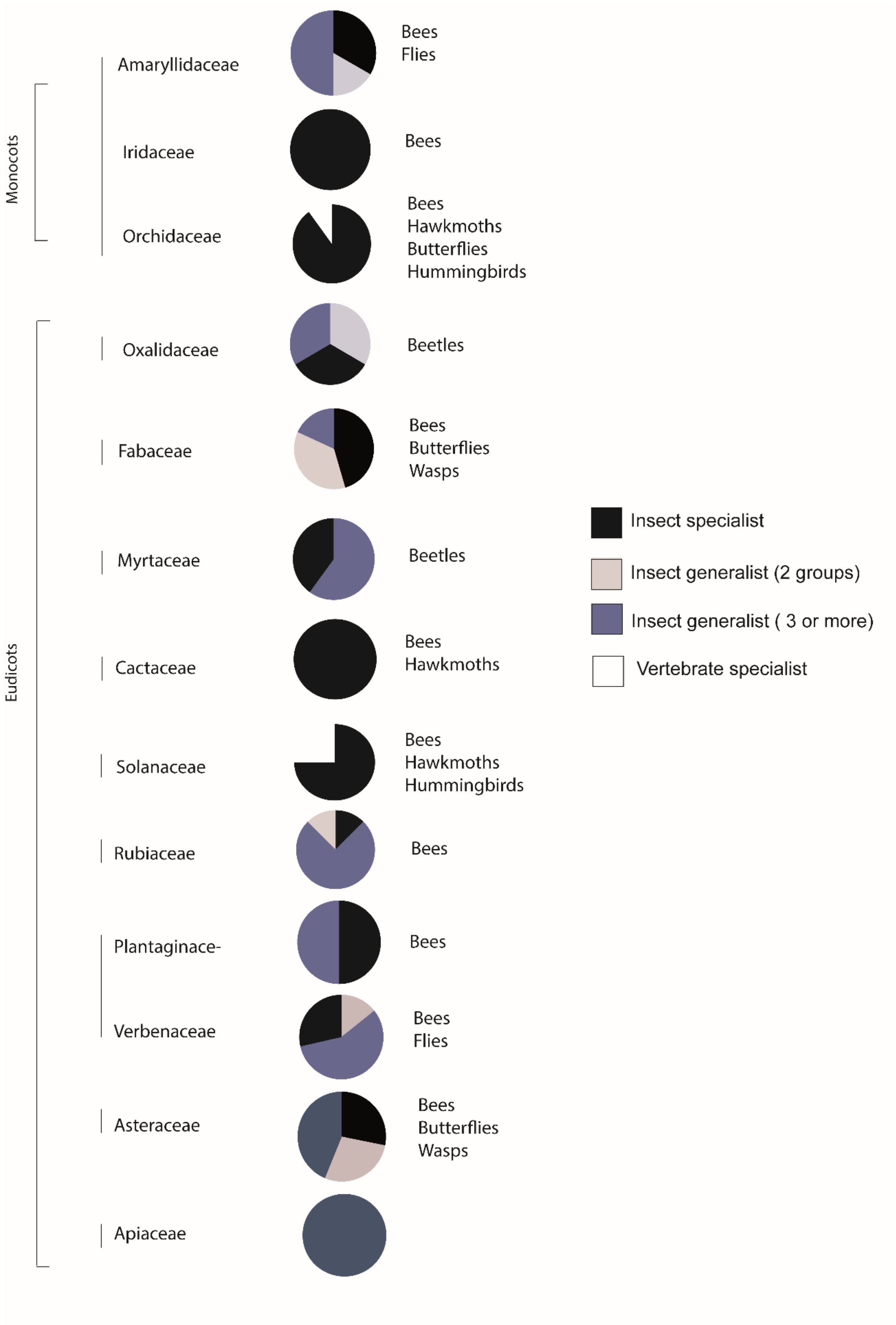
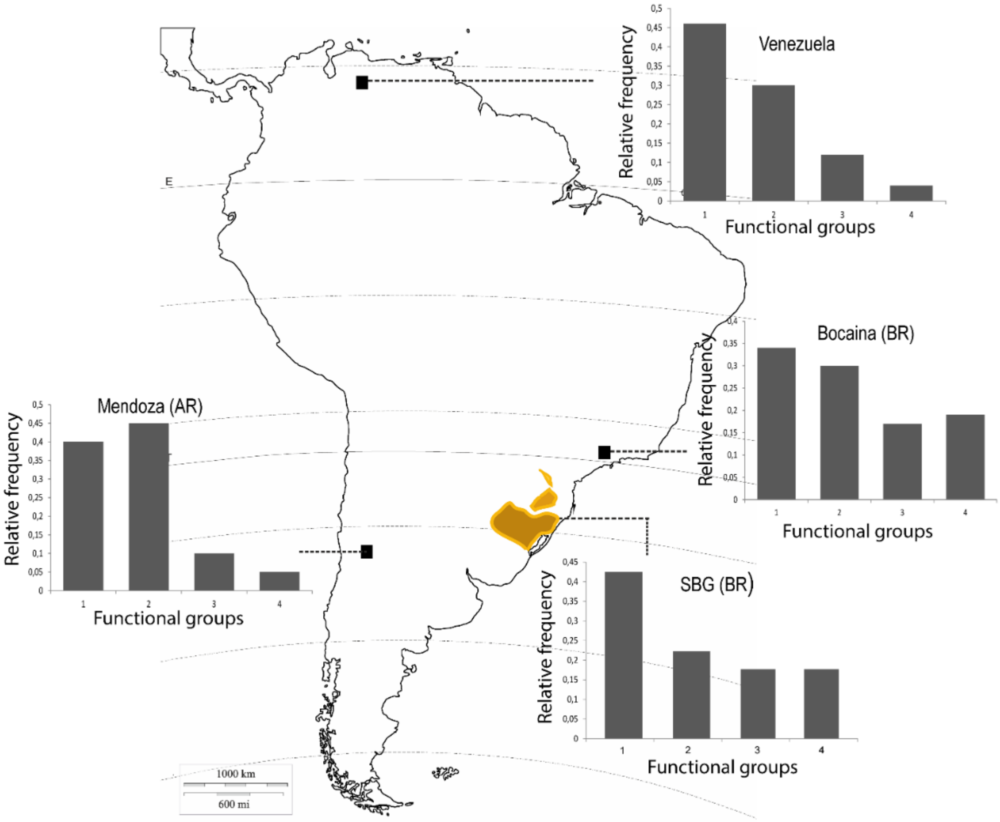

Monocots
Eudicots
Amaryllidaceae
Bees
Flies
Iridaceae
Bees
Orchidaceae
Bees
Hawkmoths
Butterflies
Hummingbirds
Oxalidaceae
Beetles
Fabaceae
Bees
Butterflies
Wasps
Myrtaceae
Beetles
Cactaceae
Bees
Hawkmoths
Solanaceae
Bees
Hawkmoths
Hummingbirds
Rubiaceae
Bees
Plantaginace-
Bees
Verbenaceae
Bees
Flies
Asteraceae
Bees
Butterflies
Wasps
Apiaceae
Insect specialist
Insect generalist (2 groups)
Insect generalist (3 or more)
Vertebrate specialist

Fig. 5



Venezuela
Relative frequency
0
0,05
0,1
0,15
0,2
0,25
0,3
0,35
0,4
0,45
0,5
1
2
3
4
Functional groups
Bocaina (BR)
Relative frequency
0
0,05
0,1
0,15
0,2
0,25
0,3
0,35
0,4
1
2
3
4
Functional groups
Mendoza (AR)
Relative frequency
0
0,05
0,1
0,15
0,2
0,25
0,3
0,35
0,4
0,45
0,5
1
2
3
4
Functional groups
SBG (BR)
Relative frequency
0
0,05
0,1
0,15
0,2
0,25
0,3
0,35
0,4
0,45
1
2
3
4
Functional groups
E
1000 km
600 mi