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POLINIZAÇÃO, FUNCIONAMENTO FLORAL E AMEAÇAS À CONSERVAÇÃO DE CACTOS MELITÓFILOS DO RIO GRANDE DO SUL

Belo Horizonte
2020

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do título de doutor em Ciências Biológicas – Biologia Vegetal

Orientador: Prof. Dr. Clemens Schlindwein

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RESUMO

Cactaceae é uma família altamente diversa, com cerca de 1450 espécies distribuídas predominantemente no continente americano. O Brasil é um dos centros de diversidade da família e grande proporção das espécies estão ameaçadas de extinção. A alteração dos habitats, a coleta ilegal e a introdução de animais exóticos nas áreas de ocorrência dos cactos induzem o declínio populacional de várias espécies. O conhecimento sobre a biologia das cactáceas em habitat natural pode auxiliar na execução de ações para a conservação de espécies. Dessa maneira, este trabalho teve como objetivo ampliar o conhecimento sobre a biologia de cactáceas melitófilas através do diagnóstico reprodutivo e identificação das principais ameaças em habitat natural. O trabalho foi desenvolvido no Pampa do Rio Grande do Sul, um dos centros de diversidade de cactáceas no Brasil. *Parodia neohorstii* (S.Theun.) N.P.Taylor e *Frailea gracillima* (Lem.) Britton & Rose foram estudadas na perspectiva da biologia da polinização e funcionamento floral. Ambas as espécies apresentam floração sazonal, longevidade floral curta e polinização por abelhas especialistas em pólen (oligoléticas). As abelhas sincronizaram a atividade de voo com o horário de abertura das flores e depositam grandes quantidades de pólen nos estigmas, o que resulta na produção eficiente de frutos e sementes. *Frailea gracillima* apresenta hercogamia temporal, ou seja, quando as flores se abrem, estigma e anteras estão espacialmente separados. Ao longo da antese, lentos movimentos autônomos dos estames e dos lobos estigmáticos diminuem a separação espacial entre as estruturas reprodutivas, o que possibilita a autopolinização autônoma tardia durante o fechamento das flores. Já *P. neohorstii* é uma espécie auto incompatível e suas flores exibem movimentos tigmonásticos dos estames, induzidos pelo contato físico entre as abelhas e os filetes. Os movimentos dos estames escondem momentaneamente parte do conteúdo polínico da flor (75%) e atua como um mecanismo de particionamento de pólen. Mostramos experimentalmente que somente a execução dos movimentos dos estames induzem a senescência floral em *P. neohorstii*, e não a polinização. Na ausência de contato físico com os filetes, os estames não se movimentam de forma autônoma, a longevidade floral é estendida por até 5 dias.

Para a espécie *P. neohorstii*, avaliamos ainda os fatores que impactam a manutenção e ameaçam a conservação de *P. neohorstii* em habitat natural. Avaliamos 3677 indivíduos em 9 localidades de ocorrência. Concluímos que a principal ameaça à espécie é a criação de caprinos e bovinos, 16.9% dos indivíduos amostrados foram impactados por essa atividade. Os principais impactos foram o deslocamento e a remoção dos cactos do substrato, e danos físicos causados pelo pisoteio e pelo consumo parcial das plantas, o que resultou em 41% de mortalidade. O pastoreio dos animais de criação impactou não somente as espécies foco do estudo, mas também outras populações de cactáceas simpátricas. Para a conservação das comunidades de cactos melitófilos em habitat natural, é necessário a manutenção dos polinizadores especialistas e a redução do acesso de animais de criação nas áreas de ocorrência das espécies.

Palavras-chave: Cactaceae, polinização, tigmonastia, interação planta-polinizador, abelhas oligoléticas, movimento de estames, conservação.

ABSTRACT

Cactaceae is a highly diverse family, with 1450 species distributed predominantly on the American continent. Brazil is one of the cacti diversity centers and huge proportion of species are threatened with extinction. Habitat alteration, illegal collection and the introduction of exotic animals induce population decline of several cactus species. Knowledge about the biology of cacti in natural habitat can assist in the execution of conservation actions for the threatened species. Thus, this study aimed to expand knowledge about the biology of melittophilous cacti through reproductive diagnosis and identification of the main threats in natural habitat. The study was developed in the Pampa of Rio Grande do Sul, one of the centers of cactus diversity in Brazil. *Parodia neohorstii* (S.Theun.) N.P.Taylor and *Frailea gracillima* (Lem.) Britton & Rose were studied from the perspective of pollination biology and floral functioning. Both species show seasonal flowering, short floral longevity and pollination by pollen specialists (oligolectic bees). The bees synchronized the flight activity with the opening time of the flowers and deposit huge amounts of pollen on the stigmas, which results in efficient fruit and seed set. *Frailea gracillima* presents temporal hercogamy, that is, when the flowers open, stigma and anthers are spatially separated. Throughout the anthesis, slow autonomous movements of the stamens and stigmatic lobes decrease the spatial separation between reproductive structures, which allows for delayed self-pollination during flower closure. *Parodia neohorstii* is a self-incompatible species and its flowers exhibit thigmonastic stamen movements, induced by physical contact by bees on the stamens. The movements of the stamens momentarily hide part of the flower's pollen content (75%) and act as a pollen partitioning mechanism. We showed experimentally that only the induction of stamen movements induces floral senescence in P. neohorstii, and not pollination. In the absence of physical contact, the stamens do not move autonomously and the floral longevity is extended for up to 5 days.

For the species *P. neohorstii*, we also evaluated the factors that impact the specie in natural habitat. We evaluated 3677 individuals in 9 locations. We conclude that the main threat to the species is livestock farming of goats and cattle, 16.9% of the individuals sampled were impacted by this activity. The main impacts were the displacement and removal of cacti from the substrate, and physical damage caused by trampling and partial consumption of plants, which resulted in 41% mortality. The grazing impacted not only the species that were the focus of the study, but also other populations of sympatric cacti. For the conservation of melittophilous cactus communities in natural habitat, it is necessary to maintain specialist pollinators and reduce access for farm animals in the areas where the species occur.

Keywords: Cactaceae, pollination, thigmonasty, plant-pollinator interaction, oligolectic bees, stamen movement, conservation.

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INTRODUÇÃO GERAL

Cactaceae é uma família predominantemente americana e altamente diversa, composta por 1450 espécies distribuídas em 4 subfamílias, Pereskioideae, Opuntioideae, Maihuenioideae e Cactoideae (Anderson, 2001; Hunt *et al.,* 2006, 2013). As espécies são amplamente distribuídas em quatro principais centros de diversidade, sendo eles: 1) México e a região Sul dos Estados Unidos; 2) região central da cordilheira dos Andes, principalmente na Bolívia e Peru; 3) região Leste do Brasil; 4) região Sul do Brasil e áreas adjacentes do Uruguai (Oldfield, 1997; Ribeiro-Silva *et al.,* 2011). Apesar da maior diversidade de espécies de Cactaceae ser encontrada em regiões áridas e semiáridas (Oldfield, 1997; Anderson, 2001), suas espécies ocorrem em diferentes habitats, como florestas tropicais úmidas e campos arbustivos (Martin & James, 2009; Anceschi & Magli, 2018).

No Brasil, ocorrem 261 espécies pertencentes a 39 gêneros, distribuídos em todos os biomas (Zappi, 2020). No bioma Mata Atlântica ocorrem predominantemente cactos epífitos, enquanto que no Pampa existe alta diversidade de cactos rupícolas globosos e cilíndricos (Ribeiro-Silva *et al.,* 2011; Carneiro *et al*., 2016) na Caatinga e Cerrado, os cactos colunares e arborescentes são os mais abundantes (Oldfield, 1997). No Pantanal e Chaco, os cactos colunares e globosos são os mais representativos (Gomes & Araújo 2015, Zappi *et al.*, 2018). Como nas demais regiões do mundo, uma grande proporção das espécies brasileiras são endêmicas e têm distribuição restrita (Forzza *et al.*, 2010).

O bioma Pampa, que ocupa a porção sul do Rio Grande do Sul é um dos principais centros de diversidade de cactáceas no Brasil, com 65 espécies pertencentes a 11 gêneros, onde destacam-se espécies dos gêneros *Parodia* Speg. e *Frailea* Britton & Rose (Ribeiro-Silva *et al*., 2011; Carneiro *et al*., 2016; Saraiva *et al*., 2014). A região é marcada por grande heterogeneidade ambiental e alta disponibilidade de hábitats favoráveis à ocorrência das espécies. Uma característica marcante dos cactos gaúchos é o endemismo, que chega a 16% nas espécies da região (Carneiro *et al*., 2016). Os cactos do Rio Grande do Sul são coletados e estudados desde a primeira metade do século XIX por naturalistas europeus (Pontes *et al*., 2017a). Apesar disso, até os dias atuais pouco se sabe sobre a distribuição geográfica, o tamanho e o estado de conservação das populações da maioria das espécies (Ribeiro-Silva *et al*., 2011). Além disso, existem poucos estudos sobre a biologia reprodutiva e da polinização das espécies desse importante centro de diversidade de cactáceas no país (Schlindwein & Wittmann, 1995, 1997).

Breve histórico sobre os cactos do Rio Grande do Sul

A singularidade e a diversidade de formas das espécies de cactos atraíram a atenção de botânicos e naturalistas europeus que visitaram o continente americano no início do século XIX (Engelamnn, 1856; Marshall & Bock, 1941). Em longas expedições de campo, registradas a partir de 1818, diversas espécies de cactos globosos foram descobertas e coletadas no Rio Grande do Sul e em áreas adjacentes do Uruguai e Argentina. Esses exemplares foram enviados à Europa e serviram como material tipo para a descrição de diversas espécies (Pontes *et al*., 2017a), que posteriormente serviram de subsídio para a publicação de livros e chaves de identificação (Förster & Rümpler, 1886; Schumann, 1890; Schumann & Hirscht, 1899; Spegazzini, 1905; Pontes *et al*., 2017a).

A partir de 1950, cidadãos gaúchos, principalmente descendentes de alemães, passaram a se interessar pelos cactos da região e a estabelecer coleções com espécimes coletados em expedições ao interior do estado (Pontes *et al*., 2017b). Com conhecimento acumulado sobre espécies novas, os brasileiros forneciam informações e enviavam sementes e cactos para a Europa em troca de literatura especializada, além de serem guias em expedições no Brasil (Pontes *et al*., 2017b). A partir dos anos 1960, iniciou-se uma grande exportação de cactos para Europa com finalidade comercial (Pontes *et al*., 2017b). Apesar do ponto negativo da exploração comercial dos cactos no RS, esse período foi marcado por grande quantidade de descrição de espécies e um aumento do conhecimento sobre a diversidade das cactáceas no sul do Brasil (Pontes *et al*., 2017b). No início dos anos 80, leis ambientais começaram a restringir a exportação de espécies nativas da flora (Pontes *et al*., 2017b) e nos anos 90, a Convenção sobre Comércio Internacional de Espécies Ameaçadas publicou um documento proibindo o comercio internacional de diversas de espécies de cactáceas, o que resultou em um aumento das apreensões de cactos enviados para o exterior nos anos subsequentes (Hunt, 1992).

Apesar de grandes expedições em busca de novas espécies, pouco se sabia sobre a biologia dos cactos em habitat natural. Os primeiros registros sobre a história natural dos cactos do Rio Grande do Sul datam do início da década de 1990 com estudos da polinização de espécies melitófilas (Schlindwein & Wittmann, 1995). O levantamento detalhado das espécies de abelhas da região de Caçapava do Sul demonstrou uma rica apifauna com diversas abelhas especialistas em pólen (oligoléticas), muitas delas ainda não descritas (Schlindwein, 1998). As extensas horas de observação da floração dos cactos em habitat natural revelou interações especializadas com polinizadores (Schlindwein & Wittmann, 1995, 1997). Apesar da grande riqueza de interações entre cactos e polinizadores, até o momento poucos estudos foram desenvolvidos com as espécies em habitat natural.

As expedições em busca de novas espécies e populações continuaram, assim como a constante atualização da taxonomia e sistemática do grupo (Carneiro *et al*., 2016; Pontes *et al*., 2017c; Anceschi & Magli, 2018). Atualmente, no Rio Grande do Sul são registradas 65 espécies pertencentes a 11 gêneros, sendo *Parodia* o maior gênero (Carneiro *et al*., 2016). A descrição mais recente de uma espécie de *Parodia* do Rio Grande do Sul, *Parodia gaucha* M. Machado & Larocca é do ano de 2008 (Machado & Larocca, 2008).

Apesar da grande riqueza de espécies do estado, muitas estão ameaçadas de extinção e grande proporção está em declínio populacional (Carneiro *et al*., 2016; Anceschi & Magli, 2018; IUCN, 2020). Um grande esforço para a conservação *ex situ* no Brasil é a coleção de cactáceas do Jardim Botânico de Porto Alegre, onde estudos de germinação e desenvolvimentos de cactos são desenvolvidos com as espécies do Rio Grande do Sul (Carneiro *et al*., 2016).

Conservação de cactos

Os cactos são plantas que possuem forte apelo ornamental e por isso muito admiradas e cultivadas em várias parte mundo. A grande procura de cactos para fins ornamentais e a coleta excessiva de indivíduos e sementes para horticultura tornou-se uma grande ameaça para a família (Oldfield, 1997; Ribeiro-Silva *et al*., 2011). Cerca de 86% dos cactos ameaçados de extinção que estão disponíveis para cultivo são coletados diretamente do habitat natural (Goettsch *et al*., 2015). Dessa maneira, populações de diversas espécies foram drasticamente reduzidas e até mesmo dizimadas (Anderson, 2001).

Naturalmente, os cactos são vulneráveis a alterações em seu habitat devido distribuição restrita, dependência de polinizadores para reprodução e de agentes facilitadores para germinação e estabelecimento (Godínez-Alvarez *et al*., 2003; Ortega-Baes & Godínez-Alvarez, 2006; Mandujano *et al*., 2010; Saraiva & Souza, 2012). Esses fatores somados à pressão antropogênica tornaram Cactaceae uma das famílias de plantas mais ameaçadas do planeta (Goettsch *et al*., 2015; IUCN, 2020). A criação de animais impacta fortemente as populações, seja pelo consumo direto dos cactos por cabras ou pelo pisoteio por gado, que causa altas taxas de mortalidade (Schlosser, 1979; Warrick & Krausman, 1989; Machado *et al*., 2005; Machado, 2007; Anceschi & Magli, 2018). Além disso, a conversão de habitat para silvicultura e outras lavouras diminuiu drasticamente as áreas de ocorrência de diversas espécies (Goettsch *et al*., 2015; Carneiro *et al*., 2016; Anceschi & Magli, 2018).

As mudanças climáticas impactam as interações entre planta-polinizador, uma vez que variações na temperatura média entre os anos podem alterar a fenologia das plantas e o período de atividade de polinizadores (Wall *et al*., 2003; Memmott *et al*., 2007; Petanidou *et al*., 2014). Essas alterações são mais graves para as espécies de cactos auto incompatíveis e que mantêm interações com polinizadores especialistas, que normalmente tem períodos restritos de atividade de voo ao longo do ano (Simpson & Neff, 1987; Schlindwein *et al*., 1995; McIntosh, 2005; Blair & Williamson, 2008; Eggli & Giorgetta, 2017).

Em nível global, cerca de 31% das espécies de cactos estão ameaçadas de extinção, e dessas, apenas 6% estão amplamente protegidas dentro de unidades de conservação (Goettsch *et al*., 2015, 2019). O Brasil é um dos países com maior proporção de espécies de cactáceas endêmicas e ameaçadas de extinção (Ortega-Baes & Godínez-Alvarez, 2006; Ortega-Baes *et al*., 2010), além de ser um país com grande quantidade de espécies fora de áreas protegidas (Oldfield, 1997; Goettsch *et al*., 2019). Assim, é considerado um país prioritário para a conservação de cactos (Ortega-Baes & Godínez-Alvarez, 2006).

Visando a conservação das espécies brasileiras, o Plano de Ação Nacional para Conservação das Cactáceas propõe ações para conservação, incluindo estudos demográficos, reprodutivos, genéticos, entre outros (Ribeiro-Silva *et al*., 2011).

Estudos sobre as espécies são importantes para subsidiar as ações de conservação direcionadas. O levantamento de dados fenológicos, reprodutivos e de interação com polinizadores são importantes para compreender a capacidade das espécies de se reproduzirem *in situ*, principalmente aquelas que não se reproduzem vegetativamente. A baixa produtividade de frutos e sementes, além da baixa viabilidade de sementes, pode influenciar negativamente no recrutamento de novos indivíduos e consequentemente afetar a estrutura populacional das espécies (Godínez-Alvarez *et al*., 2003). Dados sobre a distribuição espacial e abundância de indivíduos em habitat natural podem auxiliar na escolha de áreas prioritárias para a conservação das espécies.

Interações entre cactos melitófilos e polinizadores especialistas

As espécies da família Cactaceae são polinizadas por diferentes grupos funcionais de visitantes florais, como beija-flores, morcegos, abelhas e mariposas (Porsch, 1938; Grant &

Grant, 1979; Rowley, 1980; Anderson, 2001; Barthlott *et al.*, 2015). A diversidade de atributos florais nas cactáceas, como cor, forma, odor, tipo de recursos e tamanho estão diretamente relacionados com os grupos de polinizadores que interagem (Grant & Grant, 1979; Mandujano *et al*., 2010). As flores podem medir de 5 milímetros a 40 centímetros de diâmetro, com estames numerosos, grande quantidade de grãos de pólen e néctar (Anderson, 2001). Muitas flores apresentam forma de disco ou sino, com recursos florais acessíveis aos visitantes florais (Grant & Grant, 1979). Porém, apesar das flores apresentarem morfologia aparentemente generalista com recursos abundantes, podem exibir mecanismos de restrição ao acesso dos recursos florais pelos visitantes, resultando em interações especializadas com polinizadores (Schlindwein & Wittmann, 1997).

As interações cactos-polinizadores e aspectos da biologia reprodutiva foram estudadas em aproximadamente 5% das espécies em nível global e 10% das espécies brasileiras (Mandujano *et al*., 2010; Ribeiro-Silva *et al*., 2011). Porém, muitas vezes estes estudos foram conduzidos sem observação de visitantes florais e polinizadores em habitat natural. A maioria das espécies de cactos apresentam características florais que favorecem a polinização cruzada, como hercogamia, dicogamia e mecanismos de auto incompatibilidade, tornando-as completamente dependentes de polinizadores para se reproduzirem (Anderson, 2001; Mandujano *et al*., 2010). Portanto, estudos detalhados sobre o comportamento dos polinizadores são importantes, uma vez que nem todos os visitantes florais contribuem para formação de frutos e sementes (Ollerton *et al*., 1996; Alves-dos-Santos *et al*., 2016).

Flores melitófilas, polinizadas por abelhas (Faegri & van der Pijl, 1979), são as mais comuns dentre as cactáceas (Anderson, 2001) e diversas espécies podem manter interações especializadas com abelhas oligoléticas (Simpson & Neff, 1987; Schlindwein *et al*., 1995; McIntosh, 2005; Blair & Williamson, 2008; Eggli & Giorgetta, 2017). A oligoletia corresponde a especialização da dieta polínica de abelhas, na qual as fêmeas coletam pólen exclusivamente em plantas de um mesmo gênero ou família (Robertson, 1925). No Brasil, a oligoletia ocorre em espécies de pelo menos 12 tribos de abelhas, que se relacionam com pelo menos 16 famílias de plantas (Schlindwein, 2004). Devido a especialização na coleta de pólen, as abelhas oligoléticas podem apresentar adaptações morfológicas, fisiológicas e comportamentais relacionadas a interação com sua planta hospedeira (Linsley, 1958; Danforth *et al*., 2019a). Uma das adaptações é a sincronia temporal entre a emergência das abelhas dos ninhos e a época de floração de suas plantas hospedeiras, além da sincronia da atividade de voo diária com o momento da abertura das flores (Linsley, 1958; Wcislo & Cane, 1996; Schlindwein &

Wittmann, 1997; Danforth *et al*., 2019a). A sincronia de voo com a antese floral é importante na interação com cactáceas, uma vez que maioria das flores são efêmeras e a longevidade floral pode ser de apenas algumas horas (Schlindwein & Wittmann, 1995; Mandujano *et al*., 2010; Valverde *et al*., 2015). Outra característica que abelhas oligoléticas podem apresentar são estruturas adaptadas ao manuseio e transporte de grãos de pólen com tamanho grande (Linsley, 1958; Thorp, 1979; Danforth *et al*., 2019a), uma característica recorrente do pólen de cactáceas (Leuenberger, 1976). O tamanho do pólen pode impedir a coleta dos grãos por abelhas que não apresentam estruturas especializadas (Roberts & Vallespir, 1978; Thorp, 1979).

Estudos sobre o comportamento de forrageio de abelhas oligoléticas demonstram altas taxas de visitação e constância na coleta de pólen, contribuindo para a polinização efetiva das suas plantas hospedeiras e formação de frutos e sementes com sucesso (Schlindwein *et al*., 2005; Milet-Pinheiro & Schlindwein, 2010; Siriani-Oliveira *et al*., 2018). A eficiência de coleta de pólen está diretamente relacionada ao sucesso reprodutivo das abelhas oligoléticas, uma vez que quanto mais pólen for coletado por unidade de tempo, maior a quantidade de células de cria para aprovisionamento larval (Strickler, 1979; Schlindwein *et al*., 2005). Ninhos de abelhas oligoléticas em flores de *Opuntia* mostram elevada pureza no conteúdo polínico, cerca de 99.5 a 100% é composto por pólen de cactáceas (Schlindwein & Wittmann, 1997). Apesar das grandes quantidades de pólen coletadas pelas abelhas oligoléticas e transportadas para o ninho, a deposição de pólen no estigma é alta e suficiente para atingir a produção máxima de sementes. Muitas abelhas utilizam o estigma dos cactos como plataforma de pouso, o que favorece a deposição de pólen xenógamo previamente depositado na superfície do corpo do polinizador (Schlindwein & Wittmann, 1997; Blair & Williamson, 2008; Lenzi & Orth 2011).

Abelhas oligoléticas podem exibir comportamento reprodutivo atrelado a flores de cactos (Schlindwein & Wittmann, 1997; Oliveira, 2015). Ao aguardarem dentro das flores, machos de diversas linhagens de abelhas solitárias têm a oportunidade de acasalar com fêmeas que visitam flores em busca de recursos (Danforth *et al*., 2019b). Os machos podem se camuflar entre estames para esperar pelas fêmeas (Schlindwein & Wittmann, 1995; Oliveira, 2015) ou patrulhar uma sequência de flores em busca de parceiras e defender territórios de machos invasores (Oliveira & Schlindwein, 2010). Cópulas podem ocorrer dentro das flores, o que potencializa o contato entre as abelhas e as estruturas reprodutivas florais e contribui para o fluxo polínico entre fores coespecíficas (Grant & Hurd, 1979; Schlindwein & Wittmann, 1995, 1997; McIntosh, 2005).

Devido à grande demanda de pólen por abelhas, a coleta excessiva sem transferência polínica entre indivíduos pode ser prejudicial às plantas em algumas interações (Westerkamp, 1996; Hargreaves *et al*., 2009). Abelhas pequenas podem coletar recursos em flores de cactos sem tocar nos estigmas, assim, muitas vezes podem atuar como pilhadoras de pólen (McFarland *et al*., 1989; Osborn *et al*., 1988; Schlindwein & Wittmann, 1997). Como flores de cactos produzem grandes quantidades de pólen em numerosos estames (Anderson, 2001; Mandujano *et al*., 2010), algumas espécies de abelhas podem preencher as escopas em uma única visita floral sem contribuir para polinização, como observado na interação entre *Opuntia phaeacantha* (Engelm.) (Cactaceae) e a abelha oligolética *Perdita texana* (Cresson) (Andrenidae) (Barrows *et al*., 1976). Em contrapartida, alguns mecanismos florais podem limitar a quantidade de pólen coletado pelos visitantes e favorecer a polinização cruzada (Westerkamp, 1996; Harder & Wilson, 1997; Schlindwein & Wittmann, 1997), por exemplo, através de movimento tigmonástico de estames. Os estames sensitivos de cactos, especialmente do gênero *Opuntia*, se movem em direção ao estilete após o contato do visitante floral com os filetes (Schlindwein & Wittmann, 1997; Lenzi & Orth, 2011; Cota-Sánchez *et al*., 2013). Esse movimento atua como um mecanismo de particionamento na oferta de pólen, uma vez que os estames maiores recobrem os estames menores, escondendo cerca de 80% do recurso disponível de uma flor (Schlindwein & Wittmann, 1997). Com a limitação a quantidade de grãos de pólen coletado a cada visita floral, há um aumento da quantidade de eventos de exportação polínica e consequentemente um aumento do sucesso masculino da planta (Harder & Thomson, 1989; Westerkamp, 1996; Harder & Wilson, 1997).

O movimento dos estames em cactos também tem outros papeis importantes na interação com polinizadores. Pode atuar como um mecanismo de deposição de grãos de pólen no corpo de visitantes florais (Toumey, 1899; Lenzi & Orth, 2011), direcionar o movimento dos insetos dentro das flores (Negrón-Ortiz, 1998) e favorecer o uso do estigma como plataforma de pouso (Grant & Hurd, 1979). Esses movimentos ocorrem em pelo menos 10 gêneros de cactáceas em duas subfamílias (Jeffries, 1966; Grant & Hurd, 1979; Rauh, 1979; Kiesling, 1982; Jeffries, 1991), mas apenas espécies de *Opuntia* foram estudadas sob ponto de vista da polinização (Schlindwein & Wittmann, 1997; Lenzi & Orth, 2011).

Enquanto grande proporção das cactáceas é dependente de polinizadores, uma pequena porção é independente de vetores de pólen para se reproduzir sexualmente (Mandujano *et al*., 2010). Algumas espécies apresentam mecanismos de autopolinização autônoma, que pode garantir a reprodução em ambientes com baixa densidade de polinizadores (Nassar *et al*., 2007;

Mandujano *et al*., 2010). Pequena proporção de espécies apresenta cleistogamia, uma estratégia reprodutiva que garante independência de vetores de pólen, uma vez que ocorre a autopolinização sem que as flores se abram (Lord, 1981; Richards, 1997). A cleistogamia foi reportada em espécies de quatro gêneros de cactos, sendo mais frequente em *Frailea* (Cota-Sánchez, 2004; Barthlott & Hunt, 1993; Taylor, 1991; Putnam, 1968).

Diante do exposto, essa tese teve como objetivo estudar aspectos da biologia da polinização e do funcionamento floral de duas espécies de cactáceas melitófilas do Rio Grande do Sul, *Parodia neohorstii* (S.Theun.) N.P.Taylor e *Frailea gracillima* (Lem.) Britton & Rose, bem como avaliar as principais ameaças a conservação de *P. neohorstii* em habitat natural.

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CAPÍTULO 1

THE COST OF FIDELITY: FORAGING OLIGOLECTIC BEES GATHER HUGE AMOUNTS OF POLLEN IN A HIGHLY SPECIALIZED CACTUS–POLLINATOR ASSOCIATION[1]

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THE COST OF FIDELITY: FORAGING OLIGOLECTIC BEES GATHER HUGE AMOUNTS OF POLLEN IN A HIGHLY SPECIALIZED CACTUS–POLLINATOR ASSOCIATION

ABSTRACT

Plant-pollinator interactions vary along a specialization-generalization continuum. Advances in understanding the evolutionary and ecological consequences of different degrees of specialization depend on precise data on plant-pollinator interdependency. We studied the association of *Parodia neohorstii* (Cactaceae) and its bee pollinators focusing on pollinator foraging behavior, flower functioning, female and male reproductive success, and pollen fate. *Parodia neohorstii* showed synchronized flower opening and pollen presentation but discontinuous blooming. The apparently generalized flowers partition pollen through thigmonastic stamen movements that function as mechanical filter against generalized bees by restricting access to the major pollen reservoir to bees with flower handling “know-how”, favouring thereby the oligolectic bee *Arhysosage cactorum*. This pollinator adjusted its pollen foraging to flower opening, removed pollen hurriedly and promoted maximal fruit and seed set, which was minimal in its absence. Estimates of pollen fate revealed that a huge amount of pollen flows to specialized pollinators (86.5%), and only 0.9% reaches conspecific stigmas. The specialized interaction between *P. neohorstii* and *A. cactorum*, both threatened species, is efficient but fragile. Any environmental modification that causes a mismatch between the partners is likely to result in reproductive failure.

Keywords – *Ahrysosage cactorum* - Cactaceae – *Parodia neohorstii* – Plant-pollinator interaction - Pollen dispersal – Pollen fate - Specialized pollination – Melittophily

INTRODUCTION

Despite numerous adaptations of flowering plants to improve pollen transfer, only a small portion of the pollen produced by a flower reaches the stigmatic surface of a conspecific individual, while a huge amount is excluded from plant reproduction (Inouye *et al*., 1994). In bee-pollinated pollen-flowers, the majority of pollen is transported to bee nests as larval food while only a minor portion reaches stigmas (Harder & Wilson, 1996; Schlindwein *et al*., 2005; Müller *et al*., 2006). Some plants are pollinated by oligolectic bees, which are pollen specialists that collect pollen exclusively from the same genus or family (Robertson, 1925). In these pollination systems, the bees acquire for larval supply often more than 85% of the pollen produced by a flower (Schlindwein & Martins, 2000; Schlindwein *et al.,* 2005; Carvalho & Schlindwein, 2011; Pick & Schlindwein, 2011). Oligolectic bees exhibit high flower fidelity often with high flower visitation rates and, despite the removal of huge amounts of pollen from host flowers, generally deposit a sufficient amount of conspecific pollen grains on the stigmatic surface for maximal fruit and seed set (Schlindwein *et al.,* 2005; Milet-Pinheiro & Schlindwein, 2010; Carvalho & Schlindwein, 2011; Pick & Schlindwein, 2011; Siriani-Oliveira *et al.,* 2018). Pollinator behavior between collecting pollen from a donor flower and arrival at the stigmas of conspecific individuals is crucial, since the fate of pollen grains directly affects reproductive success (Johnson *et al.,* 2005). In self-incompatible plant species that are threatened with extinction, pollinator effectiveness is vital for maintaining viable populations over time (Bond, 1994).

Cactaceae is the fifth most threatened botanical family, with almost one third of the species being classified as endangered (Goettsch *et al.,* 2015). The majority of studied cacti species are self-incompatible and depend on pollinators to reproduce (Osborn *et al.,* 1988; McFarland *et al.,* 1989; Boyle, 1997; McIntosh, 2002; McIntosh, 2005; Strong & Williamson, 2007; Mandujano *et al.*, 2010; Martinez-Peralta *et al.*, 2014; Martins & Freitas, 2018). Melittophilous cacti apparently have phenotypically generalized flowers — they possess floral traits that are attractive and accessible to a wide range of floral visitors (Ollerton *et al.,* 2007), due to radial symmetry, and wide-open bowl-shaped corollas that make abundant pollen rewards accessible. Surprisingly, however, several melittophilous cacti species maintain relationships with specialized pollinators, such as oligolectic bees (Grant & Hurd, 1979; Simpson & Neff, 1987; Osborn *et al.*, 1988; McFarland *et al.*, 1989; Schlindwein & Wittmann, 1995, 1997; McIntosh, 2005; Blair & Williamson, 2008; Roig-Alsina & Schlumpberger, 2008) that exhibit foraging and reproductive behaviors in association with cactus flowers, and which have been reported as the main pollinators for several genera (Simpson & Neff, 1987; Schlindwein & Wittmann, 1995, 1997; Roig-Alsina & Schlumpberger, 2008; Eggli & Giorgetta, 2017).

The genus *Parodia* contains 62 species, 33 of which are native to Rio Grande do Sul, the southernmost state of Brazil (Anceschi & Magli, 2018). This region was identified as a hotspot for threatened cactus species (Goettsch *et al.*, 2015), most of which grow outside of protected areas (Goettsch *et al.*, 2018). Oligolectic bees were found to be effective pollinators of three species of *Parodia* Speg. in the region (cited as *Notocactus* (K.Schum.) Frič) (Schlindwein & Wittmann, 1995), and recorded as floral visitors of other *Parodia* species not yet studied (Schlindwein, 1998).

We studied the relationships between the endemic red-listed cactus *Parodia neohorstii* (S.Theun.) N.P.Taylor and oligolectic bees in the Pampa region of South Brazil. *Parodia neohorstii* is classified as Critically Endangered (IUCN) due to its restricted distribution, population decline and fragmented populations (Larocca, 2013). We focused on the role of specialist bees for the reproduction of this species and asked: 1) What is the breeding system of *P. neohorstii*? 2) Are oligolectic bees more effective pollinators and more efficient pollen collectors than other floral visitors? 3) If so, what are the set of floral traits and feeding behaviors of oligolectic bees that mediate this relationship? 4) What is the fate of pollen during dispersal by flower visitors (stigmatic deposition, pollen removal by bees or removal failure)? 5) Is there pollinator limitation and, as a consequence, an insufficient supply of pollen to the stigmas that contributes to threatening the survival of the species?

To answer these questions, we characterized the floral biology and breeding system of *P. neohorstii*, analyzed the behavior of its flower visitors, assessed the fate of its pollen and quantified the deposition of heterospecific pollen grains on the stigmatic surface.

MATERIAL AND METHODS

Study site and plant species

The study was conducted in the Serra do Sudeste in the municipalites of Santana da Boa Vista and Caçapava do Sul in the state of Rio Grande do Sul, Brazil, from October to December in 2016 and 2017. The study sites are located in the region of Guaritas near the mining town

Minas do Camaquã (30°53'36.3"S 53°25'14.3"W), where steep blocks of sandstone and conglomerate of up to 100 meters in height are common (Fig. 1A). The region is part of the Pampa domain, while the local vegetation is characterized by a grassland matrix with shrubs and small trees and a rich flora of herbaceous species. The local bee fauna is diverse and contains a high proportion of oligolectic species (Schlindwein, 1998). The climate is sub-temperate and humid (Maluf, 2000). The area has an average annual precipitation of 1509 mm and an average annual temperature of 18.7 °C, with frost occurring occasionally in the winter, while summer is hot (INMET, 2018). *Parodia neohorstii* is a local endemic cactus restricted to the region of Guaritas. When the study was undertaken, only two populations were known for the species, with one being chosen for study based on the number and accessibility of individuals. The cacti grow on sun-exposed rock substrate almost without soil with their roots fixed between crevices and small loose stones (Fig. 1B, C). Neighboring vegetation is limited to terrestrial bromeliads (*Dyckia maritima* Baker) and agglomerations of bryophytes. The cacti grow as solitary individuals, and do not branch or form clones. The body of the cactus is globular when young and vertically cylindrical at advanced ages, reaching up to 11 cm height. It possesses 18-26 obtuse ribs and areoles with central straight stiff spines (Anceschi & Magli, 2018). At apex of the cactus, long spines and apical lanosity surround the flowers and protect ovaries and fruits, which remain hidden inside the wool. The nectarless flowers have bright yellow petals with a pinkish inner floral tube and produce numerous stamens.

Vouchers of the species were registered at BHCB herbarium - Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (BHCB 194347 and 194073).

Anthesis and flowering

The studied population contained 248 individuals, 93 of which were in reproductive stage. We numbered all reproductive stage individuals (n = 93 plants) with small aluminum tags, and documented flower production over time and the time of flower opening and closure. During 25 days of the same period in 2016 and 2017 (October 27 to November 20), which corresponded to the flowering period of *P. neohorstii,* we recorded the number of open flowers daily to characterize the temporal pattern of flowering. To measure flowering intensity and flowering synchronization among individuals, we counted the number of flowering individuals per day in relation to the total number of reproductive individuals of the population. We related the flowering pattern to the amount of precipitation over time, which was measured daily with a pluviometer. Time of anther opening was determined by directly examining flower buds and flowers.

Breeding system

To determine the breeding system of *P. neohorstii* we performed controlled pollination experiments with individuals with one open flower per day as follows: (1) hand self-pollination — flowers were hand-pollinated with their own pollen (n = 31); (2) autonomous self-pollination — flowers were bagged without manipulation (n = 33); (3) hand cross-pollination — flowers were cross-pollinated with pollen from other individuals (n = 20); and (4) natural pollination — non-manipulated marked flowers were maintained accessible to flower visitors (n = 31).

Flowers used for hand self-pollination, spontaneous self-pollination, and hand cross-pollination were bagged in the bud stage and remained bagged after the treatments until senescence. We determined fruit set by calculating the percentage of formed fruits in relation to the flowers used in each treatment. We determined seed set by calculating the percentage of seeds formed in relation to the average number of ovules per flower, which was determined from 32 flowers of 32 individuals.

Floral visitors

We recorded the frequency of floral visitors of 30 cactus flowers of different individuals throughout the entire process of anthesis (75 hours of observation) in 2016. We noted the following during 30-min intervals throughout anthesis: (1) bee species; (2) bee sex; (3) landing place on arrival at flower (stamens, petals or stigma); (4) whether or not the bee touched the stigma during the visit; (5) whether or not the bee crossed the bundle of stimulated thigmonastic stamens to access hidden pollen at the base of the flower; and (6) visit duration. The 30-min intervals were used to characterize the frequency of visitation over time.

To evaluate pollinator effectiveness, we considered whether the bees touched the stigmas and the frequency of visitors. Bee behavior was also analyzed from video recordings (6 hours) made with a camcorder (Panasonic HC-X929) and digital camera (Canon EOS T5 and 5D) mounted on a tripod. Specimens of bees were deposited in the Entomological Collection of UFMG (Centro de Coleções Taxonômicas da UFMG, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil).

Impact of the absence of the main pollinator on cactus reproduction

At the beginning of the flowering season in 2017, when the main pollinator, *Arhysosage cactorum* Moure 1999 (Andrenidae, Calliopsini), was still inactive, we measured fruit and seed set in 33 flowers to assess the impact of this species on the pollination of *P. neohorstii.* These flowers were all accessible to other flower visitors, which were identified through observations of flowers for 10 hours.

Pollen fate

To characterize pollen fate (and pollen flow promoted by flower visitors), we counted pollen grains inside the flower at different stages of anthesis: (1) "pre-anthesis" — flower buds one hour before opening; (2) "early stage anthesis" — period of high frequency of flower visits, one hour after opening; and (3) "post-anthesis" — recently closed flowers, approximately three hours after opening.

Bee movements inside flowers deposited pollen grains on various flower parts during visits. Thus, we counted the pollen grains adhering to (a) anthers and petals and (b) the stigmatic surface for the three previously described stages. To quantify pollen grains adhering to the stigma surface, we collected the styles of 10 flowers in each stage of anthesis mentioned above (N = 30 styles total). Each style was transferred to a microscope slide containing glycerine gelatin stained with alcoholic fuchsine solution. The gelatin was melted by heat with an alcohol flame, covered with a coverslip and sealed with paraffin wax. We analyzed the samples under a microscope by identifying and counting all the pollen grains present.

To measure the quantity of cross-pollen and self-pollen deposited on the stigmatic surface, we emasculated 10 flowers and compared the number of pollen grains adhering to the stigma of emasculated and intact flowers at the end of anthesis. Because pollen is deposited on the stigma after it is touched by bees, we compared the number of stigma touches by floral visitors to emasculated flowers with that of intact flowers (3 hours of observation for pairs of emasculated and intact flowers). The number of conspecific pollen grains adhering to the stigma of emasculated flowers was considered cross-pollen flow because emasculated flowers are only pollen receptors. The difference in mean number of pollen grains on stigmas in emasculated flowers and stigmas in intact flowers was considered self-pollen flow. Differences in mean pollen content of flowers throughout anthesis (pre-anthesis, early stage anthesis, post-anthesis) were considered as the quantity of both pollen collected by flower visiting bees and pollen remaining in the flowers at different time intervals.

For flowers and flower buds that had styles removed, we washed petals and stamens with 70% ethanol solution and separated the pollen grains present with a nylon sieve (mesh size 70 µm). The solution with the pollen grains was centrifuged for five minutes at 4500 RPM. The suspended solution was withdrawn and 500 µl of 3:1 glycerol lactic acid was added followed by 2 minutes of shaking with a vortex shaker. We extracted an aliquot from this solution for counting pollen grains in a Neubauer chamber (Maêda, 1985).

Statistical analyses

We performed Student's t-test to compare: (1) the number of seeds produced in natural pollination and hand cross-pollination; (2) number of seeds produced in natural pollination with presence and absence of main pollinator; and (3) number of stigma touches received by emasculated and intact flowers. We used the non-parametric Mann-Whitney Rank Sum Test to compare: (1) the amount of pollen deposited on the stigmatic surface between one hour after flower opening and at the end of anthesis; and (2) the amount of pollen deposited on the stigmatic surface of emasculated and intact flowers. All statistical tests were performed with SigmaStat 3.5 software for Windows.

RESULTS

Anthesis and flowering

Parodia neohorstii blossomed predominantly in November. We identified flowering synchronization among individuals in the population during 2016 and 2017. We identified four flowering peaks each year in the same period, between October 27 and November 20, when up to 61% of the individuals set flowers on the same day (Fig. 2). Flowering seems to have been triggered by rainfall since in both years flowers opened on average 2.6 ± 1.0 (N = 11) days after rain, but only on days with high solar intensity (Fig. 2). Flowering, thus, was not continuous and concentrated to just a few days; there were no cactus flowers present during 56% of the flowering period (N = 25 days) in 2016 and 39% (N = 41 days) in 2017.

Flowers opened synchronously in the population at around 12:44h and closed 3 hours later (188 ± 18 min; N = 41). There was little variation in the time of flower opening within the population (on average 30 ± 13 minutes) (N = 7 days). Under natural conditions, all flowers accessible to pollinators opened for only one day (N = 136). Bagged flowers without pollen deposition on the stigmas opened for two (55.4%) or three days (44.6%) (N = 56). Flowering individuals had on average 1.5 ± 0.8 open flowers per day; 65.8% had one, 25.1% two, 6.1% three, and 3.0% four to seven simultaneously open flowers per day (N = 700). Individual plants produced from 1 to 26 flowers (5.3 ± 4.3, N=332) per year.

When petals unfolded, all anthers were already dehisced and the stigma receptive. Hermaphrodite flowers had on average 206 ± 20 (N = 10) sensitive stamens inserted in 10 or 11 layers (N = 20) along the floral tube. When floral visitors contacted the stamens, the stamens responded to the mechanic stimulus by bending inward until they attached to the style. Thus, the layer of the uppermost stamens formed by a set of 53 ± 4 stamens (N = 20), covered the rest of the stamens that were inserted in the lower part of the floral tube. In the stimulated condition, only 25% of the total pollen content of the flower was freely accessible to floral visitors.

Breeding system

The studied population of *P. neohorstii* was predominantly xenogamous. All marked non-bagged flowers accessible to pollinators, as well as all hand cross-pollinated flowers, had similar fruit and seed set (t = -0.513, d.f. = 49, P = 0.610, N = 51, Table 1). Fruit set was low for self-pollination treatments, (32% after hand self-pollination and 10% after autonomous self-pollination), while seed set was minute (2 seeds per fruit on average in both treatments) (Table 1).

Floral visitors

We recorded a total of 2284 bee visits to flowers of *P. neohorstii* representing seven species of four families, all of which were solitary species (Table 2). Only two species were frequent flower visitors: *Arhysosage cactorum* with 81.2% of the visits, and *Anthrenoides micans* Urban 1955 (Andrenidae, Protandrenini) with 16.9% (Fig 3, Video 1). Other bee species were sporadic flower visitors and together accounted for only 1.9% of the visits (Table 2).

Bees landed on flowers at three sites: stigmatic surface, petals and stamens. Females of *A. cactorum* used the stigmatic surface as their main landing platform while *A. micans* landed mainly on petals (Table 3). The flowers were also searched by male bees of both species, who mainly used the stigmatic surface to land. The bees synchronized their flight activity with flower opening and actively collected pollen from the first minutes opening onward (Fig. 4A). Females of *A. cactorum* forced their entry when the petals unfolded before the stamens were visible. Males of both bee species patrolled flowers in search of females, and flowers served as mating sites. Males of *A. cactorum* occasionally hid between the stamens waiting for females. When females arrived in flowers in search for pollen, males seized them with their mandibles and tried to copulate. Couples in copulatory position flew between flowers while the females continued foraging for pollen even *in copula*, which resulted in high frequencies of pollen transfer and stigma touches (Fig. 3A). Couples contacted stigmas repeatedly during almost all flower visits (Table 3). Females of *A. cactorum* touched stigmas more frequently than females of *A. micans*. Visits of males of *A. micans* were rare because they frequently flew over the flowers without touching them; of 13 flyovers the flower was touched only once (N = 413), and most of these contacts were to the stigma surface (Table 3). Couples of *A. micans* frequently touched the stigma, but they were very rare in the flowers (0.4% of the visits).

The sensitive stamens moved to the center of the flower after mechanic stimuli such as body contacts of bees during pollen collection. The dense layer of the stimulated uppermost stamens hindered the bee access to the pollen of the lower stamens, thus forming a pollen chamber containing 75% of the pollen grains of a flower. Females of *A. cactorum* crossed the stamen barrier in stimulated flowers during 94% of the visits (N = 83) and continued to collect the hidden pollen, while females of *A. micans* reached the pollen chamber during only 39% (N = 98) of the visits to flowers with stimulated stamens. Larger bees of *Cephalocolletes rugata* Urban 1995 and *Ancyloscelis romeroi* Holmberg 1903, which visited the flowers only sporadically, accessed the hidden pollen on all of their recorded visits. Small bees, like those of *Dialictus* sp., never crossed the bundle of stamens.

Fifty-two percent of all visits occurred during the first hour of anthesis. The frequency of female bees decreased drastically thereafter, and about two hours after flower opening females were practically absent. Males of *A. cactorum*, on the other hand, continued patrolling flowers in search of females even when female flower visits had ceased (Fig. 4B). Visits were very infrequent during the last 30 minutes of anthesis and represented only 1.4% of the total.

Impact of the absence of the main pollinator on cactus reproduction

In the beginning of the flowering season in 2017, the most frequent visitor, *A. cactorum*, was completely absent for 13 days. During this period only 33% of the flowers set fruits (N = 33). Seed set differed dramatically between the presence and absence of this main pollinator (t = -8.199, d.f = 40, P = <0.001, N = 42) with only 18% of ovules setting seed in its absence. In addition, flower longevity increased to 2.25 ± 0.44 days (N = 16). The flowers were visited by bees of *A. micans* (72.1%), *Dialictus* sp. (24.5%), *C. rugata* (1.7%), and *Callonychium* sp. (1.7%) (N = 61 visits).

Pollen fate

Flowers of *P. neohorstii* produced on average 100,200 ± 13,614 (N = 10) pollen grains. Bees collected 58% of the total pollen from the flowers during the first hour of anthesis (Fig. 5A), after which the stigmatic surface was already covered with pollen grains, with an average of 1,721 ± 695 grains (N = 10) adhered to the stigmas. There was no difference in the number of pollen grains adhering to the stigma in the first hour and at the end of anthesis (U = 55,000, P = 0.734, N = 20, Fig. 5B, Fig. 6). Heteroespecific pollen grains (i.e., from other species) represented 0.34% of the total pollen deposition on the stigma (5.9 ± 6.9; N = 10). This pollen content came from Asteraceae, Poaceae, Iridaceae and Euphorbiaceae (*Croton* sp.). Autonomous pollen deposition (i.e., by the flower on its own stigma) was minimal. Only 106 ± 97 (N=10) grains were deposited on stigmas of fresh open flowers.

Emasculated and intact flowers received a similar number of stigma touches by bees (t = -1.294, d.f. = 34, P = 0.204, N = 36), which promoted the same chance of pollen deposition on the stigma surface in both treatments. Emasculated flowers received on average 2.2 ± 1.2 stigma touches per 10-minute interval, while intact flowers received on average 1.8 ± 0.7 (N = 18 pairs). The number of pollen grains deposited on the stigmatic surface of the two treatments, however, differed (U = 84.000, P = 0.011, N = 20), with emasculated flowers having on average 929 ± 577 (N = 10) pollen grains on the stigmatic surface while intact flowers had on average 1,730 ± 747 (N = 10). The difference between treatments was considered autogamous pollen deposition during the movement of bees inside the flower (801 ± 314 grains, N = 10).

Only 1.7% of the pollen produced by a flower was deposited on a stigmatic surface by the end of anthesis (Fig. 7). Thus, bees collected 86.5% of the total pollen produced by flowers.

At flower closure, 11.8% of the total pollen content (11,890 ± 3.813 grains, N = 10) remained adhering to floral parts inside the flower.

DISCUSSION

Pollination of Parodia neohorstii

Parodia neohorstii depends on specialized pollinators to reproduce and only sets fruit and seeds after cross-pollination. The oligolectic bee *Arhysosage cactorum* is by far its most important pollinator due to the following attributes: (1) high rate of stigma touches; (2) effective pollen transfer among individuals; (3) frequent floral visits; (5) high flower constancy during foraging flights; and (6) high fruit and seed set only in its presence.

The use of the stigmatic surface as a landing platform dramatically increases cross-pollen transfer because xenogamous pollen adhering to a bee's body from previously visits is deposited directly on the stigma before the bee makes contact with autogamous pollen. When floral visitors land on petals or stamens, and directly collect pollen before touching the stigma, autogamous pollen grains are deposited on their body, adhering to the superficial layers and covering xenogamous pollen. According to layering models (Harder & Wilson, 1996), such covering decreases the chance of xenogamous pollen deposition on the stigma because it is located under layers of autogamous pollen on the bee's body. Other cactus-oligolectic bees have also been reported to land on stigmas (Schlindwein & Wittmann, 1997; Blair & Williamson, 2008; Lenzi & Orth, 2011).

Besides *A. cactorum* being the most frequent floral visitor, females of the species have additional stigma touches during movements within the flowers and touch the stigmatic surface twice as much as females of *A. micans* and four times more than females of *Dialictus* sp. Moreover, also males of *A. cactorum* most likely contribute to efficient cross pollen flow because while searching for females, they inspect each flower of *P. neohorstii* on the routes of their patrolling flights, frequently use the stigma as landing platform and touch anthers. In the absence of *A. cactorum*, other bee species promote only very low fruit set, and especially low seed set, probably due to low xenogamous pollen flow. As is the case for *P. neohorstii*, other self-incompatible cacti species have been found to be dependent on specialized bee pollinators, such as *Astrophytum asterias* Lem., *Ferocactus cylindraceus* (Engelm.) Orcutt and *Ferocactus wislizeni* Britton & Rose, even when numerous other floral visitors are present (McIntosh, 2005;

Blair & Williamson, 2008). In these cases, the focal cacti only set seed in the presence of oligolectic bee pollinators.

We consider our findings to indicate that the interaction between *A. cactorum* and *P. neohorstii*, as is likely true with interactions of other melittophilous *Parodia* cacti, corresponds to an "ecological specialization", *sensu* Armbruster (2017). Thus, we corroborate the idea that analyzing flower morphology alone, decoupled from analyses of pollinator feeding behavior, is not sufficient to determine the degree of "realized specialization" of flower-pollinator interactions.

Controlled pollen removal enhances male success

Synchronized mass flowering with pollen presentation by a population is an extraordinary attractive to pollinators. Oligolectic bees time their visits to flower opening already when petals start to unfold and concentrate their pollen collection during the first hour of anthesis. *Parodia* floral traits, such as wide-open corollas and pollen-at-once presentation, promote this fast pollen removal, but otherwise decrease cross-pollen flow (Harder & Wilson, 1996). In this context, we interpret pollen partitioning mediated by thigmonastic stamen movements as a mechanism to limit pollen removal by individual bees that do not have adequate flower handling ability. Stimulated flowers hide about three quarters of their total pollen, thus restricting pollen access for generalist bees to the upper layers of stamens. This floral functioning improves male function because unspecialized floral visitors remove less pollen per visit, and thus pollen availability is maintained for more time, attractiveness to pollen seeking females is extended and movements of bees among flowers is enhanced, all of which contribute to increasing cross-pollen flow. Geitonogamy is largely avoided since that the majority of individual cacti open only one flower per day.

Specialization of Arhysosage cactorum *on cacti flowers*

Besides the high frequency of visits and stigma touches, some behavioral characteristics of *A. cactorum* make it a specialized pollinator of *P. neohorstii*.

(i) Pollen diet — females of this species restrict the diet of their larvae to only cacti pollen. As shown by scopa pollen load analyses, females always carry monospecific pollen content of the cactus species in flower during a foraging trip (Schlindwein & Wittmann, 1995).

(ii) Timing foraging activity with flower opening — females of *A. cactorum* are capable of adjusting their pollen foraging to the exact moment of flower opening. This finding is surprising because whole populations of *P. neohorstii* have synchronized, but episodic, flowering during only five to six days per month. The fine-tuned synchronization of foraging on a sporadic flowering cacti species maybe related to (a) perception of specific chemical and/or visual cues that attract the specialist bees to the flowers (Burger *et al.*, 2010; Dobson *et al.*, 2012; Milet-Pinheiro *et al.*, 2012; Carvalho *et al.*, 2014) ; (b) recognition of changes in weather conditions that induce flowering (changes in air humidity, rainfall regime, temperature, sunshine intensity); or (c) monitoring and inspection of cactus sites.

(iii) Behavioral adaptation, flower and pollen handling — females of *A. cactorum* have the most efficient pollen collection behavior among the flower visiting bee species in *P. neohorstii*. During the first hour of anthesis, period with high rates of bee visitation, the position of stimulated stamens makes access to pollen difficult for floral visitors, except for females of *A. cactorum*, which are the only bees with the capacity to always cross the bundle of stamens to access the hidden pollen. Thus, the formation of this pollen chamber can also be interpreted as a mechanical filter against generalized bees by restricting access to the major pollen reservoir to only bees with flower handling "know-how". The same functional interpretation of thigmonastic stamen movements in cacti flowers was previously proposed for flowers of *Opuntia* cacti, where stimulated stamens hide about 80% of the pollen grains inside the pollen chamber and also where only oligolectic specialist bees gained access to this pollen (Schlindwein & Wittmann, 1997).

(iv) Mating behavior of *A. cactorum* — similar to many other oligolectic bee species, the reproductive strategy of male *A. cactorum* is associated with the pollen host plants of females (Wittmann *et al.*, 1990; Medeiros & Schlindwein, 2003; Oliveira & Schlindwein, 2010; Oliveira *et al.*, 2012; Oliveira, 2015). Males of *A. cactorum* wait and search for females in cactus flowers, while couples flying *in copula* among cacti flowers strongly enhance cross pollination (Schlindwein & Wittmann, 1995). This mating behavior is only known for a few other species of Andrenidae (Alcock *et al.*, 1978; Wittmann *et al.*, 1990).

Pollen fate

Pollen flow is complex with many factors influencing pollen destination (Harder & Wilson, 1996). We estimated the main pollen fates here by integrating extensive field observation of pollinator behavior and flower functioning. Our analysis of pollen destination for the studied population of *P. neohorstii* revealed that less than 2% of the pollen grains produced in a flower reach stigmas, and about half of this comprises xenogamous pollen. Although only a small portion of pollen is destined directly for cacti reproduction, the mean amount of cross-pollen deposited on the stigmas is about 12-times greater than the number of ovules, and thus sufficient to achieve almost optimal seed output. Thus, fruit and seed set were maximal for the cacti-bee association, but not when bees of *A. cactorum* were absent. Bees of the genus *Arhysosage* are closely related with cacti species in different areas of South America (Schlindwein & Wittman, 1995; Moure, 1999; Engel, 2000; Ramos, 2013). These bees forage for pollen only in Cactaceae and exhibit reproductive behavior associated with cactus flowers (Schlindwein & Wittman, 1995; Ramos, 2013), indicating that the evolutionary history of the genus *Arhysosage* might be tightly related to melittophilous cacti species. High frequency of flower visitation rates of theses bees in *P. neohorstii* promote high reproductive success of the female function. The male function, however, gets uncertain due the huge amount of pollen removed from plant reproduction. Because of the variation in the destiny of pollen, it can be expected that selection pressures act more deeply in the male than in the female component of fitness (Moore & Pannell, 2011). Based on our data, however, it is not possible to determine to what extent this apparently exaggerated sexual performance of male function is consequence of sexual selection via an intra-sexual competition mechanism to improve male reproductive success, or a result of natural selection via consumption of pollen grains by specialized pollinators.

Although we cannot quantify exactly the amount of pollen collected by females of the two frequent visitor species *A. cactorum* and *A. micans*, it is clear that a larger portion by far flows into the reproduction of the oligolectic *A. cactorum.* This greater flow is due the following attributes: (1) visiting *A. cactorum* females are five times more frequent, and their visits three times longer, than females of *A. micans;* (2) body and scopa size of *A. cactorum* are also somewhat greater, which likely results in a greater capacity to carry pollen.

Similar large amounts of pollen (86-95%) were found to be collected by oligolectic pollinators to feed their larvae in other specialized associations between oligolectic bees and plants (Schlindwein *et al.*, 2005; Carvalho & Schlindwein, 2011; Pick & Schlindwein, 2011). Although huge quantities of pollen flow to oligolectic bee nests, plants that maintain relationships with such bees have great chances to achieve high reproductive success due to high degree of conspecific pollen transfer among individuals (Schlindwein *et al.*, 2005). Thus, this huge quantity of pollen that goes to oligolectic bee nests is the "fidelity cost" of attracting pollen-specialist bees as main vectors of effective pollination service.

Another 11.8% can be considered pollen loss due removal failure, because uncollected pollen inside of flowers does not serve pollination nor feed future pollinators.

Analysis of stigmatic pollen loads in *P. neohorstii* revealed almost pure compositions of conspecific pollen and, thus, an efficient flower-pollinator association. Heterospecific pollen grains were negligible, and probably were carried by females of *A. micans*, because some of them arrived to the flowers carrying pollen loads of different color (Fig. 3C). Furthermore, this species has been documented to carry mixed scopa pollen loads when visiting cacti flowers (Schlindwein & Wittmann, 1995).

Interdependence of cacti and oligolectic bees: a relationship in danger?

The interaction between *P. neohorstii* and *A. cactorum* is highly seasonal and occurs only during a few weeks of the year. The flight periods of oligolectic bees usually encompass the blooming period of their host plants (Wcislo & Cane, 1996). A mismatch between the period of emergence of *Arhysosage* bees from nests and flowering of *P. neohorstii* could substantially impact the reproductive success of both the plant (without effective cross-pollen transfer) and the pollinator (without rendezvous and mating sites).

We noted that other cacti species from the genera *Parodia, Gymnocalycium* and *Frailea* that occur at the study site, and which are also intensely visited by the oligolectic cacti bees (Schlindwein & Wittmann, 1995), blossomed during the breaks in flowering of *P. neohorstii* (unpubl. data). This might guarantee continuous pollen supply to *A. cactorum*. Such staggered flowering among alternate days has been reported previously for other melittophilous sympatric cacti species (Giorgis *et al.*, 2015; Eggli & Giorgetta, 2017). This pattern is an effective strategy to share pollinators and maintain reproductive isolation from similar species with overlapping populations (Giorgis *et al.*, 2015). Our data indicate that the synchronized mass flowering of *P. neohorstii* was triggered mainly by rainfall, which is similar to the findings of Eggli & Giorgetta (2017). It is possible that other abiotic factors, such as temperature, air humidity and direct sunlight, also influence synchronized flower opening of cacti populations and contribute to alternating flowering of sympatric cacti species.

Arhysosage cactorum, therefore, is the key pollinator of the community of small cacti in the study region. The co-occurrence of a range of cacti species that bloom, in a staggered manner, during the flight season of *A. cactorum* may be necessary to maintain this bee species throughout the entire flowering season, as observed for cacti oligoleges in Arizona and Texas (Simpson & Neff, 1987; Blair & Williamson, 2008).

Our study shows that the specialized relationship between *P. neohorstii* and *A. cactorum* is effective but also fragile. Both partners are threatened, red listed species (Larocca & Machado, 2013; Sul, 2014). It is improbable that pollinator limitation puts the cactus in danger, but there are other factors that could disrupt the interaction between *P. neohorstii* and *A. cactorum.* Any environmental impact, such as climatic change, that causes phenological variation in the periods of plant flowering and pollinator activity (Memmott *et al.,* 2007), may promote a mismatch between partners and lead to reproductive failure. Changes in land use, driven by agriculture and livestock farming, affect cactus habitats, availability of nesting sites for pollinators, and the conservation of the threatened partner species.

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TABLES

Table 1. Measures of fruit and seed set for pollination treatments of *Parodia neohorstii*: (1) autonomous self-pollination; (2) hand self-pollination; (3) hand cross-pollination; (4) natural pollination; and (5) *Arhysosage cactorum* absence — non-manipulated marked flowers visited by *Anthrenoides micans, Dialictus* sp., *Cephalocolletes rugata and Callonychium* sp. during the 13 day period of absence of *A. cactorum.*

Treatments	No. of flowers	Fruit set % (N)	Seed set mean ± SD	Seed set in relation to ovule number (%)
Autonomous self-pollination	40	10 (4)	2.0 ± 1.4	2.7
Hand self-pollination	31	32.2 (31)	2.3 ± 1.2	3.2
Hand cross-pollination	20	100 (20)	66.2 ± 27.9	91.7
Natural pollination	31	100 (31)	62.8 ± 18.4	87.1
Arhysosage cactorum absence	33	33.3 (33)	13.1 ± 13.2	18.2

Table 2. Flower-visiting bees of *Parodia neohorstii*. Frequency of floral visits were measured only in 2016 during 75 hours of observation (N = 2284 visits). (*) Species not recorded in 2016 and not included in the calculation of the frequency. We only observed *Ancyloscelis romeroi* in 2017 as a sporadic visitor (N = 4 visits).

Bee species	**Tribe / Subfamily**	**Sex**	**Frequency (%)**
Andrenidae			
Arhysosage cactorum Moure 1999	Calliopsini	♀♂	81.21
Callonychium sp.	Calliopsini	♀	0.04
Anthrenoides micans Urban 1955	Protandrenini	♀♂	16.90
Apidae			
Ancyloscelis romeroi Holmberg 1903	Emphorini	♀	*
Ceratina sp.	Ceratinini	♀	0.17
Colletidae			
Cephalocolletes rugata Urban 1995	Neopasiphaeinae	♀	0.48
Halictidae			
Dialictus sp.	Halictini	♀	1.18

Table 3. Landing site, stigma contacts and duration of flower visits of frequent bee visitors (> 1% of visits) to flowers of *Parodia neohorstii*. Visit duration was quantified only for female bees. Landing places: Sg = stigmatic surface, P = petals, S = stamens.

Bee species	**Sex**	**Landing place**				**Stigma touches (%)**	**Visit duration (s)**
		Sg	P	S	(N)		
Arhysosage cactorum	♀	62%	24%	14%	79	87% (N = 129)	18.4 ± 14.9
	♂	64%	15%	21%	140	76% (N = 222)	-
	Couple	50%	50%	0%	14	97% (N = 84)	13.8 ± 13.4
Anthrenoides micans	♀	21%	62%	17%	128	47% (N = 182)	6.8 ± 5.5
	♂	69%	8%	23%	49	70 % (N = 79)	-
	Couple	-	-	-	-	80 % (N = 10)	-
Dialictus sp.	♀	6%	82%	12%	103	22% (N = 27)	5.5 ± 6.9

FIGURES

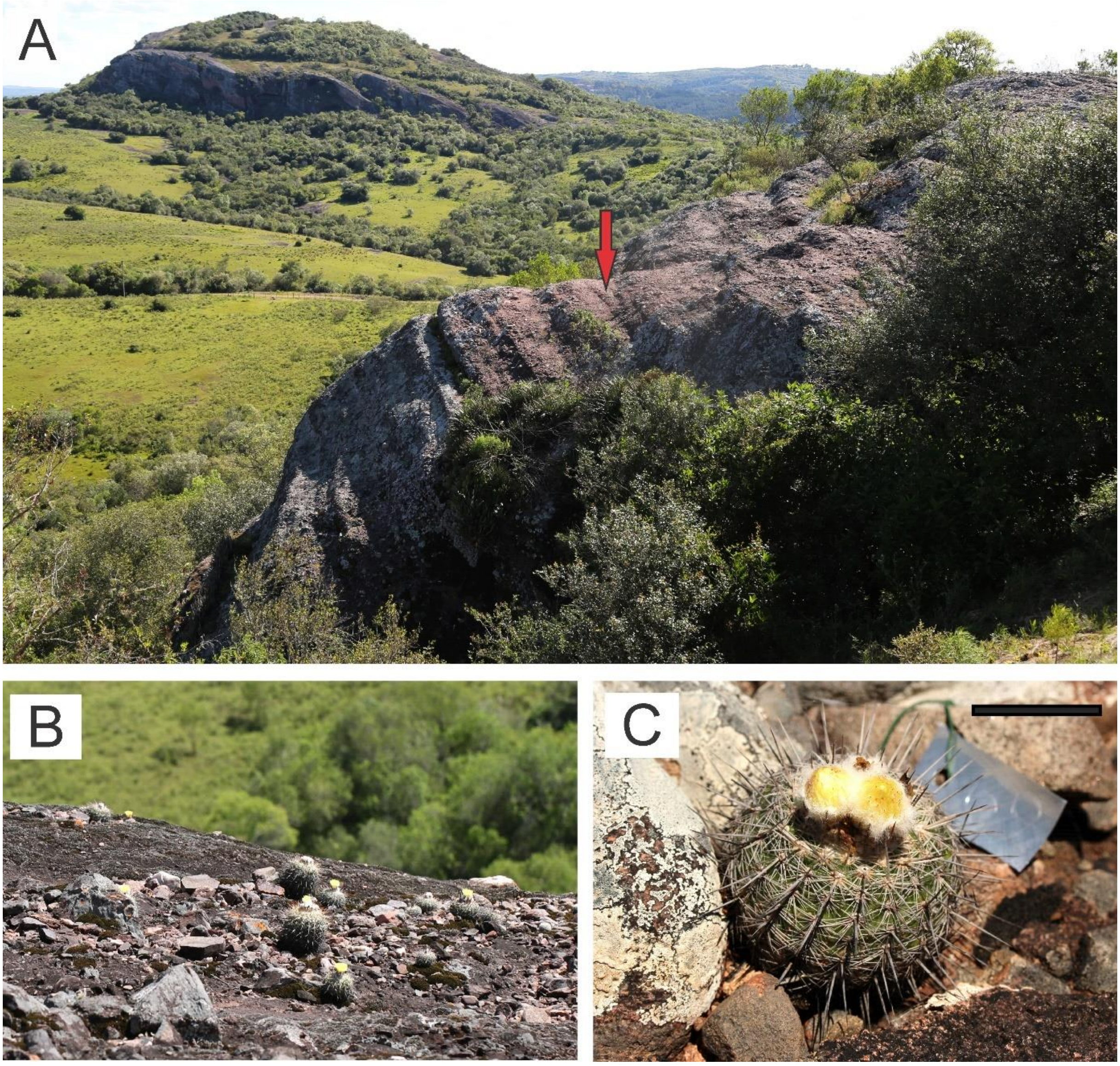

Figure 1. Study area and habitat characteristics. (A) Sandstone and conglomerate rock outcrops in the Guarita-region of the state of Rio Grande do Sul. The red arrow indicates the location of the studied population. (B) Individuals of *Parodia neohorstii* growing on rock substrate almost without soil. (C) Flowering cactus with aluminum tag. Scale bar = 35 mm.

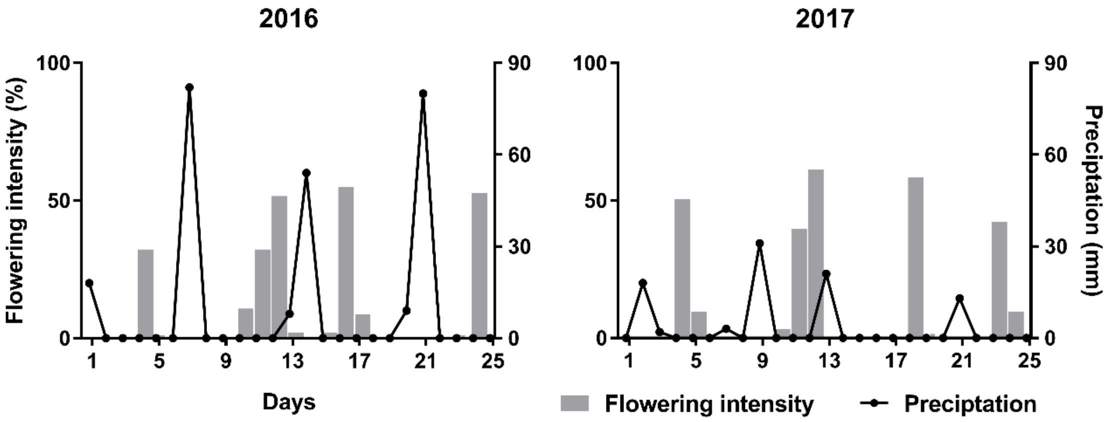

Figure 2. Flowering intensity (number of flowering individuals/total number of reproductive individuals) of *Parodia neohorstii* and precipitation from October 27 (day 1 of X axis) to November 20, during 2016 and 2017.

Figure 3. Main floral visitors of *Parodia neohorstii*. (A) A pair of *A. cactorum in copula*: male is clutching the female with its mandibles. (B) Female of *A. cactorum* collecting pollen. (C) Female of *A. mican*s collecting pollen. Note the different color of the pollen load in the scopa of *A. micans*. Stimulated stamens contacting the style in (B) and (C). Scale bars = 2 mm.

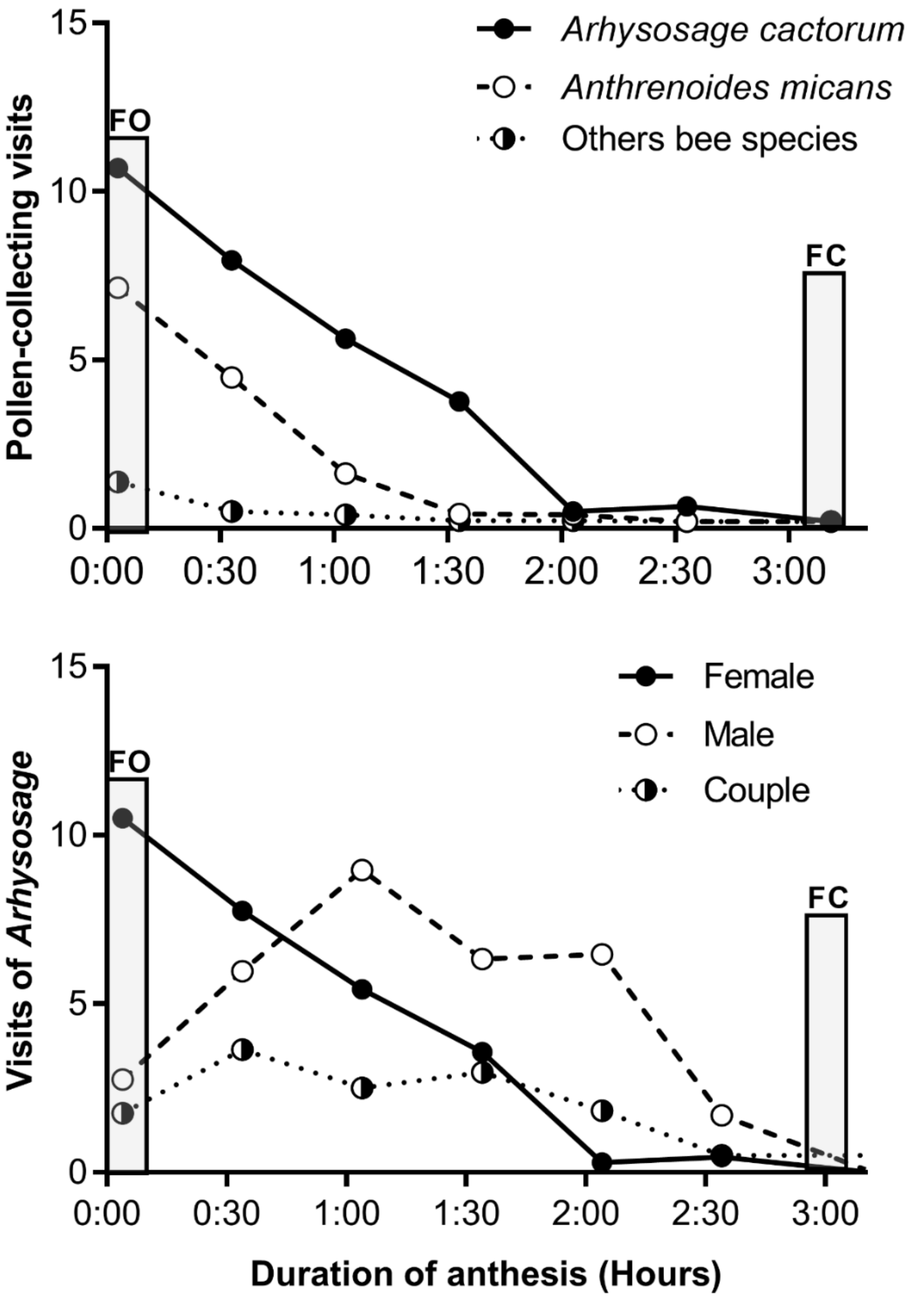

Figure 4. Flower visits for *Parodia neohorstii* throughout anthesis. (A) Mean number of pollen collection visits by female bees. (B) Mean number of visits by males, females and couples of *A. cactorum*. FO = flower opening and FC = flower closure.

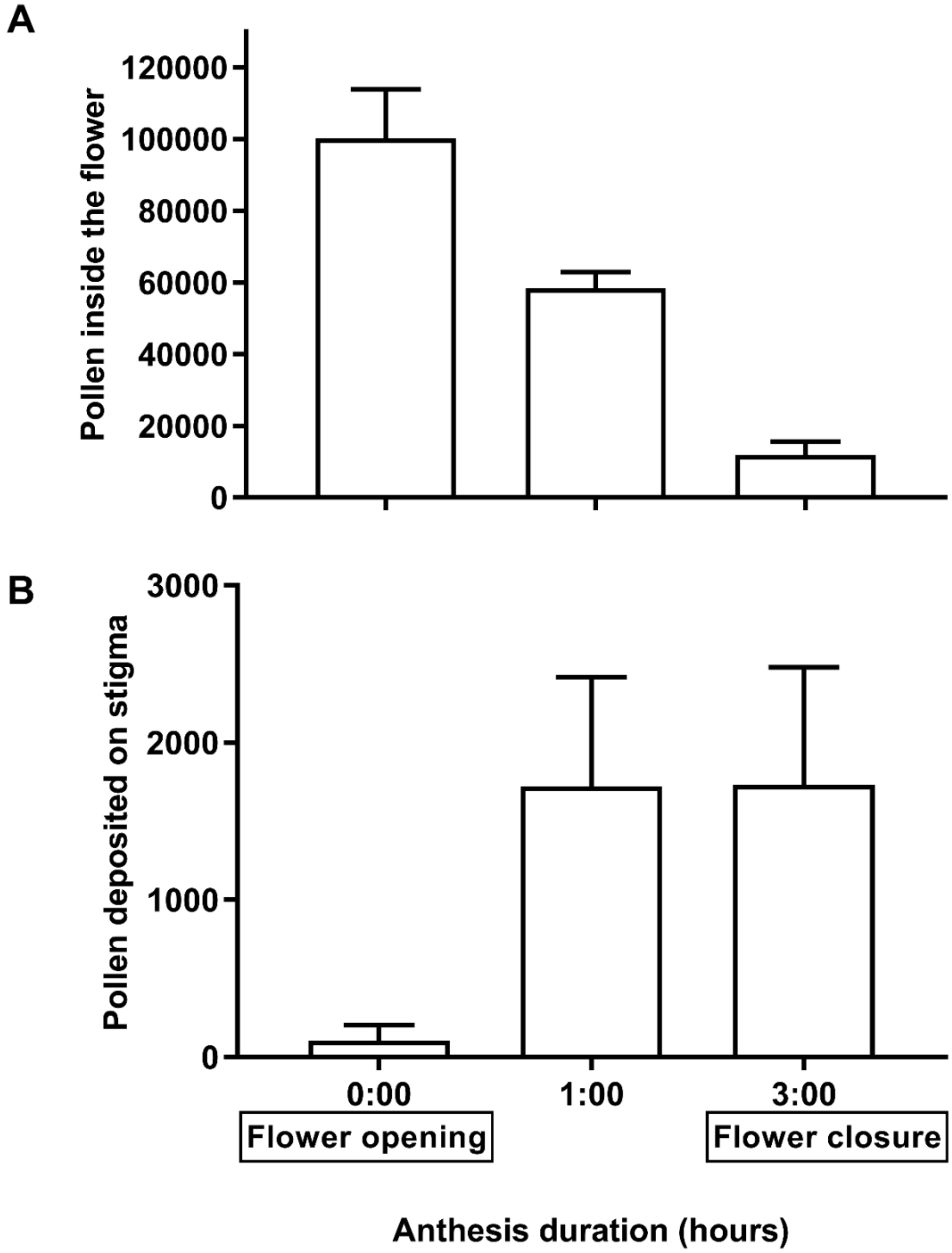

Figure 5. Pollen removal and deposition by bees in flowers of *Parodia neohorstii.* Pollen grains inside the flower (A) and pollen deposited on the stigma (B) after bee visits during different moments of anthesis (N = 10).

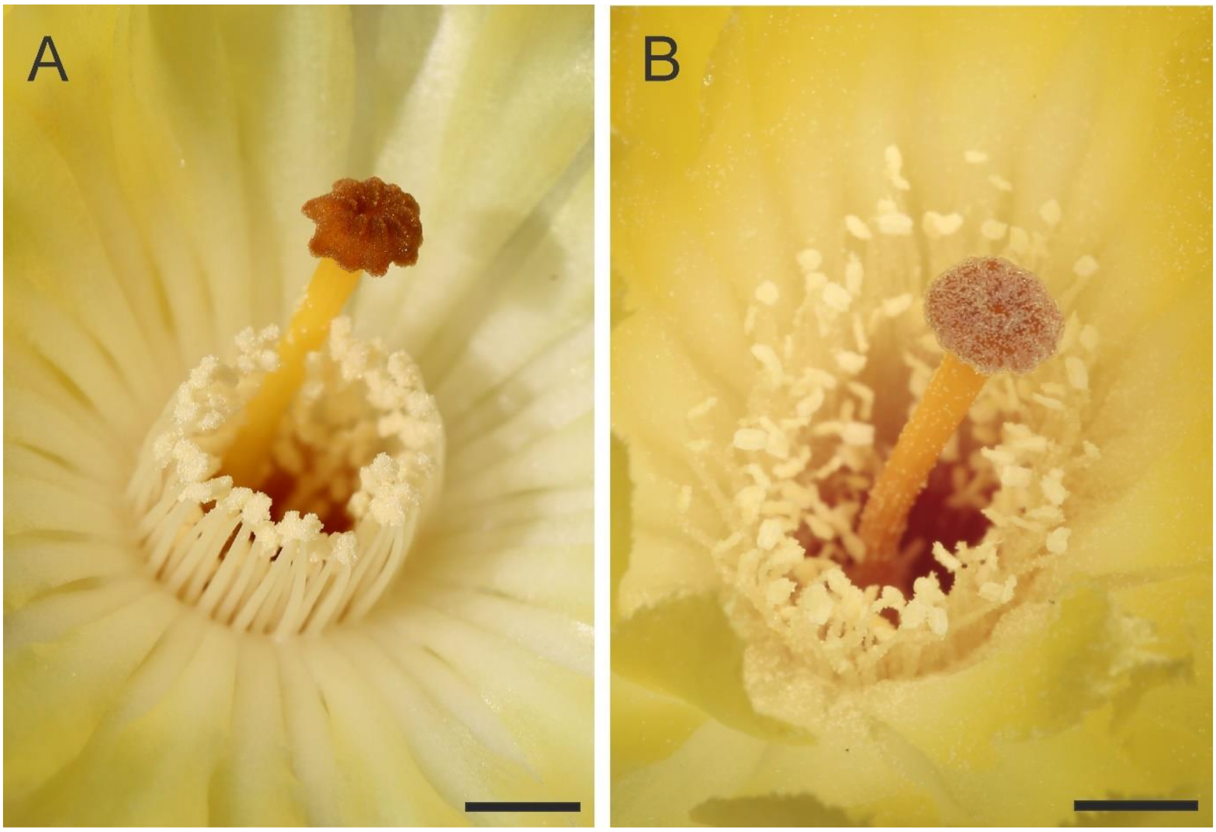

Figure 6. Deposition of pollen grains on the stigma of *Parodia neohorstii*. (A) Freshly open flower with stamens still upright and dehisced anthers. Pollen and anthers do not contact the stigmatic surface. (B) Flower at the end of anthesis near flower closure. Stigmatic surface saturated with pollen grains. Note: pollen grains adhered to petals and style. Scale bar = 2 mm

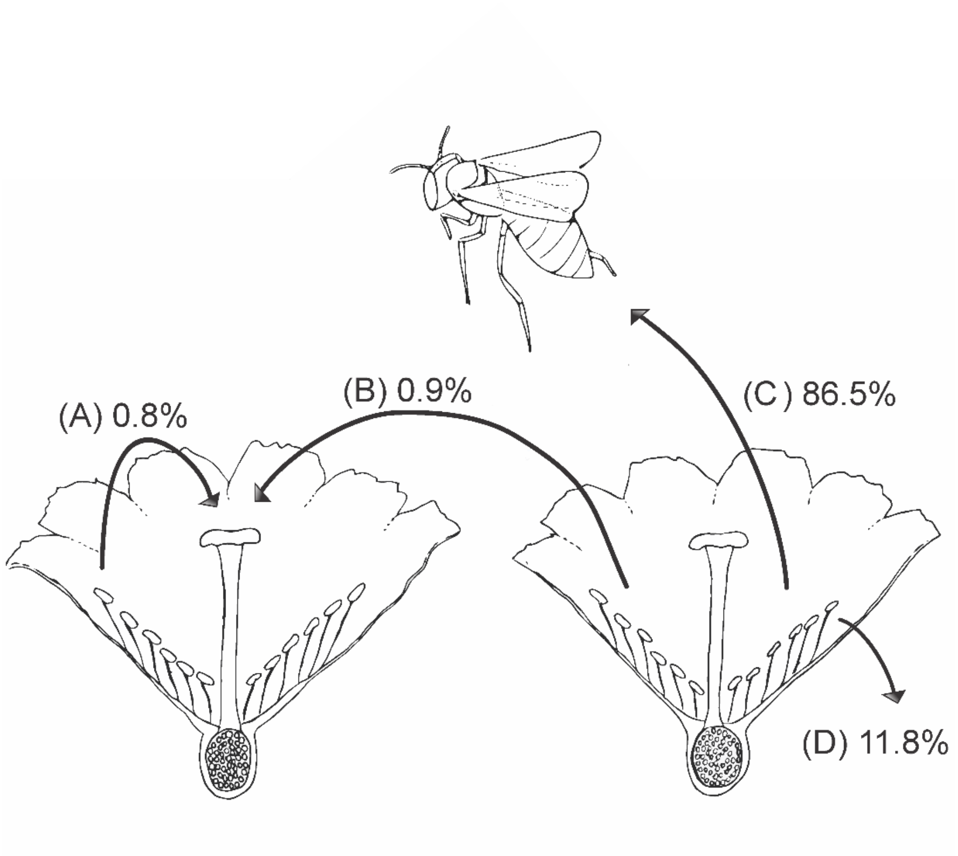

Figure 7. Pollen fate for *Parodia neohorstii.* Percentages represent the fate of all pollen produced by a flower at the end of anthesis. (A) Autogamous pollen deposited on a stigma after the movement of bees inside the flower. (B) Xenogamous pollen deposited on a stigma by bees. (C) Pollen collected by bees. (D) Pollen remaining in the flower after closure.

CAPÍTULO 2

STIMULATION OF THIGMONASTIC STAMENS BY BEES INDUCE FLORAL SENESCENCE IN CACTUS FLOWERS [2]

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STIMULATION OF THIGMONASTIC STAMENS BY BEES INDUCE FLORAL SENESCENCE IN CACTUS FLOWERS

ABSTRACT

Floral longevity, the period when flowers are open and functional, vary dramatically among plant species. In several species, flowers demonstrate plasticity in their lifespan due to abiotic and biotic factors, such as temperature, water availability and pollination events. *Parodia neohorstii*, a cactus pollinated by pollen-specialist bees, has synchronized flower opening and thigmonastic stamen movements. Flowers lasts only three hours in the presence of the specialized bees that promote maximum pollen deposition to stigmas in the first hour of anthesis and continuously trigger stamen movements to the centre of the flower after touching the filaments. When specialized bees are absent in the beginning of flowering season, flowers open again on the following days even in the presence of less efficient pollinators. To identify which factors promote the variation in floral longevity, we experimentally tested the effect of pollen deposition and induction of stamen movements promoted naturally by specialized bees.

We found that pollen deposition to stigmas has no influence on floral longevity. Only mechanical stimuli to the filaments, triggering stamen movements, shorten flower lifespan to three hours. We discovered a new function for stamen movement, directly related to the induction of floral senescence. In *P. neohorstii*, effective pollen dispersal only occurs if accompanied by movement of stamens induced by specialized bees, and the lack of these movements may indicate a scarce pollination environment. The maintenance of unstimulated flowers for extended periods in the absence of the effective pollinators that stimulate stamen movements may improve the male function by increasing chances of pollen export.

Key words: Thigmonastic stamen movements, floral biology, floral senescence, Cactaceae, oligolectic bees, thigmonasty, Sensitive stamens, *Parodia neohorstii*

INTRODUCTION

Floral longevity corresponds to the period from the beginning of anthesis until floral senescence (Primack, 1985), and its plasticity may be a feature that influences the reproductive success of pollen dispersal and ovule fertilization (Ashman & Schoen, 1994). Pollen removal, as well as pollen deposition on the stigma, can increase when flowers are open for a longer period (Ishii & Sakai, 2000; Blair & Wolfe, 2007; Aximoff & Freitas, 2010). The extension of floral longevity in situations with low pollinator activity may be an alternative to increase the chances of reproduction (Rathcke, 2003). Flower maintenance for long periods, otherwise, comes at an energy cost and may lead to the production of low-quality fruits (i.e. reduced seed number) (Ashman & Schoen, 1997; Castro *et al.*, 2008).

In general, the period in which flowers remain open and functional varies among species from a few hours up to months (Primack, 1985; Ashman & Schoen, 1994). Floral lifespan can be influenced by abiotic factors such as drought and high temperature and biotic factors (Primack, 1985; Teixido & Valladares, 2015). Moreover, there is often a relationship between floral longevity and pollination in long-lasting flowers, since pollen deposition to the stigmas can trigger physiological responses that induce petal wilting and abscission (van Doorn, 1997; Rogers, 2006; Tripathi & Tuteja, 2007; Araujo *et al*., 2020).

Pollen deposition on the stigma triggers senescence in many species, e.g. *Digitalis purpurea* (Plantaginaceae), *Erythranthe guttata* (Phrymaceae) and *Epilobium angustifolium* (Onagraceae) (Stead & Moore, 1979; Arathi *et al*., 2002; Clark & Husband, 2007). Pollen removal may influence floral longevity like in protandrous Campanulaceae, where the duration of the staminate phase diminishes after pollen removal by bee pollinators (Devlin & Stephenson, 1984; Richardson & Stephenson, 1989, Evanhoe & Galloway, 2002). In flowers of *Blumembachia* (Loasaceae), nectar uptake by specialized bee pollinators accelerates pollen presentation and shortens the staminate phase (Siriani-Oliveira *et al.,* 2018, 2020). Moreover, in many orchid species, the potentially long-lived flowers rapidly wilt after pollinarium deposition on the stigma (Ashman & Schoen, 1994; Proctor & Harder, 1995; Martini *et al*., 2003; Abdala-Roberts *et al.,* 2007).

Parodia neohorstii (Cactaceae) a globular cactus from southern Brazil, is an example of a species with strong plasticity in flower longevity (Cerceau *et al.,* 2019). Flowers open synchronously at about 13 p.m. and present thigmonastic stamens that move to the center of the flower after mechanic stimuli at the filaments. Flowers are pollinated by pollen-specialist bees.

Floral longevity is ephemeral, lasting only three hours in the presence of the specialized pollinators, which promote maximum pollen deposition to stigmas in just one hour and while continuously triggering stamen movements (Cerceau *et al.*, 2019). When pollinators are absent, or when flowers are bagged, flowers open again on the following days (Cerceau *et al.*, 2019).

Having discovered this variation in floral longevity in *P. neohorstii* according to the presence or absence of pollinators and, the shortening of floral longevity after pollen deposition on the stigmas in many plant species (Stead & Moore, 1979; Arathi *et al*., 2002; Clark & Husband, 2007), we hypothesized that floral longevity in *P. neohorstii* shortens through deposition of conspecific pollen deposition on the stigma. We also considered whether flower manipulation by bees influences flower longevity. We focused on two questions: 1) Does deposition of pollen on the stigma of *P. neohorstii* flowers shortens floral duration? 2) Does triggering of thigmonastic stamen movements during pollen collection by bees influence the floral longevity?

To answer these questions, we performed controlled pollination experiments with different pollen sources (autogamous and xenogamous pollen), determined which bees trigger stamen movements, and applied mechanical stimuli to the stamens, simulating bee visits to flowers, with and without pollen deposition to the stigmatic surface.

MATERIAL AND METHODS

Study site

The study was conducted in the Serra do Sudeste in the state of Rio Grande do Sul, Brazil. We studied three populations of *Parodia neohorstii* located in the region of Guaritas near the mining town of Minas do Camaquã (30°53'36.3"S, 53°25'14.3"W) between October and December of 2016, 2017 and 2018. The region is situated in the Pampa biome and is characterized by steep blocks of sandstone and conglomerate of up to 100 meters height. The local vegetation is composed of a rich herbaceous flora with shrubs and small trees (Carneiro *et al.*, 2016). The local bee fauna is diverse and contains a high proportion of oligolectic species (Schlindwein, 1998). Oligolectic bees are pollen specialists that collect this resource only in plants of the same genus or family to feed their larvae (Robertson, 1925). The climate is sub-temperate and humid (Maluf, 2000), with an average annual temperature of 18.7 °C and an average annual precipitation of 1509 mm (INMET, 2018).

Plant species

Parodia neohorstii is an endangered cactus endemic to the region of Guaritas. The small globose cacti grow as solitary individuals on sun-exposed rock substrate. The flowering season is between end of October and early December. The cacti produce on average five flowers per year but generally only one flower opens per day from 13:00 to 16:00h, which is intensely visited by solitary bees. Oligolectic *Arhysosage cactorum* (Andrenidae, Calliopsini) and *Anthrenoides micans* (Andrenidae, Protandrenini) are the commonest flower visitors and pollinators (Cerceau *et al.,* 2019). *Parodia neohorstii* has thigmonastic stamens that bend inwards toward the style after mechanic stimuli of the filaments. The stamens exhibit two main positions: (1) Original position, where stamens are sprawled erect and flower base and style are freely accessible; (2) Stimulated position, where stamens bent inwards or bent forwards towards to the center of the flower and lean onto the style after mechanical stimuli. After some minutes, the stamens bend back to the original position ("unstimulated stamens").

Vouchers of the studied species were deposited at BHCB herbarium - Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (BHCB 194347 and 194073).

Description of stamen movements in Parodia neohorstii

To describe in detail the stamen movements, we touched the filaments with a paintbrush and measured the time stamens took to move to the center of the flower and the time interval to return to the original position (N = 30).

Floral longevity and senescence in natural conditions

Under natural conditions, floral longevity of *P. neohorstii* increases in bagged flowers and in the absence of pollinators (Cerceau *et al.*, 2019). To quantify changes in floral longevity according to the visiting bee species, we marked flowers and observed floral visitors throughout the anthesis during the flowering periods of *P. neohorstii* during three years. Observations were made at the beginning of the flowering season in 2017 and 2018 (N = 21 flowers), when the main pollinator species, *Arhysosage cactorum,* was still inactive and in the middle of the flowering period in 2016, 2017 and 2018 (N = 187 flowers), when bees of this species were the most frequent floral visitor. Marked flowers were accompanied daily to check for flower opening, and consistence of the petals (turgid or wilted).

Factors that alter floral longevity

To verify which factors influence floral longevity, we performed eight treatments involving deposition of pollen grains on the stigmas and simulation of bee visits through experimental induction of thigmonastic stamen movements by mechanic stimuli. We individually marked floral buds according to the treatment they received and monitored the flowers over time until senescence. All flowers were bagged before and after treatments. We checked whether flowers opened in subsequent days, the opening hours and the consistence of the petals (turgid or wilted). We compared the results with non-manipulated flowers accessible to pollinators.

To know if pollination alters floral longevity, we performed four manual pollination treatments: (1) hand cross-pollination with one pollen donor from another individual (n = 49); (2) hand cross-pollination with five pollen donors from five individuals (n = 31); (3) hand self-pollination (n = 40); (4) autonomous self-pollination (n = 60).

We performed four treatments simulating bee visits to find out whether mechanic contacts of the pollinators to the filaments alter floral longevity. For this, we gently touched filaments with a paintbrush to trigger stamen movements. The stimulation of the stamens was repeated every five minutes (roughly the period stamens took to return to their original position). The flowers submitted to these treatments were bagged again after the hand stimulations to keep them inaccessible to floral visitors.

In order to verify the effect of stamen movements on floral longevity without the influence of pollination, we performed paired treatments with or without pollen deposition on the stigmas: (1) five stimuli to stamens with xenogamous pollen deposition on the stigmas (n = 40); (2) five stimuli to stamens without pollen deposition on the stigmas (n = 40); (3) ten stimuli to stamens with xenogamous pollen deposition on the stigmas (n = 40); (4) ten stimuli to stamens without pollen deposition on the stigmas (n = 40).

Bee species that trigger stamen movements

To determine which bee species triggers stamen movements during flower visits, we observed flowers with unstimulated stamens in the field (15 h) and made video recordings of bee visits to flowers (10 h) with digital cameras (Canon EOS T5i and 5D) and camcorder (Panasonic HC-X929) in November of 2016, 2017 and 2018. We recorded the duration of bee visits and verified whether stamens moved to the flower center after the visits. We classified floral visitors by sex and noted whether they were in copula position.

To correlate body size of the bees with successful stamen movements, we collected bee individuals in the flowers and measured nine regions of the insect body (N=20 individuals per sex) (see below) in the laboratory. To obtain a more precise measure of the body volume of floral visitors, we used the mathematical formula of ellipsoid volume. For this, we divided the body of the bees into the three sections: head, mesosoma and metasoma, and each of the sections was measured for height, diameter and width. The measurements of each body section were inserted in the formula:

$$\text{Volume of each body section} = 4/3 * \pi * (\text{height}/2) * (\text{diameter}/2) * (\text{width}/2)$$

To calculate the total volume of the bees' body, we summed the volume of each section:

$$\text{Volume of bee body} = (\text{Volume of head}) + (\text{Volume of mesosoma}) + (\text{Volume of metasoma})$$

For bees in copula position, we summed the body volume of females and males.

Bee specimens were deposited in the Entomological Collection of UFMG (Centro de Coleções Taxonômicas da UFMG, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil).

Statistical analyses

To compare floral longevity under natural conditions between flowers visited and non-visited by the main pollinator, we performed a Mann-Whitney Rank Sum Test.

To compare floral longevity in different pollination treatments, we performed the test Kruskal-Wallis One Way Analysis of Variance on Ranks.

To compare floral longevity in flowers receiving 5 or 10 manual stimuli in stamens, we performed a Mann-Whitney Rank Sum Test. The same test was performed to compare flowers that received the same number of manual stimuli in stamens but differed in pollen deposited on the stigma or not.

To compare the body volume of different bee species, we used the following tests: 1) Kruskal-Wallis One Way Analysis of Variance on Ranks to compare more than two species; and 2) Student's t-test to compare only two species.

RESULTS

Stamen movements in Parodia neohorstii

After mechanical touches of the filaments, stamens immediately bent inward and reached the style (Figure 1) on average after 2.1 seconds (± 0.4, N = 30 flowers). Stamens returned to the original position on average after 280.7 seconds (± 69.4; N = 30). Under natural conditions, the stamens are stimulated after visits by bees (Figure 2).

Floral longevity

Anthesis of new flowers of *P. neohorstii* started around 1239 h (± 26 minutes) and flowers closed at 1539 h (± 32 minutes) (N=68 flowers). Bagged flowers, not visited by bees, reopened at the subsequent day at 1138 h (± 29 min) and closed at the same time as first-day flowers (1533 h ± 17 minutes) (N=23). Flowers, thus, opened for 3 hours on the first and for 4 hours on the second day of anthesis.

Pollination and stamen stimulation experiments

All pollination treatments with bagged flowers resulted in similar floral longevity of the *Parodia* flowers (H = 0.232, d.f. = 3, P = 0.972, N = 180, Figure 3A). Self-pollinated flowers had similar floral longevity that cross-pollinated flowers. Floral longevity was on average 2.3 ± 0.7 days for all pollination treatments, and 84.4% of the flowers opened 2 or 3 days. In some of these hand cross-pollinated flowers, floral longevity reached up to 5 days.

Floral longevity was short only in flowers whose filaments received experimental mechanical stimuli that resulted in thigmonastic stamen movements (Figure 3B), and 94.3% of its flowers lasted only one day (N = 160).

There was no difference in the floral longevity among the hand stimulated flowers that were hand cross-pollinated from those that were not pollinated. Pollen deposition on the stigma surface, therefore, had no influence on the floral longevity (stamens five times hand-stimulated: U = 774.5, P = 0.477, N = 80 / stamens ten times hand-stimulated: U = 800, P = 0.099, N = 80). Moreover, flower longevity did not differ in flowers, whose stamens were stimulated experimentally five or ten times (flowers with pollen deposition on stigmas: U = 740, P = 0.240, N = 80 / no pollen deposition on stigmas: U = 720, P = 0.115, N = 80).

Bee species that trigger stamens movement

Visiting female bees in flowers of *P. neohorstii* search only for pollen, since flowers do not produce nectar (Cerceau *et al.,* 2019). The flowers of *P. neohorstii* were visited by four species of bee (Table 1). Of the two panurgine species *Arhysosage cactorum* and *Anthrenoides micans*, which were the most common visitors, both females and males visited the flowers. For both species, we found bees copulating in the flowers or visiting the flowers in copula position, which was very common for *A. cactorum* (Figure 4, Table 1).

The body volume of the four species differed significantly among the species (P = <0.001, H = 156.81, d.f.= 8, N=155, Figure 5). The triggering of stamen movements in a flower visit was related to the body volume of the bees: larger bees triggered movements in most visits, while the smaller bees (*Dialictus sp.*) never triggered stamen movements. Visits of couples, with around double body volume, triggered more stamen movements when compared to visits of solitary females of the same species.

The behavior of the bees inside the flower also influenced the triggering of stamen movements. Pollen collecting females stimulated more stamen movements than males of the same species (*A. micans*: t = -10.263, d.f.= 33, P = <0.001, N = 35; *A. cactorum*: t = -6.021, d.f.= 38, P = <0.001, N = 40). Males remained for a shorter time in a flower than females and rarely touched the stamens. Males of *A. cactorum* made quick flower visits on their patrol flights and sometimes hid between the stamens, remain immobile with their head among anthers looking outward and, in general, induce movements of only a few stamens. On their patrol flight, males of *A. micans* predominantly fly over flowers and seldom visit them. On their few quick visits, they alight on the petals, rarely touch stamens, and therefore never triggered stamen movements. Females of *A. cactorum* actively collect pollen, moving their legs quickly between the bundle of stamens and stay longer inside the flowers (on average 17.9 ± 14.6 seconds), which promoted stamen movements during almost all visits. During flights in copula position, females of *A. cactorum* carried the male actively but continued to collect pollen in a uniform manner. In the few flower visits observed, females of *Cactocolletes rugatus* always triggered stamen movements due to their intense movements during pollen collection. Females of the tiny *Dialictus sp*. generally landed on the petals and collected pollen grains fallen down from anthers to the petals or the flower tube after flower visits of larger bees and rarely removed pollen directly from anthers. Therefore, they had little contact with the filaments and never triggered stamen movements (Table 1). Females of *A. micans* collect pollen actively from the anthers, nevertheless, they stimulated stamens in only one third of the visits. However, when females of *A. micans* were in copula position within a flower, the rate of stamen movements rose to 90.9% (Table 1). Considering that copulations lasted a long time (up to 7 min), couples remained immobile within the flowers during most of the time.

Flower longevity in different natural conditions

Under natural conditions, there are two scenarios of abundance of main flower visitors. In the flowering peak, when females and males of *A. cactorum* were the most frequent floral visitors, floral longevity lasted only one day (1.0 ± 0.0 d, N = 187). In this period, flower visits of other bee species were rare. In the beginning of the flowering period, when bees of *A. cactorum* were absent, flower longevity lasted on average 2.2 ± 0.4 days (N=21), demonstrating that floral longevity varied under natural conditions (T = 4158.000, U= 0.000 P = <0.0001, N = 208, Figure 6). In this period, flowers were visited mainly by bees of *Anthrenoides micans* and *Dialictus sp.* (Figure 7).

DISCUSSION

Our results reveal that only the stimulation of the thigmonastic stamens is responsible for the shortening of the floral longevity in *Parodia neohorstii* to the three-hour lifespan. Neither self nor cross-pollen deposition to the stigma surface cause any effect on the duration of the cacti flowers. This is surprising, because in most species with variable flower longevity, the deposition of pollen to the stigmatic surface is one of the most important factors that induces floral senescence, once the female function has been fulfilled (Arathi *et al.*, 2002; Clark & Husband, 2007; Niu *et al.*, 2011). In *P. neohorstii*, pollen dispersal to the body of effective pollinators occurs together with the induced movements of stamens, and the lack of these movements may indicate a scarce pollination environment. Consequently, the increased floral longevity favors male function and enhances the probability of pollen export to conspecific flowers.

Pollination effectiveness of stamen movement triggering bees

Some species can alter floral longevity according to the abundance of floral visitors and extend flower lifespan to increase the chances of pollination in scenarios of pollinator scarcity (Harder & Johnson, 2005). It is interesting that even receiving pollen on the stigma, floral longevity of *P. neohorstii* does not vary with the abundance of floral visitors, but with the quality of bee species, being regulated by its effective pollinators.

Whether flower visiting bees trigger stamen movements or not is related to two main factors:

1. Body volume and strength of bees: Small bees do not trigger stamen movements because they do not have enough strength and body mass to induce thigmonastic stamen movements. The insects require a minimum force and weight to surpass the stimulus threshold to activate the movement of stamens. In *Opuntia polyacantha* flowers, thigmonastic stamen movements are activated only by larger floral visitors (Cota-Sánchez *et al.,* 2013), but the force required to trigger the movements has not been measured. The strength of bees that is closely related with body mass was shown to be the key factor to open keels in papilionid flowers (Córdoba & Cocucci *et al.*, 2011) or activate the catapult mechanism in a species of *Cornus* for explosive pollen dispersal (Edwards *et al.,* 2005).
2. Flower handling: Males exhibit different flower handling, because they do not search for floral resources in the nectarless flowers, which reduces the triggering of stamen movements. On their search for females, males make short inspection visits in the flowers of *P. neohorstii* and touch the stamens only slightly. Females of *A. cactorum, A. micans* and *C. rugatus*, however, intensely contact filaments and anthers during pollen collection, stay much longer inside the flowers and rashly move around the stamen ring, but females of the small *Dialictus sp*. rarely touch the stamens during pollen gleaning and never trigger stamen movements.

Females and males of *A. cactorum* frequently fly in copula position from flower to flower. While the male-carrying female continues pollen foraging (Schlindwein & Wittmann, 1995; Oliveira, 2015; Cerceau *et al.*, 2019), the couples stimulated stamen movements in all visits, given their large body volume and the intense prolonged pollen collection by the females. This is also true for pairs of *A. micans*. However, flowers visits in copula position of this species are rare, the couples often remain in the same flower for minutes, and the females suspends pollen collection in this period (Dutra *et al.*, 2020).

In pollen rich cactus flowers, small bees can easily fill their scopae in a single flower visit without contact with receptive stigmas, as occurs in cacti flowers of *Parodia*, *Gymnocalycium* (Schlindwein & Wittmann, 1995) and *Opuntia* (Barrows *et al.*, 1976, Osborn *et al.*, 1988). Due to the restricted foraging of small bees on the same flower and neighboring flowers, the contribution to pollination is minimal (Schlindwein & Wittmann, 1995).

Larger bees, however, forage on various flowers to fill the scopae. They usually use the stigma as a landing platform, which favors xenogamous pollen deposition (Cerceau *et al.*, 2019). Besides that, the flowers with stimulated stamens reduce the amount of pollen collected by individual bees (Schlindwein & Wittmann, 1997, Cerceau *et al.*, 2019), which encourages bees to move to flowers of other plant individuals, enhancing their pollination effectiveness.

In *P. neohorstii*, the triggering of the stamen movements seems to indicate the presence of effective pollinators and likely pollen export, which thus could be interpreted as the signal to close flowers after the first day of blooming.

Enhanced flower lifespan in the absence of bees that trigger stamen movements

In most species with short-lived ephemeral flowers, pollination does not induce floral senescence (Ahmad & Tahir, 2016) and flower lifespan seems to be fixed and regulated by internal factors (Stead, 1992). Floral longevity of *P. neohorstii* is ephemeral only after triggering of stamen movements. The indication of a scarce pollination environment through missing or weak thigmonastic stamen movements linked to increased flower lifespan worked well under natural conditions: when bees of oligolectic *A. cactorum* were absent at the beginning of the flowering period in two years (2017 and 2018), flowers opened again on subsequent days. This occurs due to the presence of almost exclusively small bees and the absence of stamen movement triggering bees.

Curiously, flower opening of second- and third day flowers is one hour earlier than that of new flowers. The one-hour advanced flower opening of the old in comparison to first-day flowers might favor the old flowers by anticipated flower visits and pollination. The flower visitation rate of the effective pollinator *A. cactorum* is highest at the opening time of first-day flowers, and a one-hour time interval was enough for these bees to deposit the maximum amount of pollen to the stigma to fertilize all ovules (Cerceau *et al.,* 2019).

The function of stamen movements

Stamen movements occur in at least 15 plant families (Sibaoka, 1969; Simons, 1981; Schlindwein & Wittmann, 1997a, 1997b; Ren, 2010; Du *et al.,* 2012; Ren & Bu, 2014). In some cases, it was demonstrated that stamen movements enhance pollen deposition on the pollinator body (Rathcke & Real, 1993; Angulo *et al.,* 2014) and pollen flow among conspecific plants through pollen partitioning (Schlindwein & Wittmann, 1997a; Henning & Weigend, 2012; Siriani-Oliveira *et al.,* 2018, 2020).

In Cactaceae, thigmonastic stamen movement occurs in at least 10 genera (Jeffries, 1966; Grant & Hurd, 1979; Rauh, 1979; Kiesling, 1982; Jeffries, 1991). It is not yet known whether the stamen movement influences the floral longevity in other species of the genus *Parodia* and in other genera. The function of thigmonastic stamen movements is still not clearly understood. In species of *Opuntia* it was shown that stimulated thigmonastic stamens form a pollen chamber that leads to that effective pollinators touch the stigma to access the hidden pollen (Schlindwein & Wittmann, 1997), promoting pollen deposition on the pollinator body (Lenzi & Orth, 2011) and that triggering of the stamen movements is closely related to the insect body size (Cota-Sánchez *et al.,* 2013). This is similar to *P. neohorstii*, where stamen movements result in pollen partitioning that limit the amount of pollen removal per floral visit and form a mechanical barrier that prevents unspecialized bees to access the hidden pollen reservoir (Cerceau *et al.,* 2019).

However, our results point to a new function for stamen movements. The induction of floral senescence after successive stamen movements seems to signal to the flower that the pollinators visited the flowers and pollen was exported.

These results bring new hypotheses for future research to elucidate the complex functioning of this mechanism and clarify how it evolves in cacti lineage, once stamen movement occurs in 2 subfamilies (Jeffries, 1966; Grant & Hurd, 1979; Rauh, 1979; Kiesling, 1982; Jeffries, 1991). In *P. neohorstii*, the induction of floral senescence after visits of oligolectic pollinators may be related to the highly specialization in plant-pollinator interaction, however, further studies are needed to clarify these hypotheses.

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TABLES

Table 1: Floral visitors of *Parodia neohorstii*, their body volume, duration of flower visits and percentage of visits that resulted in stamen movement.

Bee specie	**Sex**	**Body volume (mm^3)**	**Visit duration (s) (N)**	**% stimuli (N)**
Arhysosage cactorum Moure 1999	Female	27.3 ± 3.8	17.9 ± 14.6 (101)	98.0 (50)
	Male	18.8 ± 5.0	2.1 ± 1.2 (41)	23.6 (55)
	Couple	46.1 ± 7.1	13.6 ± 13.44 (103)	100.0 (60)
Anthrenoides micans Urban 1995	Female	15.4 ± 1.8	6.9 ± 5.4 (111)	30.1 (113)
	Male	9.2 ± 4.3	1.2 ± 0.5 (4)	0.0 (16)
	Couple	24.62 ± 2.4	132.6 ± 159.2 (5)	90.9 (11)
Cactocolletes rugatus (Urban)	Female	52.1 ± 6.4	19.0 ± 1.8 (4)	100 (4)
Dialictus sp.	Female	4.9 ± 0.9	4.1 ± 4.5 (71)	0.0 (59)

FIGURES

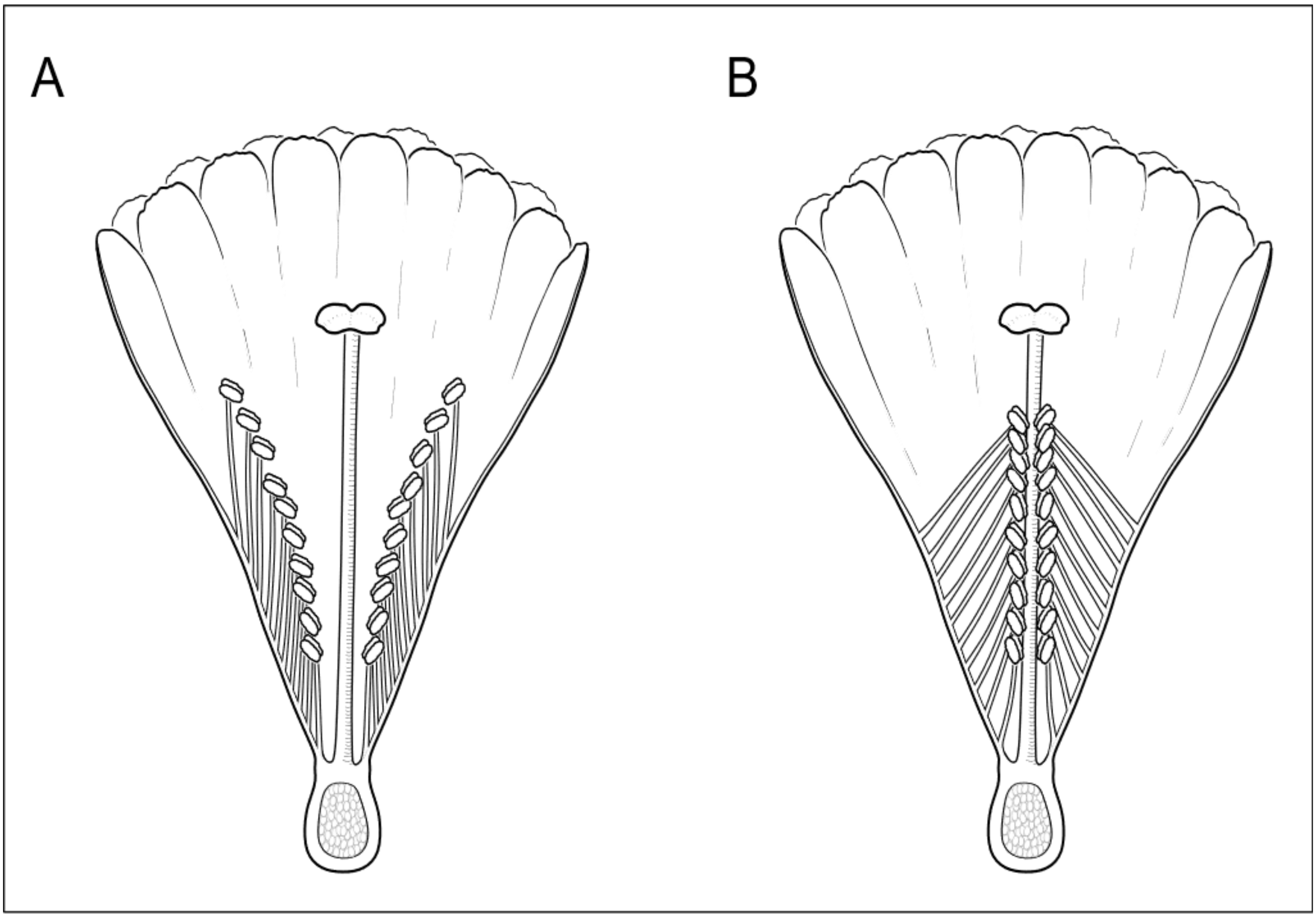

Figure 1: Flower of *Parodia neohorstii*, longitudinal section, schematic drawing of stamen movements. (A) Unstimulated stamens in the original position, sprawled erect; anthers, flower base and style are free accessible; (B) Stamens in the stimulated position. After mechanical stimulation, stamens bend to the center of the flower and lean to the style. Only anthers of the uppermost stamens are accessible to flower visitors. After four to five minutes, the stamens bend back to the original position.

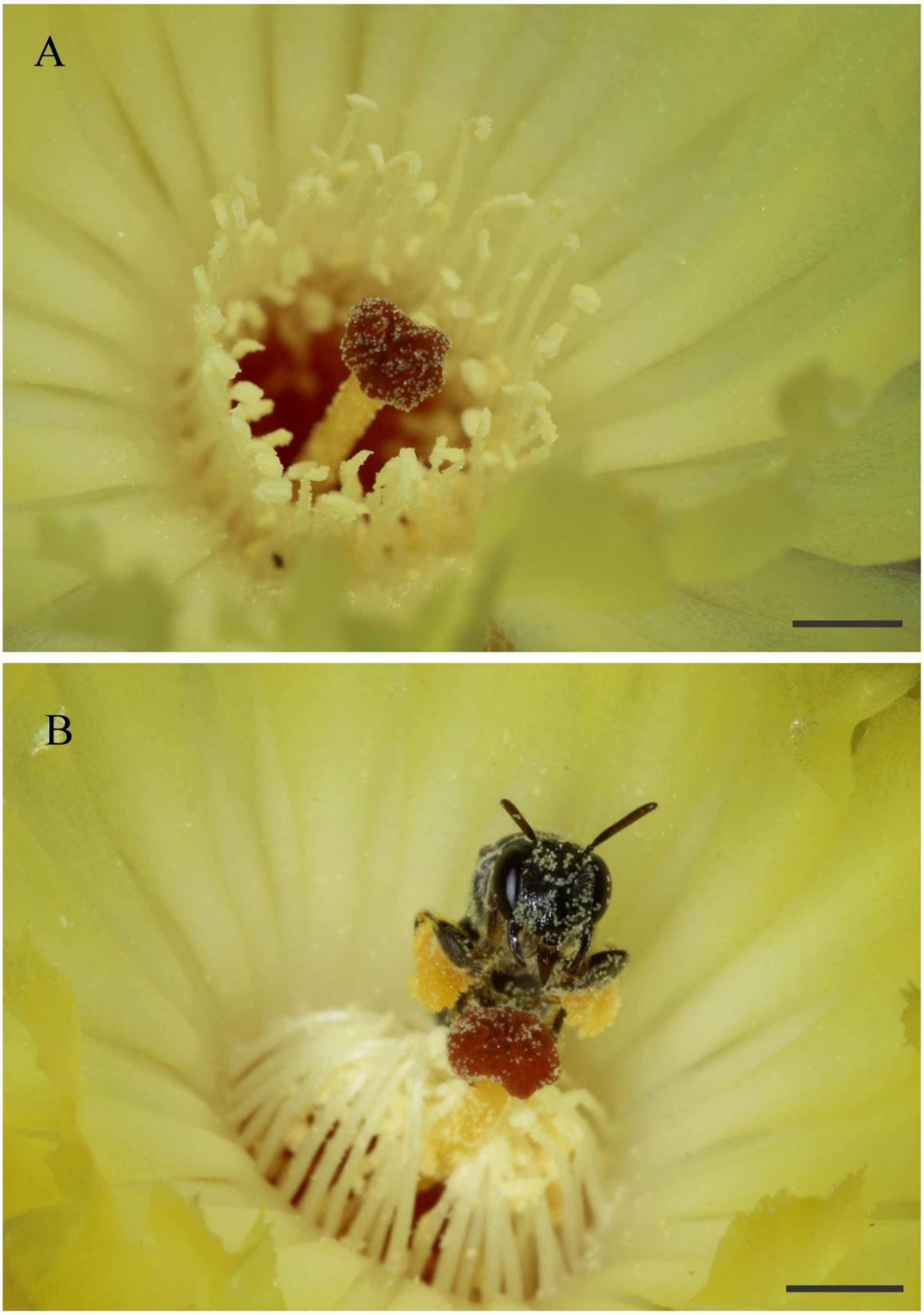

Figure 2: Flowers of *Parodia neohorstii* before and after flower visits of bees. (A) Flower with the stamens in original position before bee visits. The free style and the dark red to violet inner floral tube are visible. (B) Flower with stimulated stamens after bee visits. Stamens lean to the style and hinder the access to the anthers of lower stamens. A pollen collecting female of *Anthrenoides micans* is grooming pollen. Scale bars = 2 mm.

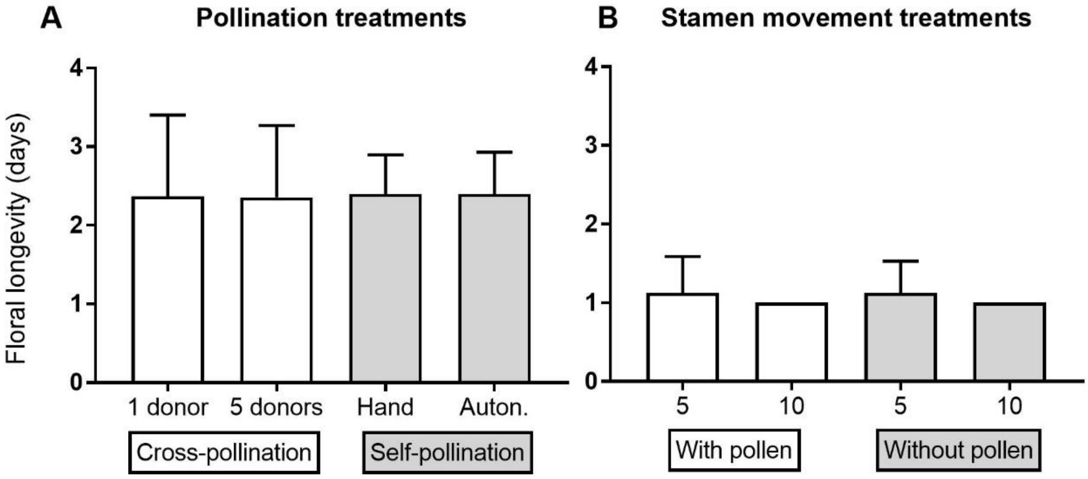

Figure 3: Floral longevity of *Parodia neohorstii* in different treatments. (A) Pollination treatments. Cross-pollination treatments differed in the number of pollen donors: a single conspecific donor (1 donor) or five different conspecific donors (5 donors). In self-pollination treatments, flowers were hand self-pollinated or maintained bagged (autonomous pollination). (B) Stamen stimulation treatments. Stamen movements were experimentally stimulated five or ten times in intervals of 5 minutes. The flowers were hand-cross pollinated (with pollen from conspecific donors) or not pollinated.

Figure 4: Couple of *Arhysosage cactorum* inside a flower of *Parodia neohorstii*. The male clinches to the female with its mandibles during copulation. The female continues pollen foraging in copula position. Scale bars = 2 mm.

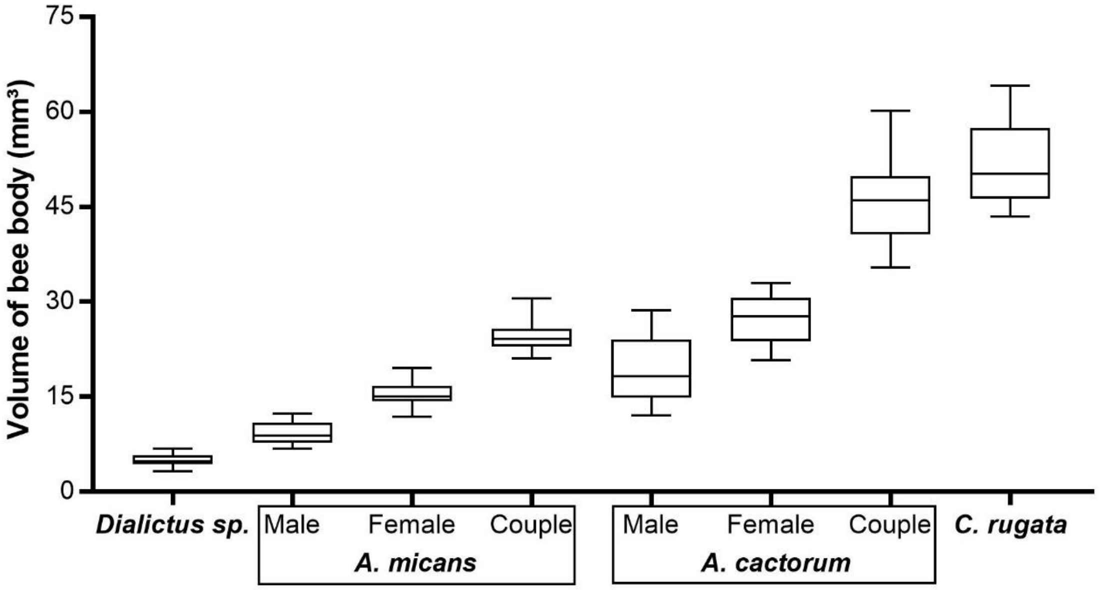

Figure 5: Body volume of flower visiting bees of *Parodia neohorstii*. The number of individual bees measured were: *Dialictus sp.* (N = 20 females), *Anthrenoides micans* (N = 20 females and 15 males), *Arhysosage cactorum* (N = 20 females and 20 males) and *Cactocolletes rugatus* (N = 20 females).

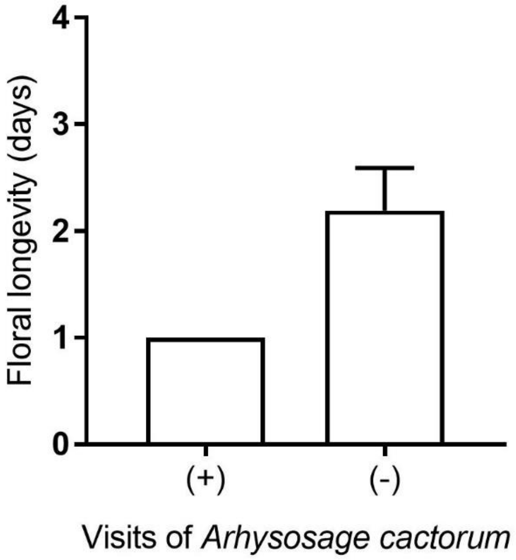

Figure 6: Floral longevity of *Parodia neohorstii* under natural conditions, with the presence (+) or absence (-) of the main pollinator *Arhysosage cactorum.*

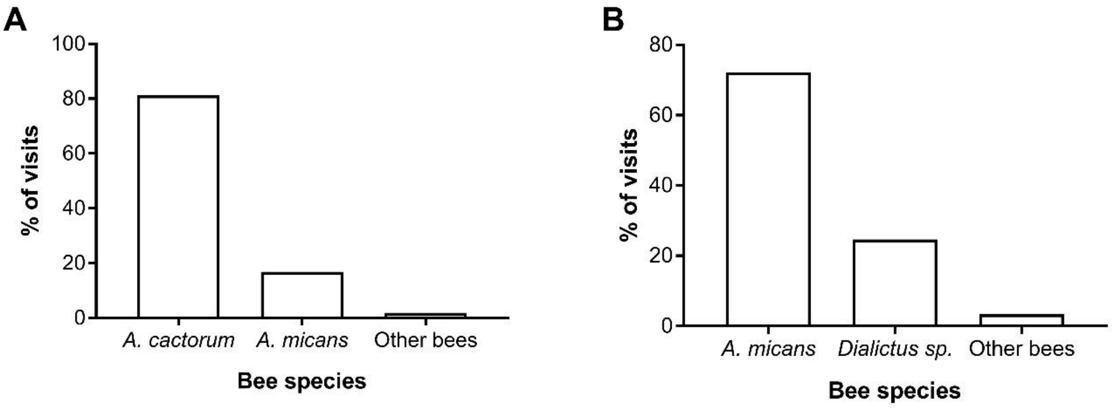

Figure 7: Visiting bees in *Parodia neohorstii* flowers in two different time scenarios. (A) Frequency of bee visitation during the peak of flowering, where the bee specie *A. cactorum* is the dominant floral visitor. (B) Frequency of bee visitation during the beginning of the flowering period, where *A. cactorum* was still inactive.

CAPÍTULO 3

AMEAÇAS À CONSERVAÇÃO DE *PARODIA NEOHORSTII* (CACTACEAE), ESPÉCIE CRITICAMENTE AMEAÇADA DE EXTINÇÃO

AMEAÇAS À CONSERVAÇÃO DE *PARODIA NEOHORSTII* (CACTACEAE), ESPÉCIE CRITICAMENTE AMEAÇADA DE EXTINÇÃO

RESUMO

Dentre as 1450 espécies da família Cactaceae, aproximadamente um terço é ameaçada de extinção. A pressão antropogênica é o principal fator de ameaça às populações naturais, uma vez que áreas de ocorrência dos cactos podem ser convertidas em áreas de pastagem e agricultura. A espécie *Parodia neohorstii* é classificada como criticamente ameaçada e ocorre em afloramentos de arenito no Pampa do Rio Grande do Sul. Com o objetivo de identificar as ameaças à conservação dessa espécie, percorremos as áreas de ocorrência, quantificamos as populações encontradas e classificamos os indivíduos quanto ao tamanho, fase reprodutiva e integridade das plantas. Contabilizamos 3677 indivíduos em 9 localidades, sendo 1225 juvenis e 2452 reprodutivos. Os cactos iniciaram a fase reprodutiva ao atingirem 25.3 mm de altura e a produção de flores está positivamente correlacionada com a altura dos indivíduos. Dos cactos avaliados, 16.9% foram impactados por animais de criação. Os impactos corresponderam ao deslocamento dos indivíduos do substrato e a danos físicos causados por mordidas e por pisoteio. Cabras e gado foram os principais animais de criação observados nas áreas de ocorrência dos cactos. As cabras foram os principais agentes responsáveis pelo consumo direto dos indivíduos de *P. neohorstii* enquanto o gado foi responsável pelo pisoteio. Os cactos impactados apresentaram taxa de mortalidade de 41% e os indivíduos reprodutivos foram sete vezes mais impactados quando comparado a juvenis. Dos 9 afloramentos avaliados, 4 estão localizados dentro de áreas de pastagem. Nos afloramentos de mais fácil acesso aos animais, as populações de *P. neohorstii* apresentaram maior proporção de cactos danificados e mortos, além de menor representatividade de indivíduos jovens, quando comparadas aos locais de difícil acesso. Concluímos que medidas de contenção de animais domésticos nos locais de ocorrência das populações de *P. neohorstii* é primordial para garantir a manutenção da espécie.

Palavras-chave: Cactaceae, ameaças à conservação, impacto de animais, levantamento populacional

INTRODUÇÃO

A família Cactaceae é representada por cerca de 1450 espécies, distribuídas em diversos hábitats nas Américas (Anderson, 2001; Hunt *et al.*, 2006, 2013). Aproximadamente um terço das espécies é classificada em algum grau de ameaça de extinção (Goettsch *et al.,* 2015). A pressão antropogênica é a maior ameaça aos representantes da família e causa destruição dos habitats, principalmente devido a expansão urbana e agropecuária (IUCN, 2020). Características intrínsecas das cactáceas, como altas taxas de mortalidade na fase juvenil, requerimentos específicos de habitat e distribuição restrita tornam as espécies vulneráveis a mudanças no habitat (Godínez–Álvarez *et al.*, 2003). Diversas espécies de cactos globosos ameaçados de extinção estão em declínio populacional, o que significa que a exposição continuada a fatores de risco agrava ainda mais o declínio das populações naturais (Mandajuno *et al.,* 2015; Martínez-Ramos *et al.,* 2016; Anceschi & Magli, 2018).

A criação de animais em áreas de ocorrência dos cactos impacta negativamente muitas populações devido ao pisoteio por bovinos e consumo de indivíduos por caprinos (Schlosser, 1979; Machado *et al.*, 2005; McIntosh *et al.*, 2020). Além disso, os animais podem remover as plantas que agem como facilitadoras na germinação e estabelecimento de cactos (Bowers, 1997). A germinação de sementes e sobrevivência de indivíduos juvenis normalmente está relacionada com microhabitats específicos ou interações de facilitação com plantas ou objetos, por exemplo rochas (Godínez-Alvarez *et al.,* 2003; Saraiva & Souza 2012), que promovem condições favoráveis para o desenvolvimento inicial das plântulas (Gurvich *et al.*, 2017).

Em Cactaceae, de uma maneira geral, somente uma pequena proporção dos indivíduos jovens atinge a fase reprodutiva e contribui para o crescimento populacional através da produção de frutos e sementes (Godínez-Alvarez *et al.,* 2003). Simulações de dinâmica populacional de cactos demonstraram que pequenas modificações na estrutura populacional, como remoção de indivíduos ou o aumento da mortalidade em determinada faixa de tamanho podem gerar impactos negativos a longo prazo (Mandujano *et al.,* 2015; Martínez-Ramos *et al.*, 2016).

Dessa maneira, é importante identificar as principais ameaças às populações naturais de cactos para implementar ações de conservação direcionadas para a realidade de cada local.

O Brasil é um dos países com maior riqueza e diversidade de cactáceas, e assim como em várias regiões das américas, grande proporção de espécies estão ameaçadas de extinção (Goettsch *et al.*, 2019). Um dos centros de diversidade de cactáceas no Brasil é o estado do Rio Grande do

Sul, onde estão concentrados aproximadamente 30% das espécies de cactos brasileiros (Carneiro *et al.*, 2016). No estado, cada região apresenta uma composição única das espécies e os campos do Pampa concentram a maior diversidade (Saraiva *et al*., 2015).

Para identificar as principais ameaças aos cactos em habitat natural, escolhemos *Parodia neohorstii* (S.Theun.) N.P.Taylor como modelo. Trata-se de um cacto globoso, criticamente ameaçado de extinção, com ocorrência restrita em afloramentos areníticos dos campos do Pampa brasileiro (Larocca & Machado, 2013). Identificamos as principais ameaças e analisamos os impactos causados pelos animais de criação (gado e cabras) nas populações de *P. neohorstii*. Perguntamos: (1) Quais são os principais danos causados por animais de criação nas populações naturais de *P. neohorstii?* (2) Qual a taxa de mortalidade de indivíduos em habitat natural?

Para responder a essas perguntas, percorremos afloramentos rochosos em busca de *P. neohorstii* e avaliamos detalhadamente as populações encontradas. Em cada localidade, classificamos todos os indivíduos em faixas de tamanho, fase reprodutiva e verificamos a integridade do corpo dos cactos. Além disso, avaliamos a acessibilidade de animais de criação ao local de ocorrência de cada população.

MATERIAIS E MÉTODOS

Área de estudo

O estudo foi conduzido na Serra do Sudeste, entre os municípios de Santana da Boa Vista e Caçapava do Sul, no sul do Rio Grande do Sul (30°53'17.1"S 53°24'03.6"W). A região faz parte do bioma Pampa, caracterizado pela fitofisionomia de campos arbustivos, onde há abundância de afloramentos rochosos conhecidos como Guaritas, encontrados em meio a uma matriz vegetacional. A vegetação é predominantemente herbácea e arbustiva, com ocorrência de diversas espécies de cactáceas ameaçadas de extinção (Carneiro *et al.,* 2016). O clima é subtemperado (Maluf *et al.,* 2000) com média de precipitação anual de 1509 mm e média de temperatura anual de 18.7 °C. No inverno ocorrem geadas esporádicas (INMET, 2018). As expedições de campo foram realizadas entre outubro e dezembro de 2017 e 2018.

Espécie estudada

Parodia neohorstii é uma espécie de cacto globoso e solitário, endêmico da região das Guaritas. A espécie ocorre em áreas pedregosas expostas no topo de afloramentos de arenito e conglomerado (Figura 1). Antes do estudo, apenas duas populações eram conhecidas. A espécie floresce entre o final de outubro e início de dezembro. A antese dura normalmente apenas três horas na presença dos polinizadas. Os polinizadores são abelhas solitárias, que visitam exclusivamente flores de Cactaceae (Schlindwein, 1998; Cerceau *et al.*, 2019).

Definição de população

Neste estudo, as populações foram definidas em conformidade com o proposto por Pianka (1978): grupo de indivíduos que ocorrem em uma área geográfica e que tem maior probabilidade de reproduzirem entre si do que com indivíduos fora desta área de ocorrência.

De acordo com Cerceau et al., (2019), *P. neohorstii* é uma espécie autoincompatível e dependente de abelhas especialistas para se reproduzir. A floração sincronizada e a antese de apenas 3 horas promovem a intensa visitação por abelhas e polinização efetiva em curto intervalo de tempo. Individualmente, as abelhas polinizadoras são féis às flores dos cactos que visitam. Estudos recentes demonstraram que abelhas marcadas são frequentes nas mesmas flores durante toda a antese (Dutra, 2015; Dutra *et al*., 2020). Devido ao fato de *P. neohorstii* ocorrer em afloramentos rochosos ilhados em meio à vegetação, há maior probabilidade de fluxo polínico entre indivíduos do mesmo afloramento. Dessa maneira, consideramos que cada afloramento abriga uma população.

Produção de flores e tamanho reprodutivo

Para avaliar a produção de flores por indivíduos de *P. neohorstii*, medimos a altura de 661 indivíduos com paquímetro digital e contabilizamos o número de flores produzidas por cada indivíduo durante a estação reprodutiva. A partir desses dados, identificamos o tamanho mínimo dos indivíduos reprodutivos. Verificamos se existe correlação entre da altura do indivíduo com o número de flores produzidas durante a estação reprodutiva.

Classificação dos indivíduos em faixas de tamanho

A partir dos dados de correlação entre a produção de flores e altura dos indivíduos, definimos oito faixas de tamanho, sendo elas: (1) 0-25 mm; (2) 25-50 mm; (3) 50-75 mm; (4) 75-100 mm; (5) 100-125 mm; (6) 125-150mm; (7) 150-175 mm; (8) 175-200 mm. Confeccionamos um instrumento de medição rápida de altura, composto por um bastão de bambu com códigos de cores. Cada cor correspondeu a uma faixa altura pré-definida (Figura 2).

Levantamento populacional e as principais ameaças a Parodia neohorstii

Para ampliar o conhecimento sobre a distribuição da espécie, percorremos alguns afloramentos de arenito da região em busca de novas populações de *P. neohorstii*. Ao encontra-las, classificamos todos os indivíduos em: 1) juvenil ou reprodutivo; 2) faixa de tamanho (altura); 3) morto ou vivo; 4) íntegro ou impactado por animais. Os dois principais impactos causados por animais foram: i) remoção do cacto do substrato e ii) danos físicos. A remoção dos indivíduos do substrato ocorre após a passagem de animais, o que ocasiona no desprendimento das raízes do substrato e deslocamento do cacto do local de estabelecimento. Os danos físicos corresponderam a perfurações no corpo dos cactos causados por mordidas ou pisoteio por animais. Os indivíduos com danos físicos foram encontrados amassados, com espinhos quebrados, marcas de dentes ou grande parte do parênquima exposto.

Classificamos as áreas de acordo com a acessibilidade de animais de criação: 1) acessibilidade alta, 2) acessibilidade média, e 3) acessibilidade baixa. As áreas de alta acessibilidade foram consideradas aquelas onde os afloramentos estão situados dentro de uma área de pastagem e de fácil acesso aos animais de criação (gado e cabras). Áreas de média acessibilidade corresponderam àquelas parcialmente cercadas, impedindo o acesso de gado, porém, com acesso de cabras pelas áreas íngremes, não cercadas, dos afloramentos. Os afloramentos de baixa acessibilidade foram aqueles muito íngremes, considerados inacessíveis a animais de criação.

Avaliamos a proporção de indivíduos jovens nas populações para inferir o índice de regeneração, uma vez que a presença de indivíduos na primeira faixa de tamanho (0-25 mm) indica que o recrutamento de novos indivíduos está ocorrendo através da germinação de sementes. Para isso calculamos a proporção de cactos no primeiro estágio de tamanho (0-25 mm) em relação a quantidade total de indivíduos em cada população.

Através de imagens de alta resolução espacial do Google Earth, medimos a área total de cada afloramento, bem como a área ocupada pelas populações dentro de cada afloramento. Através desses dados, calculamos a densidade de cactos por área de ocorrência (nº de indivíduos / área). Além disso, registramos a altitude dos pontos de ocorrência das populações.

Análises estatísticas

Para verificar se existe correlação entre altura dos cactos e quantidade de flores produzidas por estação, utilizamos o teste de Pearson.

RESULTADOS

Classificação dos indivíduos em faixas de tamanho

Os cactos começaram a produzir flores a partir de 25.3 mm de altura (N = 661). Identificamos 343 cactos reprodutivos e verificamos que quantidade de flores produzidas por indivíduo está positivamente correlacionada com a altura dos cactos (r = 0.7475, P < 0.0001, N = 661, Figura 3). Os cactos produziram em média 4.1 ± 3.4 flores por ano (N= 343) e apenas 6% (22 indivíduos) produziram mais de 10 flores e apenas 1% (4 indivíduos) produziram 20 flores ou mais flores por ano, sendo o máximo de 23 flores.

Levantamento populacional de Parodia neohorstii

Encontramos 3677 indivíduos distribuídos em 9 populações, sendo 3428 indivíduos vivos. Todas as ocorrências foram em afloramentos de arenito conglomeráticos com grânulos e seixos pequenos a médios, em altitudes variando de 147 a 300 metros (Tabela 1).

Os cactos ocorreram em áreas restritas dos afloramentos, apenas em áreas com substrato com grânulos e seixos, uma vez que fixam as raízes entre rochas e frestas. A área ocupada pelas populações varia entre 4.4 a 23.7% da área total do afloramento, o que corresponde a uma área de ocupação entre 184 a 12890 m^2. A quantidade de indivíduos nas populações variou entre 93 até 1056 (Tabela 1). A maioria das populações é encontrada em áreas particulares, com exceção de uma população que ocorre em um afloramento às margens de uma estrada não asfaltada. Cinco populações tem acessibilidade alta para animais de criação, onde os afloramentos ocorreram dentro de áreas de pastagem e à beira de estrada. Três populações tem acessibilidade média, onde eram parcialmente cercadas e impossibilitavam a passagem do gado, porém, caprinos acessavam percorrendo áreas íngremes não cercadas do afloramento (Figura 4). Apenas uma população é inacessível aos animais devido ao afloramento ser muito íngreme (Tabela 1).

A estrutura populacional variou entre as localidades (Figura 5). Indivíduos juvenis, com até 25mm de altura foram os mais representativos (32.1%; 1181 indivíduos). O índice de regeneração, que representa a proporção de indivíduos nesta faixa de tamanho nas populações, variou de 9.6 a 82% e foi menor onde houve maior acesso de animais (Tabela 1). A população sem acesso aos animais foi caracterizada principalmente pela abundância de indivíduos juvenis e ausência de cactos nas maiores faixas de tamanho (Figura 5.C).

Apenas 26 indivíduos foram encontrados na maior faixa de tamanho (175-200 mm) (0.7%) e apresentaram a maior taxa de mortalidade (26.9%). Indivíduos mortos ocorreram principalmente nas populações com maior quantidade de cactos.

Estado de conservação e principais ameaças das populações de Parodia neohorstii

Dentre os 3677 indivíduos avaliados em todas as populações, 83.1% estavam íntegros e 16.9% foram impactados por animais (Figura 6). Os impactos por animais se revelaram tanto por danos físicos em 8.2% dos cactos, que apresentaram marcas de mordidas e de pisoteio, quanto por deslocamento de 8.7% dos cactos do substrato.

Adultos e filhotes de cabras removeram os cactos do substrato com as patas dianteiras e se alimentaram tanto do sistema radicular quanto do parênquima dos cactos (Figura 7). Esses danos foram aproximadamente sete vezes mais frequentes em indivíduos reprodutivos quando comparado a indivíduos juvenis (Tabela 2).

Populações com alta e média acessibilidade aos animais apresentaram maior proporção de indivíduos com danos e indivíduos mortos (Tabela 2). Dentre os indivíduos avaliados, 249 estavam mortos (6.7%). A faixa de tamanho com maior proporção de indivíduos impactados e mortos correspondeu aos indivíduos maiores, com altura entre 175-200 mm (Figura 8). A maior causa da morte em todas as faixas de tamanho foram os danos físicos causados por mordidas e pisoteio (Figura 8B). Dos cactos que apresentaram esse tipo de dano, 73% estavam mortos.

Apenas 5 indivíduos íntegros estavam mortos e apresentaram o corpo seco sem nenhum dano ou deslocamento, que representou apenas 0.2% dos indivíduos amostrados.

DISCUSSÃO

Nosso estudo revelou que a principal ameaça à espécie *P. neohorstii* é o impacto direto causado por animais de criação, principalmente cabras e gado. As áreas de fácil acesso a esses animais apresentaram maior proporção de cactos danificados e mortos, além de menor representatividade de indivíduos jovens.

Principais ameaças aos cactos

Os danos causados pelos animais de criação impactaram 16.9% dos indivíduos avaliados neste estudo. Das populações avaliadas, 88% foram acessíveis a animais de criação, sendo que metade delas ocorreram dentro de áreas de pastagem, o que aumenta a probabilidade de pisoteio e o consumo dos cactos. Reportamos que cabras se alimentam do parênquima de *P. neohorstii*. Esse tipo de comportamento também foi reportado nos Estados Unidos a espécie de carneiro selvagem (*Ovis canadensis*), que consome o conteúdo interno de *Ferocactus sp*. após quebrar e retirar o excesso de espinhos com as patas (Warrick & Krausman, 1989). A exposição do parênquima dos cactos, tanto devido a mordidas quanto ao pisoteio, leva ao ressecamento e a morte do indivíduo. Em *P. neohorstii*, 73% dos indivíduos que apresentaram perfurações no corpo estavam mortos. O ressecamento após a herbivoria foi identificado como uma das maiores causas da morte do cacto globoso *Astrophytum capricorne* (Mandujano *et al.*, 2015). Outros mamíferos herbívoros, como coelhos e esquilos, se alimentam de *Astrophytum asterias* e causam altas taxas de mortalidade (Ferguson *et al.*, 2013). Nas populações de *P. neohorstii* não observamos danos causados por animais nativos.

O consumo de cactos por cabras e o pisoteio por gado também foram relatados para outras espécies do gênero *Parodia* (Anceschi & Magli, 2018) e para outros gêneros como *Discocactus* (Machado *et al.*, 2005), *Frailea* (Machado, 2007), *Gymnocalicium* (Schlosser, 1979) e *Echinocactus* (Jiménez-Sierra *et al.*, 2007). Além dos danos físicos, os indivíduos foram frequentemente removidos do afloramento rochoso pelo impacto causado pela passagem dos animais, sem apresentarem perfurações no corpo. Após o deslocamento do substrato, as raízes foram direcionadas para cima ou para os lados, impossibilitando o enraizamento. Vale ressaltar a resiliência de alguns indivíduos, pois o deslocamento causou a mortalidade de menos de 8% dos cactos e a maioria dos indivíduos deslocados continuaram florescendo.

As populações de *P. neohorstii* onde a acessibilidade de animais é mais fácil, reportamos maior quantidade de indivíduos impactados e menor quantidade de indivíduos juvenis. Esse fato pode sugerir que caprinos se alimentam de indivíduos jovens sem deixar vestígios, uma vez que eles possuem menor quantidade de espinhos e menor tamanho corporal, o que possibilita a ingestão completa do cacto. Porém, estudos de acompanhamento de cactos juvenis marcados devem ser realizados ao longo dos anos para avaliar o real impacto real de caprinos nas persistência de indivíduos nas menores faixas de tamanho. Na área de estudo, observamos cabras ingerindo indivíduos inteiros da espécie *Frailea gracillima*, uma espécie de cacto pequeno e com espinhos macios. Dados semelhantes foram registrados no México para cactos do gênero *Astrophytum* onde os indivíduos das menores faixas de tamanho foram mais impactados pelo consumo de animais (Martínez-Ávalos *et al.*, 2007; Ferguson *et al.*, 2013). Estudos populacionais demonstram que a mortalidade afeta negativamente o equilíbrio das populações (Mandujano *et al.*, 2015), e as flutuações na sobrevivência de indivíduos nos primeiros estágios de vida impactam a população a longo prazo (Bowers, 1997; Valverde *et al.*, 2004).

Outras ameaças aos cactos do Rio Grande do Sul são conversão de áreas para a plantação de *Eucalyptus*, que ameaça de pelo menos 27 espécies (Goettsch *et al.,* 2015; Carneiro *et al.,* 2016; Anceschi & Magli, 2018) e a coleta ilegal (Ribeiro-Silva *et al.,* 2011).
Parodia neohorstii não é impactada diretamente pela conversão do habitat para plantações uma vez que ocorre em afloramentos rochosos, porém, outras espécies simpátricas são impactadas. Não observamos indícios de coleta de indivíduos de *Parodia neohorstii* para finalidade ornamental, mas é uma grande ameaça a diversas espécies de *Parodia* da região (Ribeiro-Silva *et al.,* 2011).

Habitat de Parodia neohorstii

As populações de *P. neohorstii* ocorrem em áreas restritas dos afloramentos de arenito associados a blocos de conglomerados. A ocorrência em habitats específicos é uma característica de espécies de *Parodia,* que resulta em distribuição de populações isoladas (Anceschi & Magli, 2018). Diversos cactos utilizam agentes facilitadores como plantas ou fendas de rochas ou pedras menores para germinar e se estabelecer (Godínez-Álvarez *et al.,* 2003; Peters *et al.,* 2008; Zenteno Ruíz *et al.,* 2009; Martínez-Ramos *et al.,* 2016). O crescimento à sombra de pequenas pedras pode ser auxiliado por uma redução da radiação solar e sombreamento, além da umidade prolongada no solo (Larmuth & Harvey, 1978), criando condições microclimáticas favoráveis para a germinação e desenvolvimento inicial de plântulas. A ocorrência de *P. neohorstii* parece não estar relacionada com a presença de plantas facilitadoras, porém, a presença de conglomerados no substrato pode auxiliar a fixação das raízes onde há pequeno acúmulo de solo. Os afloramentos são muito heterogêneos, apresentando porções com dominância de arbustos, bromélias e musgos, além de áreas com rocha lisa de arenito exposto, sem conglomerados. Nessas áreas sem a presença dos conglomerados que podem auxiliar na fixação das raízes, não registramos a presença de *Parodia neohorstii*.

Reprodução de Parodia neohorstii

O início da idade reprodutiva varia entre as espécies de cactáceas (Godínez-Álvarez *et al.,* 2003). Indivíduos de *Parodia neohorstii* começaram a produzir flores ao atingirem 25,3 mm de altura. Com o aumento do tamanho do indivíduo, a quantidade de flores produzidas aumentou. Esses resultados foram consistentes com aqueles encontrados para outras espécies de cactos (Godínez-Álvarez *et al.,* 2003; Mandujano *et al.,* 2015; Martínez-Ramos *et al.,* 2016).

A produção de sementes é um fator crítico na manutenção de diversas espécies de cactáceas, uma vez que muitas espécies não se reproduzem vegetativamente (Godínez-Álvarez *et al.,* 2003). A baixa produção de sementes viáveis pode reduzir o número de novos indivíduos na população e levar ao declínio populacional (Esparza-Olguín *et al.,* 2005). Projeções populacionais revelaram que apenas um pequeno aumento na germinação de sementes afeta positivamente a população de cactos a longo prazo (Mandujano *et al.,* 2015). *Parodia neohorstii* não se propaga vegetativamente e depende de abelhas especializadas para formar frutos (Cerceau *et al.,* 2019). As sementes de *P. neohorstii* coletadas em campo não apresentaram sinais de dormência e a taxa de germinação variou entre 20 – 55% em condições controladas (Dal Ri não publicado). A germinação de cactos globosos em laboratório é muito variável, e foram registrados valores entre 12-95% em várias espécies (Mandujano *et al.,* 2015; Martínez-Ramos *et al.,* 2016; Gurvich *et al.,* 2017). Apesar das taxas de germinação em habitat natural serem menores do que as observadas em laboratório (Gurvich *et al.,* 2017), a presença de indivíduos jovens nas populações de *P. neohorstii* sugere que o recrutamento de novos indivíduos está ocorrendo constantemente através da germinação das sementes. De acordo com o estudo de polinização (Cerceau *et al.,* 2019), estimamos que cada indivíduo reprodutivo de

P. neohorstii produz em média 250 sementes e os maiores cactos podem produzir até 1250 sementes por ano.

Populações de Parodia neohorstii

As estruturas populacionais de *P. neohorstii* sugerem que a maioria das populações está em constante regeneração. Essa constatação pode ser feita quando há maior quantidade de indivíduos jovens na população e a diminuição progressiva do número de indivíduos mais maiores e mais velhos (Godínez-Álvarez *et al.*, 2003). Observamos que a proporção de indivíduos jovens variou bastante entre as populações de *P. neohorstii.*

Estudos populacionais sugerem que cactos rupícolas podem apresentar menor quantidade de indivíduos jovens devido a limitações de microhabitats favoráveis para germinação e recrutamento (Martínez *et al.*, 2010). No entanto, a variação do número de indivíduos jovens entre as populações pode ocorrer devido ao impacto por animais, uma vez que as populações mais acessíveis apresentaram menor proporção de indivíduos jovens. Isso indica que a presença de animais domésticos pode influenciar na quantidade de indivíduos jovens, tanto devido aos impactos causados em indivíduos reprodutivos e consequente redução na produção de sementes ou no consumo direto indivíduos jovens inteiros que não deixaram vestígios nos afloramentos.

A presença de indivíduos maiores é rara, e observamos poucos indivíduos grandes concentrados em populações maiores. A raridade dos indivíduos pode ocorrer devido a grande mortalidade causada pelo impacto de animais.

A densidade populacional de *P. neohorstii* variou de 0,01 a 1,44 indivíduos/m^2 nos diferentes afloramentos de arenito. Estudos populacionais sugerem que a variação na densidade de indivíduos entre populações de cactos pode estar relacionada com os distúrbios nas áreas de ocorrência, onde populações mais densas ocorrem em áreas mais preservadas (Martorell & Peters, 2005; Martínez *et al.*, 2010). Nossos resultados foram consistentes com aqueles encontrados em estudos anteriores, uma vez que a população de *P. neohorstii* com maior densidade corresponde aquela com menor acesso de animais.

Ações de conservação

A área de ocorrência de *P. neohorstii* é uma área rural, onde a principal atividade desenvolvida é a pecuária de pequeno porte, principalmente ovelhas, cabras e gado. Uma ação imediata para a conservação de *P. neohorstii* seria o impedimento do acesso de animais aos afloramentos de arenito, através da construção de cercas nas áreas de ocorrência da espécie. A suspensão do uso dos afloramentos como área de pastagem não causaria danos econômicos, uma vez que poucas espécies com potencial alimentício ocorrem nessas áreas. Por outro lado, a restrição do acesso aos animais de criação nessas áreas evitaria o forte dano as populações de cactos rupícolas.

Uma solução mais eficiente e a longo prazo seria a criação de uma unidade de conservação. A região das Guaritas é rica em formações geológicas raras e já foi reportado a importância de criação de um parque geológico na região (Paim *et al.*, 2010). Além disso, a diversidade biológica da região é única, como alta diversidade de abelhas especialistas (Schlindwein, 1998) e de cactáceas raras, sendo considerada uma das áreas prioritárias para a conservação de cactos no estado (Saraiva *et al.*, 2015). O sul do Brasil é uma das principais regiões do mundo onde há uma concentração de ocorrências de cactos ameaçados de extinção fora de áreas protegidas (Goettsch *et al.*, 2019) e *Parodia neohorstii* não se encontra inserida em nenhuma unidade de conservação (Carneiro *et al.*, 2016).

Concluímos que medidas de contenção de animais domésticos nos locais de populações de *P. neohorstii* é primordial para garantir a manutenção da espécie.

A diminuição progressiva dos indivíduos nas populações naturais pode contribuir para o declínio populacional. Essa espécie foi avaliada como criticamente ameaçada de extinção por Larocca e Machado (2013), principalmente devido a ocorrência restrita, indícios de declínio populacional e pouco conhecimento sobre as populações existentes. Este estudo pode contribuir para uma reavaliação do status de conservação de *P. neohorstii*, com dados mais robustos sobre a situação das populações remanescentes e a identificação das principais ameaças em habitat natural.

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TABELAS

Tabela 1: Características das populações de *Parodia neohorstii.* A área total do afloramento e a respectiva área de ocorrência dos indivíduos foram utilizadas para calcular a porcentagem da área ocupada dentro do afloramento de arenito. Densidade de indivíduos foi calculada de acordo com a quantidade de indivíduos vivos presentes na área de ocorrência dos cactos. Índice de regeneração representa a proporção de indivíduos jovens vivos, pertencentes a menor faixa de tamanho (0 – 25mm) em relação a quantidade total de indivíduos vivos na população. A porcentagem de indivíduos impactados por animais foi calculado a partir do número total de indivíduos amostrados, mortos e vivos. Acessibilidade a animais de criação: (Alta) Afloramento dentro de área de pastagem de bovinos e caprinos; (Média) Afloramento cercado parcialmente sem acesso de bovinos, porém, com acesso de caprinos e outros animais; (Baixa) Afloramentos inacessíveis a animais.

Populações	Número de indivíduos vivos	Número de indivíduos mortos	Área total do afloramento (m^2)	Área de ocorrência do cacto (m^2)	Área ocupada do afloramento (%)	Altitude (m)	Densidade (indivíduos vivos /m^2)	Índice de regeneração (%)	Indivíduos impactados por animais (%)	Acessibilidade a animais de criação
Ferradura	1056	82	54412	5222	9.59	241	0.20	24.9	26.1	Alta
Bom passo	747	20	12530	1958	15.62	210	0.38	33.6	8.2	Alta
Serro do Umbu	390	1	3351	270	8.05	210	1.44	82.0	0.8	Baixa
Pedra 2	336	42	54660	7641	13.97	213	0.04	36.9	14.8	Média
Ferradura Secreta	310	42	4073	916	22.48	235	0.33	9.6	30.4	Alta
Ferradura Lateral	208	34	774	184	23.77	231	1.13	20.6	19.4	Alta
Pedra 4	171	0	179867	12890	7.16	274	0.01	15.7	1.2	Média
Serro do Renato	117	23	96318	4298	4.46	300	0.02	47.0	22.9	Média
Passo ideal	93	5	1648	284	17.23	174	0.32	20.4	14.3	Alta

Tabela 2: Integridade de indivíduos de *Parodia neohorstii* em diferentes populações. Todos os indivíduos da população foram contabilizados, vivos ou mortos. Os indivíduos foram classificados em íntegros quando não havia nenhum tipo de dano físico. Indivíduos apresentaram dois tipos de danos físicos causados por animais: (1) Solto do substrato com as raízes desprendidas, ou (2) Danificado devido a mordidas ou pisoteio de animais, que causaram exposição do parênquima dos cactos.

	Juvenil – N (%)			Reprodutivo – N (%)		
Populações	Íntegro	Solto	Danificado	Íntegro	Solto	Danificado
Ferradura	251 (22.1)	25 (2.2)	14 (1.2)	590 (51.8)	131 (11.5)	127 (11.2)
Bom passo	263 (34.3)	16 (2.1)	4 (0.5)	441 (57.5)	27 (3.5)	16 (2.1)
Serro do Umbu	305 (78.0)	3 (0.8)	0 (0.0)	83 (21.2)	0 (0.0)	0 (0.0)
Pedra 2	145 (38.4)	5 (1.3)	14 (3.7)	177 (46.8)	12 (3.2)	25 (6.6)
Ferradura Secreta	29 (8.2)	1 (0.3)	2 (0.6)	216 (61.4)	64 (18.2)	40 (11.4)
Ferradura Lateral	38 (15.7)	3 (1.2)	1 (0.4)	157 (64.9)	11 (4.5)	32 (13.2)
Pedra 4	34 (19.9)	0 (0.0)	0 (0.0)	135 (78.9)	2 (1.2)	0 (0.0)
Serro do Renato	46 (32.9)	1 (0.7)	0 (0.0)	62 (44.3)	8 (5.7)	23 (16.4)
Passo ideal	21 (21.4)	4 (4.1)	0 (0.0)	63 (64.3)	8 (8.2)	2 (2.0)
Total	1132 (30.8)	58 (1.6)	35 (1.0)	1924 (52.3)	263 (7.2)	265 (7.2)

FIGURAS

Figura 1: População de *Parodia neohorstii*. Indivíduos ocorrerem afloramentos de arenito e conglomerados.

Figura 2: Instrumento de medida rápida de altura de cactos. Cada cor no bastão representa uma faixa de altura: (1) Rosa: 0-25 mm; (2) Verde bandeira: 25-50 mm; (3) Verde fluorescente: 50-75 mm; (4) Vermelho: 75-100 mm; (5) Azul: 100-125 mm; (6) Amarelo: 125-150mm; (7) Preto: 150-175 mm; (8) Laranja: 175-200 mm. A figura mostra um indivíduo com altura de 75-100 (faixa vermelha).

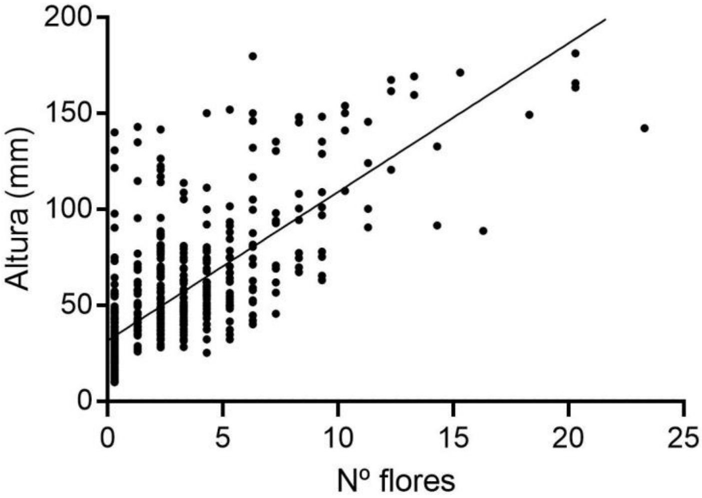

Figura 3: Correlação entre a altura dos cactos e a quantidade de flores produzidas durante a estação reprodutiva (N = 661).

Figura 4: Indivíduos de cabras na área de ocorrência de *Parodia neohorstii*. As cabras acessam o afloramento pelas partes íngremes, onde não existem cercas.

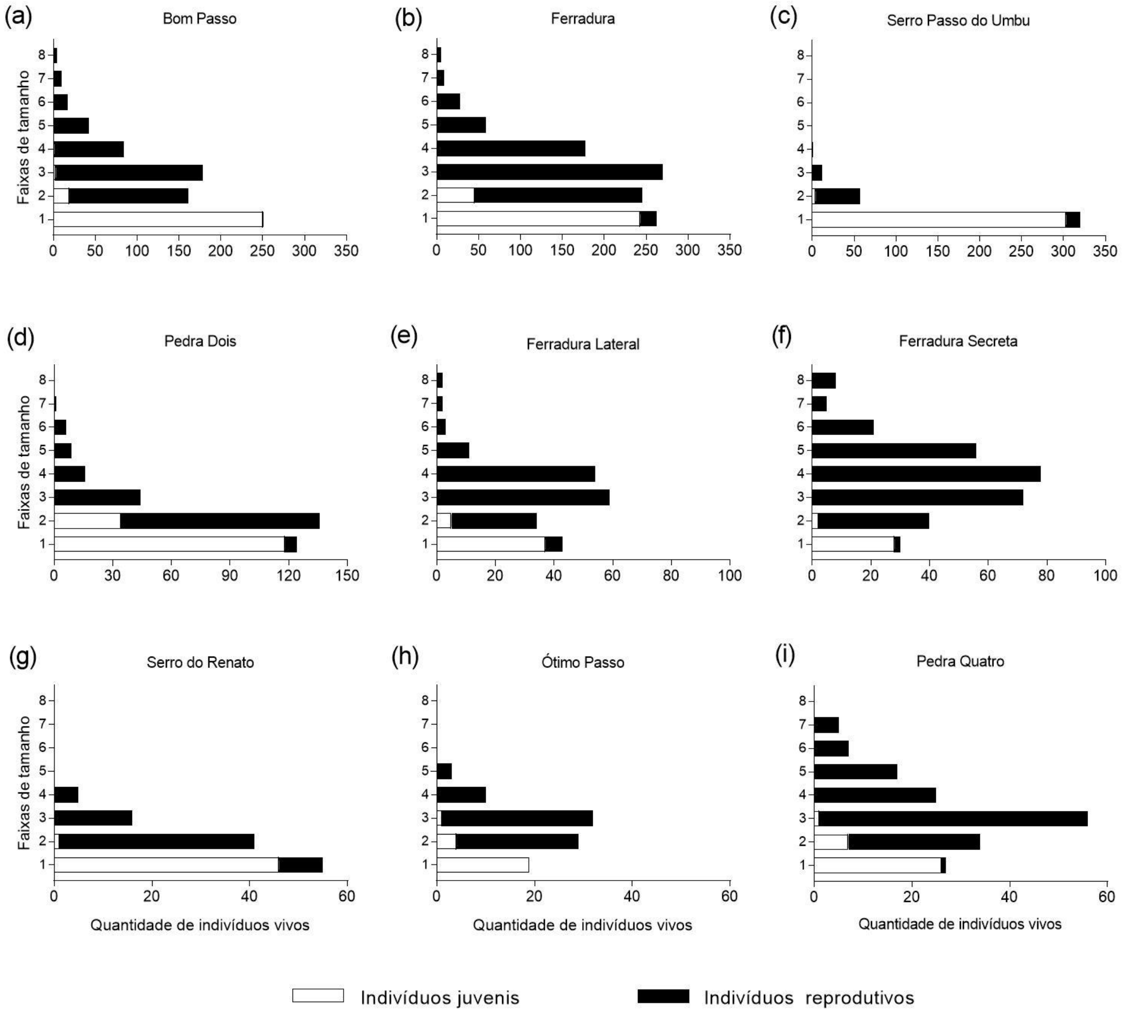

Figura 5: Levantamento populacional de indivíduos vivos de *Parodia neohorstii* em nove localidades. Os indivíduos foram classificados de acordo com faixas de tamanho: (1) 0-25 mm; (2) 25-50 mm; (3) 50-75 mm; (4) 75-100 mm; (5) 100-125 mm; (6) 125-150mm; (7) 150-175 mm; (8) 175-200 mm. Barras brancas apresentam indivíduos juvenis, que ainda não produziram flores e frutos. Barras pretas apresentam indivíduos reprodutivos.

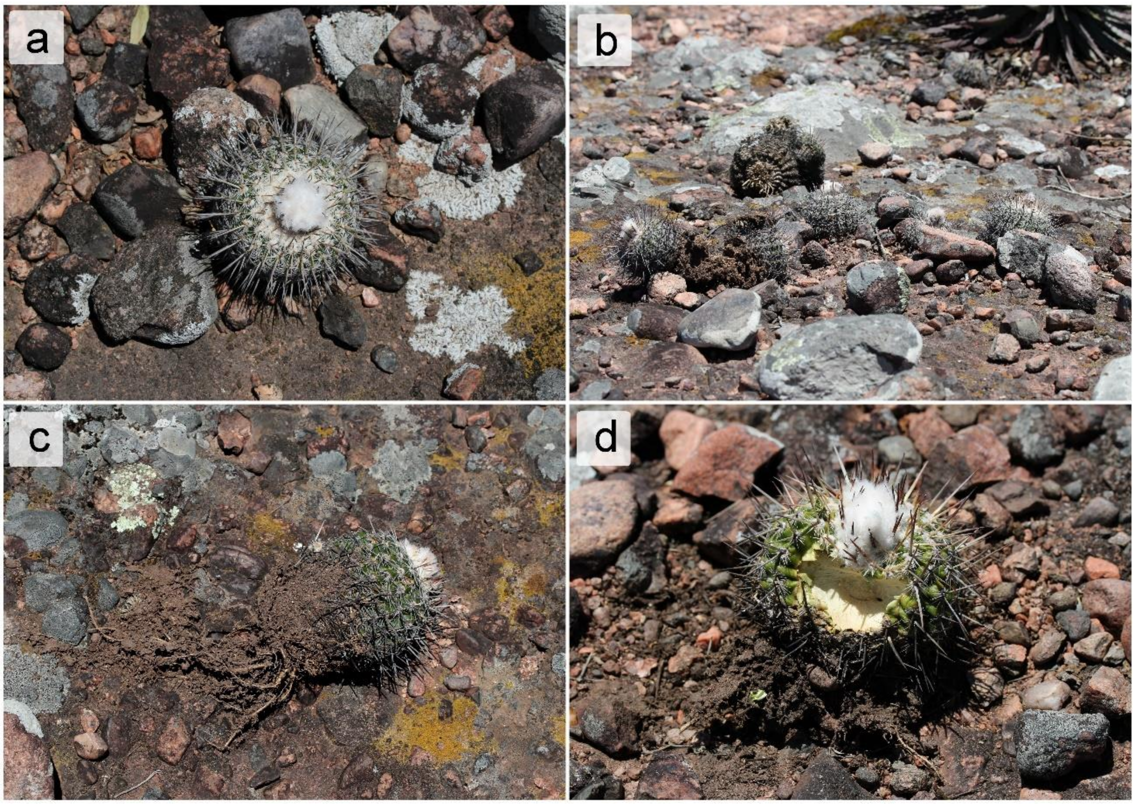

Figura 6: Indivíduos de *Parodia neohorstii*. (a) Indivíduo íntegro, com raízes fixadas entre frestas e conglomerados. (b) Grupo de indivíduos de *P. neohorstii*, indivíduos íntegros ao lado de indivíduos removidos do substrato. (c) indivíduos removidos do substrato com as raízes totalmente desprendidas. (d): Indivíduo danificado por mordida, com o parênquima exposto.

Figura 7: Filhote de cabra se alimentando de um indivíduo de *Parodia neohorstii*.

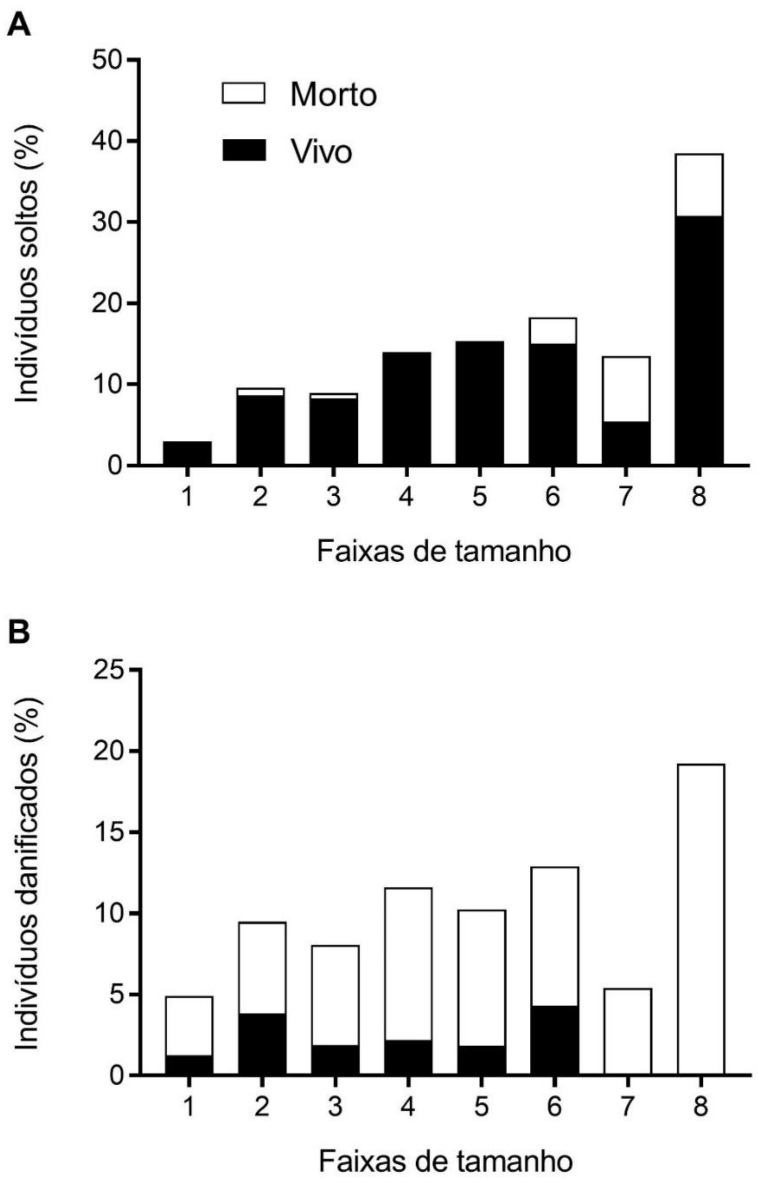

Figura 8: Porcentagem de indivíduos mortos e vivos de acordo com o tipo de impacto e faixa de tamanho em todas as populações. Faixas de tamanho correspondem a altura dos cactos: (1) 0-25 mm; (2) 25-50 mm; (3) 50-75 mm; (4) 75-100 mm; (5) 100-125 mm; (6) 125-150mm; (7) 150-175 mm; (8) 175-200 mm. (A) Porcentagem de indivíduos removidos do substrato após a passagem de animais em cada faixa de tamanho, classificados como vivos ou mortos. (B) Porcentagem de indivíduos danificados por mordidas ou pisoteio em cada faixa de tamanho, classificados como vivos ou mortos.

CAPÍTULO 4

REPRODUCTION OF *FRAILEA GRACILLIMA* (CACTACEAE) IN NATURAL HABITAT: POLLINATION BY SOLITARY BEES AND MECHANISM OF DELAYED SELF-POLLINATION

REPRODUCTION OF *FRAILEA GRACILLIMA* (CACTACEAE) IN NATURAL HABITAT: POLLINATION BY SOLITARY BEES AND MECHANISM OF DELAYED SELF-POLLINATION

ABSTRACT

The genus *Frailea* includes small cacti that predominantly cleistogamous. However, the knowledge is based on cultivated specimens and information from individuals in the natural habitat is scarce. During three seasons, we studied pollination, characteristics of flowering and breeding system of *Frailea gracillima*, a species that occurs on sandstone outcrops in the Brazilian Pampa. No cleistogamous flowers were recorded in the field. The pollen-only flowers were visited by females of several pollen collecting solitary bee species and male bees seeking for mates. Bees deposited huge amounts of conspecific pollen on the stigma, sufficient to fertilize all ovules, but also pollen from other different plant families. Flower opening was highly synchronized in the population but flowering days were interrupted by non-blooming periods and occurred in only 11% of the days in the flowering season. Lifespan of flowers visited by bees was only ~2:30h. The flowers showed temporal hercogamy, where stamens and stigma are spatially separated towards the end of anthesis. The long stigma lobes slowly move downward during anthesis and contact anthers when petals fold thus promoting delayed autonomous self-pollination. The quantity of pollen grains deposited on stigmas autonomously, however, varied between flowers, and fruit set via autonomous pollination was 56%. Flowering characteristics in *F. gracillima* strongly favor cross-pollination but, in the absence of solitary bee pollinators, set seed through delayed self-pollination.

Key words: *Frailea gracillima*, solitary bees, delayed self-pollination, Cactaceae, floral movements, Brazil, Pampa cacti.

INTRODUCTION

Among plant reproductive strategies, most species invest in floral traits to attract pollinators and in mechanisms that favor cross-pollination (Sprengel, 1793; Darwin, 1862; Faegri & van der Pijl, 1979). Autonomous self-pollination, however, can guarantee sexual reproduction in low pollinator environments (Lloyd & Schoen, 1992; Sun *et al.*, 2005). Some species reproduce through cleistogamy and promote self-pollination in closed flower buds, producing fertile seeds in the absence of pollen vectors (Uphof, 1938; Lord, 1981; Schoen & Lloyd, 1984; Richards, 1997). Cleistogamy occurs in at least 56 families in different habitats of the world (Lord, 1981), but is common at higher altitudes, regions with extreme weather conditions and poor pollination environments (Uphof, 1938; Faegri & van der Pijl, 1979). Exclusively cleistogamous species are rare (Lord, 1981) and often the same individual can produce cleistogamous and chasmogamous flowers (open flowers) according to environmental conditions such as temperature, humidity, photoperiod and soil quality (Uphof, 1938; Brown, 1952; Faegri & van der Pijl, 1979; Langer & Wilson, 1965; Schoen & Lloyd, 1984; Richards, 1997).

While floral traits in Cactaceae in general are clearly related to specific pollinator groups such as bats, bees, hawkmoths and hummingbirds (Porsch, 1938; Marshall, 1941; Grant & Grant, 1979; Rowley, 1980; Schlindwein & Wittmann, 1995; Anderson, 2001; Cerceau *et al.*, 2019) and xenogamy seems to be common (Gibson & Nobel, 1986; Mandujano, 2010), a few taxa are cleistogamous. This is reported for some species of *Rhipsalis* Gaertn. (Barthlott & Hunt, 1993), *Melocactus* Link & Otto (Taylor, 1991) and *Frailea* Britton & Rose (Putnam, 1968). Among cacti, *Frailea* is considered the genus with most cleistogamous species (Rowley, 1980). Nevertheless, both cleistogamous and chasmogamous flower production were mentioned for the genus (Britton & Rose, 1922; Köhler, 1968; Anderson, 2001; Metzing & Kiesling, 2006). However, this information is based on cacti collections found outside the natural occurrence of *Frailea* species, often where plants were grown in greenhouses. Though, in a bee-plant community survey, flowers of *Frailea phaeodisca* (Speg.) Backeb. & F.M.Knuth and *Frailea pygmaea* (Speg.) Britton & Rose were cited to receive visits of bees (Schlindwein, 1998). Up to the present moment, there was no

no need for unfortunately, no detailed information on pollination and reproductive biology of species of *Frailea* in natural habitats.

Frailea's cleistogamy seems to be strongly related to abiotic conditions. In cultivation, where climatic conditions are very different from those observed in natural habitat, cleistogamy seems to be predominant (Köhler, 1968; Metzing & Kiesling, 2006).

We studied *Frailea gracillima* (Lem.) Britton & Rose a species with a tiny solitary body that occurs in the south of Rio Grande do Sul, in sandstone rock outcrops. In the literature, the species is reported as reproducing via cleistogamy (Putnam, 1968) and chasmogamy (Britton & Rose, 1922).

In order to elucidate the breeding system and pollination of *Fraillea gracillima* we asked: 1) What are the characteristics of flowering and anthesis of this species in natural habitat? 2) What is its breeding system? 3) Who are their floral visitors and effective pollinators? 4) Is there pollen deposition on the stigmas promoted by bees and autonomously? To answer these questions, we studied a natural population of *F. gracillima* during three flowering seasons in the field, described the flower opening schedule and rhythm of flowering in three seasons, determined the breeding system, as well as the spectrum of floral visitors and quantified pollen grains on the stigma.

MATERIAL AND METHODS

Study area and species

The study was conducted in the Serra do Sudeste, in the municipalities Santana da Boa Vista and Caçapava do Sul (30°53'16.1"S 53°24'04.1"W), Rio Grande do Sul, Brazil. The region is part of the Pampa grasslands, and the study areas are characterized by rock outcrops and shallow stony and sandy soils in the region of the *Guaritas*, which are steep sandstone and conglomerate formations (Carneiro *et al.*, 2016). The local vegetation is dominated by herbaceous and shrubby species, with some small trees (Schlindwein, 1998). The region is a diversity centre for cactus species, many of them are threatened (Schlindwein, 1995; Carneiro *et al.*, 2016). The average annual precipitation in the region is 1509 mm and the average annual temperature is 18.7 ° C (INMET, 2018). In the flowering season of most cacti species, (October to December), the maximum monthly average temperature is 27.1 ° C (INMET, 2018).

With eleven among the twelve recognized species of *Frailea*, Rio Grande do Sul is the center of diversity of this genus (Ribeiro-Silva *et al.*, 2011; Metzing 2015a, 2015b). *Frailea gracillima* is a small cactus species, restricted to the Pampa, with globose to cylindrical body up to 10 cm height and 2.5 cm diameter. Individuals occur solitary, scattered or in small groups (Britton & Rose, 1922; Carneiro *et al*., 2016) on rock soils, especially in rock cracks on the sandstone outcrops (Figure 1).

Anthesis and flower longevity

In the flowering season 2016, 2017 and 2018 *of F. gracilima*, we daily recorded the precipitation with a pluviometer to check whether flowering is related to the rainfall regime. We marked flower buds in pre-anthesis with color codes and daily checked whether flowers opened and noted the day of flowering (N = 131). We recorded the opening and closing times of the flowers.

We determined and compared the floral longevity of: 1) flowers visited by bees (N = 25); 2) bagged non-visited flowers (N = 7); 3) hand cross-pollinated flowers (N = 6); 4) hand self-pollinated flowers (N = 6). Hand cross-pollinated and hand self-pollinated flowers were bagged in the bud stage, pollinated, and then bagged again.

Breeding system

To determine the breeding system of *F. gracillima* we compared the amount of fruits and seeds produced in different pollination treatments: 1) natural pollination (N = 11); 2) autonomous self-pollination (N = 9); 3) hand self-pollination (N = 6); 4) hand cross-pollination (N = 2).

To determine the number of ovules per flower, we fixed 17 flowers in 70% alcohol and counted the number of ovules under a stereomicroscope (Leica, WILD – M3Z).

Movement of stamens and stigmas

To check whether there is spatial separation of anthers and stigmas, and their movement, we followed 10 flowers throughout the anthesis and recorded: 1) position of the stigmatic lobes and stamens along the anthesis; 2) contact between stigmatic lobes and anthers along the anthesis.

We checked if stamens moved in response to touches. We also recorded the number of stigmatic lobes per flower.

Floral visitors

The frequency of flower visitors was determined throughout anthesis of 15 flowers, in 30 minutes-intervals, totaling 30 hours of observation. We record the following information during the flower visits: 1) bee species; 2) sex of the bee; 3) stigma contact. We also quantified where the bees alighted on the flowers and when they touched the stigma (beginning of visit, during, or end of the floral visit) and where they collect pollen (from anthers or adhering to petals). To analyze the behavior of the floral visitors in detail, we made video recordings (200 minutes, Canon EOS T5i).

Pollen deposition on the stigma

In flowers accessible to flower visitors (N = 10) and in bagged flowers (N = 3) we removed the stigmas at the end of anthesis to count and identify the adhering pollen grains. The stigmas were transferred to microscope slides containing fuchsine stained glycerine gelatin, the slides were heated to melt the gelatin, and the preparations were covered with a coverslip and sealed with paraffin wax. Under a microscope we counted and identified all the pollen grains that adhered to the stigmas.

Statistical analyses

To check if there was a difference in the number of bee visits received by the flowers throughout the anthesis, we used the non-parametric Kruskal-Wallis one way analysis of variance on ranks. Statistical tests were performed with SigmaStat 3.5 software for Windows.

RESULTS

Anthesis and floral longevity

Frailea gracillima bloomed between October and November. In 108 days of observation during three years (30 consecutive days in 2016, 44 in 2017 and 34 in 2018), we recorded only 12 days of flowering (3 days of flowering in 2016, 4 in 2017 and 5 in 2018). We did not find any open flowers in the field in 89% of the study period.

All previously marked flower buds opened their flowers (N = 131). Most individuals produced one flower per day (95.2%, N = 125 individuals), 6 individuals produced 2 flowers simultaneously (4.8%, N = 125) and only one individual produced 4 flowers. Flowering occurred only on sunny days. Individuals bloomed 3.0 ± 2.1 days after rain (N = 12 flowering days) (Figure 2).

Individuals of the population opened flowers synchronously. Anthesis in all marked flowers started 12:24h ± 10 min and the flowers remained open for 150 ± 13 min (N = 26). At the beginning of the anthesis the anthers were already dehisced and all pollen grains available to flower visitors. Flowers did not produced nectar.

Flowers visited by bees did not open on the next day (N = 25), but bagged flowers that received different pollination treatments without access to pollinator opened for up to 2 days. All hand self-pollinated flowers for 2 days (N = 6), 83% of hand-cross pollinated flowers opened for 2 days (N = 6), and 85% of permanently bagged flowers opened for 2 days (N = 7). We did not observe cleistogamous flowers during the study period.

Breeding system

Flowers of *F. gracillima* produced on average of 291 ± 73 ovules (N = 17). The individuals of the studied population were self-compatible and all hand-pollinated (self and cross) and open-pollinated flowers set 100% of fruits. Fruit set in unmanipulated bagged flowers was 56%, with only 5 of the 9 flowers setting fruit, with 128 ± 70 seeds. Flowers accessible to bees produced fruits with 301 ± 74 seeds (N = 11), hand-cross pollinated flowers produced 144 ± 12 seeds (N = 2) and hand-self-pollinated flowers produced 118 ± 66 seeds (N = 6).

Movement of stamens and stigma

Frailea gracillima produce hermaphroditic flowers with spatial separation of reproductive organs at the beginning of anthesis. Experimental mechanical stimulation of the stamens did not cause movement toward the style and revealed that they were not thigmonastic.

In newly opened flowers, the stamens are arranged in two positions: The outer stamens are positioned near to the petals and the inner stamens are close to the style (Figure 3A, Figure 4). Due to this arrangement, an empty space between both groups of stamens is formed along the floral tube. The stamens have different lengths, because of this, they form dense layers of anthers arranged around the style and in contact with the petals. During anthesis, from approximately 10 minutes after flower opening, the outer stamens slowly moved towards the center and the inner stamens also moved outwards (Figure 4). After around 60-80 min stamens were spread accross the floral tube, partially detaching from contact with the petals, and filled the empty space in the floral tube between inner and outer stamens.

The stigmas had on average 10 ± 1 lobes (N = 35), and were positioned vertically in relation to the style in the flower bud stage. From the first minutes after flower opening, the stigma lobes slowly moved downwards from its upward towards the stamens. The extremity of stigmatic lobes curls and increases the proximity to the anthers (Figure 3B). During flower closure, the stigma lobes can be pushed downwards by the petals and get into contact with anthers (Figure 5A). Eventually, in some flowers there is contact of the extremity of stigmatic lobes with pollen grains (Figure 5B). However, not all flowers set fruits autonomously, indicating that pollen deposition by this mechanism does not occur in all flowers.

Floral visitors

We collected 495 floral visitors, belonging 13 bee species of four families visiting flowers of *F. gracillima* (Table 1). The main floral visitor was *Anthrenoides micans* Urban, 1995, which corresponded to 70.7% of the visits observed (Figure 6A), followed by *Dialictus sp*. with 22.6% of the visits (Figure 6B). *Arhysosage cactorum* Moure, 1999 and *Chalepogenus goeldianus* Friese, 1899 had low visitation frequencies (3.3 and 2.2% respectively). Other nine bee species were sporadic floral visitors.

For three bee species *Anthrenoides micans*, *Arhysosage cactorum* and *Callonychium* sp., we recorded both male and female individuals, although females were always more frequent.

Anthrenoides micans showed reproductive behavior attached to cactus flowers. Males patrolled flowers in search of females and every minute they inspected a single flower five times (N = 341 inspections in 61 minutes). During the study period, 15 copulas were observed, which represented 3.0% of the visits received by *F. gracillima*. In this situation, couples touched the stigma in 100% of the visits. We observed only one copula of *Arhysosage cactorum* and none of *Callonychium* sp.

Female bees visited flowers throughout anthesis and there was no difference in the number of visits over the time (P = 0.690, H = 3.064, N = 141) (Figure 7). Female bees of *Anthrenoides micans* mainly used the stigma as landing platform (87% of visits, N = 30), while females of *Dialictus sp.* alighted mainly on the petals (87% of visits, N = 15).

Bees were positioned in two ways to collect pollen: directly on the petals or inside the floral tube, positioned upside down between the inner and outer stamen bundle. Females of *A. micans* landed upside down on the floral tube, perching on the style and collected pollen preferentially from stamens of the inner bundle, where anthers contact the style (76.6%, N = 30, Figure 8). Due to the position of the bee inside the flower, there was pollen deposition on the body of the bee in 86.6% of the visits. *Dialictus sp.* landed on the petals to collect pollen directly from the outer bundle of stamens or pollen grains adhering to the petals (80.0%, N = 15). Pollen deposition on the body of *Dialictus sp.* occurred in 20% of visits. When leaving the flower, *A. micans* mainly used the petals (66.6%, N = 30) while *Dialictus sp.* climbed over the style and left from the stigma (53.3%, N = 15).

In general, bees have often touched flower stigmas. Sporadic visitors such as *Arhysosage cactorum, Chalepogenus goeldianus* and *Cactocolletes rugatus* (Urban, 1995) Engel touched the stigma in all visits. Females of *Anthrenoides micans* females touched the stigma in 95.8% of the visits (N = 48), *Dialictus sp*. touched only in 21.3% of visits (N = 75).

Pollen deposition on the stigma

In flowers visited by bees, the average number of conspecific pollen grains deposited in stigma surface was 1847 ± 750 (N = 10), being six times higher than the number of ovules. Heterospecific pollen varied from 1 - 17% of the total pollen load, mainly from Asteraceae, Malvaceae, Poaceae and Euphorbiaceae (*Croton* sp.), Cactaceae (*Opuntia* sp.).

In bagged flowers, autonomous pollen deposition on stigma was on average 2318 ± 1740 pollen grains (N = 3).

DISCUSSION

In the natural habitat, *Frailea gracillima* exclusively produces chasmogamous flowers and floral traits strongly favor cross-pollination. Flowering is concentrated in just a few days during the reproductive season with periods of flowering breaks. The ephemeral and highly synchronized flowering among the individuals of the population attracts pollinators that deposit a huge amount of pollen grains on the stigma, leading to high seed set. The main pollinators are the same solitary bee species that visits sympatric globose cacti of the genera *Parodia* and *Gymnocalycium* (Schlindwein & Wittmann, 1995; Cerceau *et al.*, 2019). However, when pollinators are absent or scarce, the chasmogamous flowers reproduces through delayed autonomous self-pollination due movements of both stigma lobes and stamens during anthesis.

Flowering pattern and floral traits of Frailea gracillima *favor cross pollination*

Different to literature (Rowley, 1980; Metzing & Kiesling, 2006), our results reveal that flowering pattern and floral traits of *F. gracillima* strongly favour cross pollination, which might be also true for other species of the genus.

Most individuals open only one flower per day, which can drastically reduce geitonogamy and favours cross-pollination (de Jong *et al.*,1993). Consequently, almost all floral visits of the foraging trips of the bees lead to cross-pollen deposition on the stigma.

The entire population of *F. gracillima* blossomed in the same few days during reproductive season and the start of the anthesis was synchronized, therefore huge quantities of flowers were available for a short period of time in rock outcrops. Flowering synchronization within population may increase pollinator attractiveness, favour cross-pollination (Opler *et al.,* 1976; Augspurger, 1981) and it is an important trait in melittophilous cacti, because bees can concentrate visits at the beginning of anthesis (Cerceau *et al.*, 2019). Anthesis synchronization was previously recorded in *Frailea* species both in natural habitat (Machado, 2007) and in cultivation (Metzing & Kiesling, 2006). Both fresh pollen and receptive stigmas are exposed to pollinators in short-time, optimizing reproduction by diminishing pollen loss in the population. Anthesis synchronization may be related to abiotic factors, such as rainfall, as has been reported in other species of cacti (Eggli & Giorgetta, 2017; Cerceau *et al.*, 2019). However, precipitation does not seem to be strongly related to the flowering of *F. gracillima*, since the gap between rainy days and open-flower days was very variable.

Frailea gracillima share floral traits with other sympatric cacti species that receive visits of the same specialized effective bee pollinators, such as flower colour, shape, apical position of flowers in cactus body and erect position of flowers near the soil surface. Sympatric *Parodia* and *Gymnocalycium* also synchronized flower opening and closure within the population at a similar time schedule to *F. gracilima*, between 11:00 and 17:30 (Schlindwein & Wittmann, 1995; Cerceau *et al.*, 2019), however, surprisingly there were no flowering days overlap in rock outcrops (our unpubl. data).

Another characteristic that favours cross-pollination in *F. gracillima* is hercogamy, which prevent contact between stigma and anthers (Faegri & van der Pijl, 1979; Webb & Lloyd, 1986).

The prominent stigma composed by numerous and long lobes, positioned at flower entrance diminish the free space to flower visitors reach pollen resources below, which favour contact between bees and stigma. The stigma of *F. gracillima* is bigger than that of other sympatric melittophilous cacti, such as *Parodia* and *Gymnocalycium*, which have a tiny central almost circular stigmatic surface. However, specialist bees use the stigmatic surface of these cacti species as a landing platform (Schlindwein & Wittmann, 1995; Cerceau *et al.*, 2019), similar to *F. gracilima.*

Frailea species in Rio Grande do Sul have high habitat specificity and occur in rock outcrops of different lithologies (Pontes *et al*., 2018). Delimitation of populations on rock outrcrops can favors cross-pollen flow among individuals, since the outcrops are isolated in the landscape of the shrubby fields.

Solitary bee pollinators

The floral visitors that contributed to the pollination of *F. gracillima* were solitary bees that touched the stigma during flower visits. The position of the two bundles of stamen within the flower favored pollen deposition on the body of bees, which is transferred to conspecific flowers on the next visits, favoring cross-pollination. Several cacti species have floral traits that promote pollen deposition to insect body, such stamen movement (Lenzi & Orth, 2011; Cota-Sánchez *et al.*, 2013; Schlindwein & Wittmann, 1997). Thigmonastic stamen movements were not observed in *Frailea*, but it is frequent trait in *Parodia* and *Opuntia* flowers (Schlindwein & Wittmann 1995, 1997; Cerceau *et al.*, 2019).

The main floral visitor of *F. gracillima* is *A. micans,* registered as a frequent floral visitor of sympatric globose yellow-flowered cacti (Schlindwein & Wittmann, 1995; Schlindwein, 1998; Cerceau *et al.*, 2019). *Anthrenoides micans* exhibits reproductive behavior attached to cactus flowers, which considerably increases its presence inside the flowers. Males intensely patrol flowers in the search of pollen-collecting females and also copulate inside them (Dutra *et al.* 2020). We observed that copulating bees in flowers of *F. gracillima* frequently touch the stigmas, different to copulas in *Parodia* flowers that have smaller stigma (Schlindwein & Wittmann, 1995; Cerceau *et al.*, 2019). The reproductive behavior of specialized bees attached to host plant greatly increases flower visits and constancy, as well as effectiveness of pollination (Schlindwein & Wittmann, 1995; Oliveira & Schlindwein, 2010; Oliveira *et al.,* 2012, 2013).

Besides *A. micans,* the oligolectic bee species *A. cactorum* and *C. rugatus* also collected pollen in flowers of *F. gracillima,* however with a low visitation frequency. During the short floral lifespan of *F. gracillima,* these bees were observed flying among conspecific flowers, which provided a stigma pollen load with huge amounts of conspecific grains.

The tiny *Dialictus* bees rarely touched the stigma and little contributed to pollination similar as observed for other cacti species (Schlindwein & Wittmann, 1995, 1997; Cerceau *et al.,* 2019).

Temporal herkogamy and delayed autonomous self-pollination

The flowers of *F. gracillima* show hercogamy, which corresponds to spatial separation of reproductive organs that favors cross-pollination and reduces interference between the male and female function (Faegri & van der Pijl, 1979; Webb & Lloyd, 1986).

Hercogamy in the flowers of *F. gracillima* occurs only at the beginning of anthesis. The spatial separation of stigmas and anthers slowly diminishes during anthesis with the stigmas moving downwards, therefore contacting the pollen available in the anthers. The temporal hercogamy prevents autonomous self-pollination during the first hours of anthesis. The curving down of stigma lobes is well known in representatives of Campanulaceae and Asteraceae that have secondary pollen presentation, where stigmas can touch pollen grains that eventually still adhere to the style at the end of anthesis (Faegri & van der Pijl, 1979; Shetler, 1979). In Malvaceae also occurs curving down of un-pollinated stigma lobes during anthesis and consequently promote delayed autonomous self-pollination (Buttrose *et al.*, 1977; Klips & Snow, 1997; Ruan *et al.,* 2005; Ruan & Mopper, 2008).

In closing flowers of *F. gracillima*, where cross-pollination is no longer possible, autonomous pollen deposition result in fruit set. Delayed self-pollination occurs when floral movements at the end of anthesis promote contact between anthers and stigma (Lloyd & Schoen, 1992). This mechanism ensures reproduction when flowers were not pollinated (Faegri

& van der Pijl, 1979) and can be considered a mechanism of reproductive assurance in *F. gracillima* flowers.

Curving down of stigma lobes seems to be frequent among species of *Frailea* and often appears in photographic records (Metzing, 2015a, 2015b; Carneiro *et al.*, 2016). It would be interesting to confirm delayed self-pollination after floral movements also in other species of *Frailea* to elucidate whether this reproductive strategy is a common trait of the genus.

Cleistogamy in Frailea

Our study revealed that the individuals of *F. gracillima* do not exhibit cleistogamous flowers in the natural habitat. This contradicts information common in literature that most *Frailea* species reproduce via cleistogamy and the opening of chasmogamous flowers is rare (Britton & Rose, 1922; Rowley, 1980; Metzing & Kiesling, 2006)

In several species that produce both cleistogamous and chasmogamous flowers, cleistogamy is mainly related to abiotic conditions, such as photoperiod, temperature, soil moisture, shade and nutritional quality (Uphof, 1938; Brown, 1952; Langer & Wilson, 1964; Faegri & van der Pijl, 1979). In *Frailea* species, flowering is highly seasonal and seems to be related to temperature, humidity and day length (Köhler, 1968; Metzing & Kiesling, 2006; Machado, 2007; Gomes *et al.*, 2019). In nature, the flowering of *F. gracillima* occurs during months with longer daytime (13-14 sunlight hours), high sunlight intensity, very hot temperatures at noon and in the early afternoon when the plants bloom (12:30h -15:00h) (INMET, 2018). Moreover, days with rain are common in the flowering period. The cacti collections in greenhouses or in higher latitudes, where most *Frailea* individuals are cultivated, experience rather different environmental conditions. This might strongly influence petal unfolding in the species of *Frailea*. Thus, it has been suspected that *Frailea* species cultivated in warmer countries result in higher frequency of chasmogamous flower production. Moreover, when grown in shaded greenhouses, flowers seem to remain closed and set fruit (Köhler, 1968). Laboratory tests under controlled conditions may identify which abiotic factors influences the production of chasmogamous and cleistogamous flowers in *Frailea* species.

Conclusions

Although *Frailea* is considered a typically cleistogamous genus, *F. gracillima* presents floral traits that favor cross-pollination. Chasmogamous flower production was favored in natural habitat during three years of study. However, movements of stigma and stamens during anthesis promoted delayed autonomous self-pollination when there was no more chance of cross pollination due flower closure, which probably ensuring sexual reproduction in adverse conditions and low pollinator environments.

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TABLES

Table 1: Flower-visiting bees of *Frailea gracilima.* Frequency of floral visits were measured during 30 hours of observation (N = 495 visits). (-) Species not recorded during 30 hours of flower observation, but collected during fieldwork.

Bee species	**Tribe / Subfamily**	**Sex**	**Frequency (%)**
Andrenidae			
Arhysosage cactorum Moure 1999	Calliopsini	♀♂	3.3%
Callonychium sp.	Calliopsini	♀♂	1%
Anthrenoides micans Urban 1955	Protandrenini	♀♂	70.7%
Psaenythia sp.	Protandrenini	♀	-
Apidae			
Ancyloscelis romeroi Holmberg 1903	Emphorini	♀	-
Ceratina sp. 1	Ceratinini	♀	-
Ceratina sp. 2	Ceratinini	♀	-
Chalepogenus goeldianus Friese, 1899	Tapinotaspidini	♀	2.2%
Plebeia sp.	Meliponini	♀	-
Tapinotaspis sp.	Tapinotaspidini	♀	-
Colletidae			
Cactocolletes rugatus Urban 1995	Neopasiphaeinae	♀	0.2%
Halictidae			
Augochloropsis sp.	Halictini	♀	-
Dialictus sp.	Halictini	♀	22.6%

FIGURES

Figure 1: *Frailea gracillima* habitat and synchronized flower opening in the field. Cacti grow between rock cracks in sandstone outcrops in Santana da Boa Vista, Rio Grande do Sul.

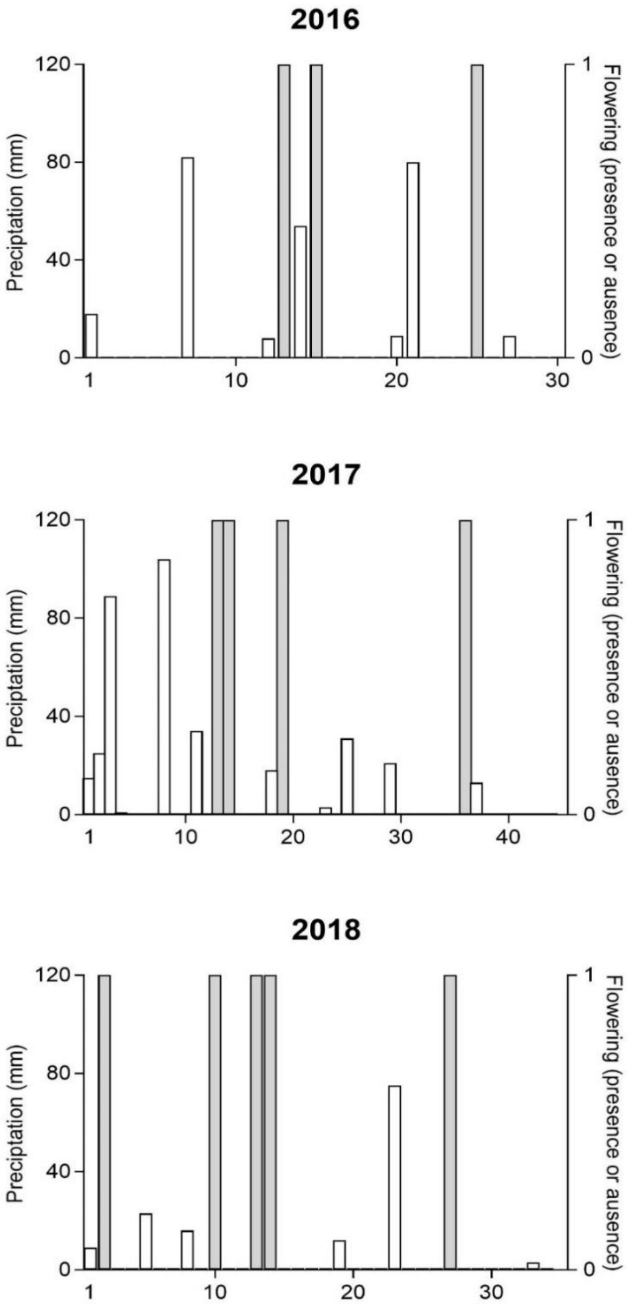

Figure 2: Daily variation in precipitation and flowering events of *Frailea gracillima* during three years. The study period in each year was: 2016 (October 27 to November 25), 2017 (October 11 to November 23) and 2018 (October 26 to November 28).

Daily rainfall (mm) represented by white bars, varied between 0 and 104 mm in three years. The events of presence or absence of flowering are represented by the gray bars. Flowering occurred from 1 to 7 days after precipitation.

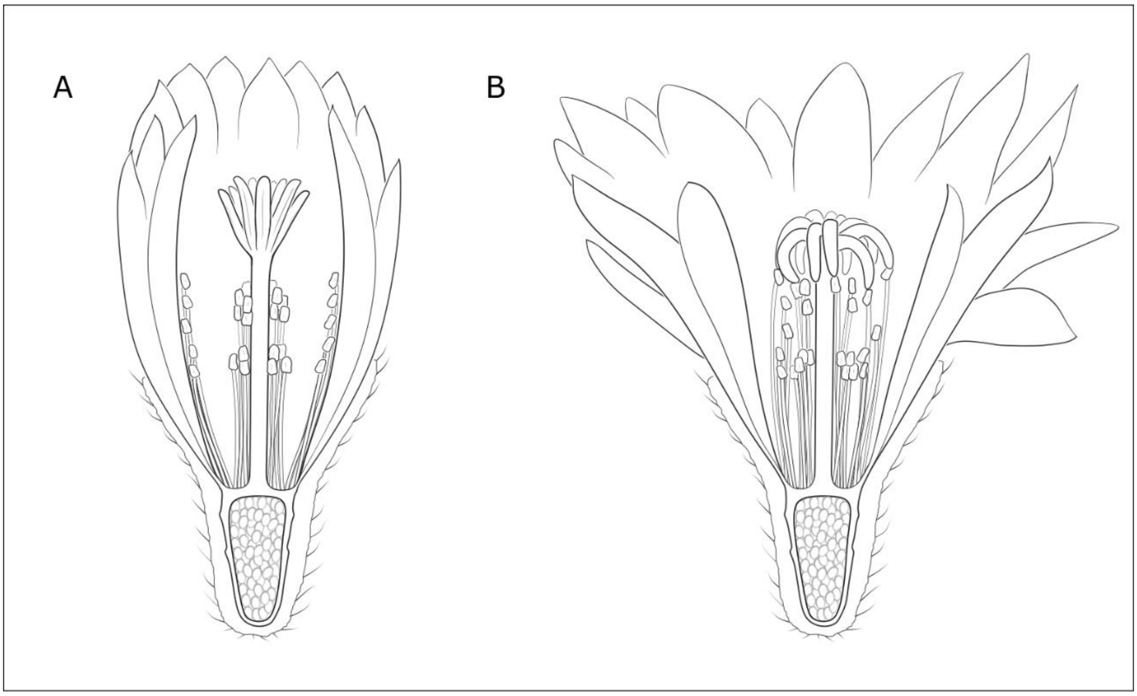

Figure 3: Movement of reproductive organs during the anthesis. (A) In the first minutes of anthesis, the stigmatic lobes are positioned vertically and the stamens are positioned in two main positions: in contact with the petals or in contact with the style. (B) At the end of anthesis, approximately 2:30 hours after opening the flowers, the stigmatic lobes move towards the stamens and come into contact with the anthers. Stamens move gradually along the anthesis and are distributed along the floral tube.

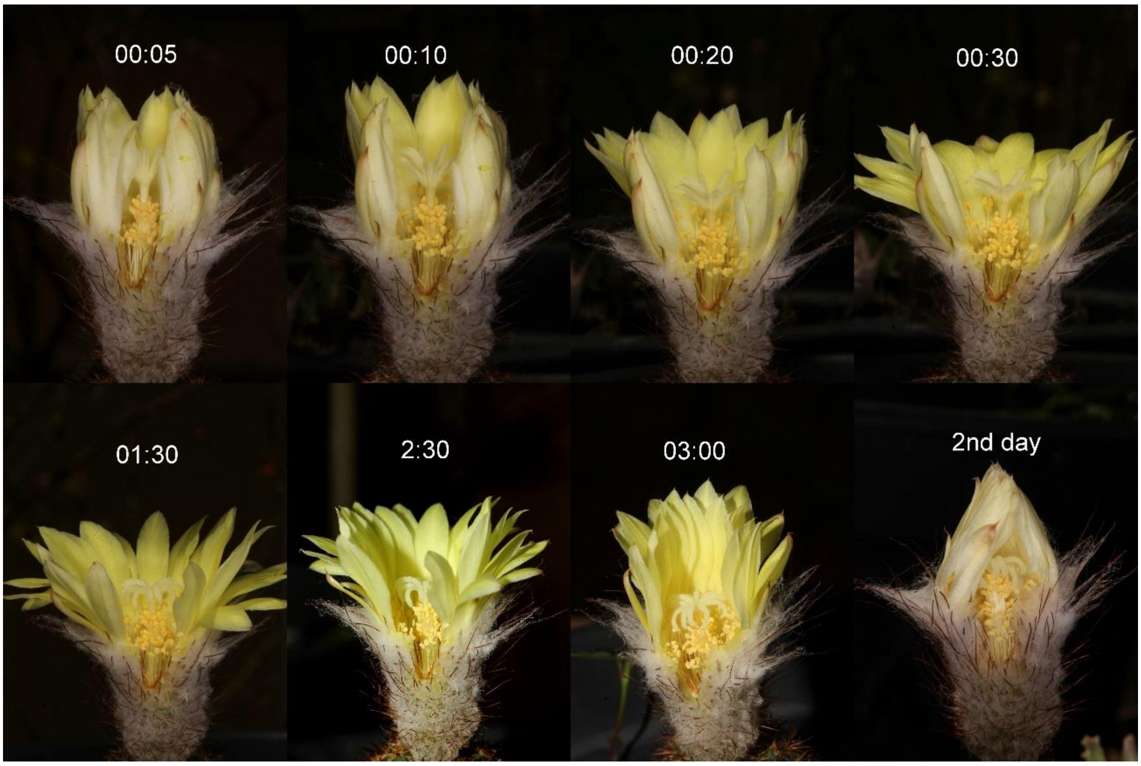

Figure 4: Movement of stigmatic lobes and stamens during anthesis of *Frailea gracilima*. The schedules in the photo correspond to the time elapsed since the beginning of the flower opening. During the beginning of anthesis, there is no contact between stamens and stigma. Throughout the anthesis, the structures move: stigmatic wolves curl up and the stamens spread to the center of the flowers. Flower at the time of 3:00 had the petals partially open with hands to demonstrate the position of the stamens and stigma inside the flower. On the second day of anthesis, the reproductive structures are in dense contact.

Figure 5: Contact of stigmatic lobes with anthers in *Frailea gracillima* flowers. (A) Flowers in the end of anthesis shows intense contact of the stigmatic lobes with anthers. (B) Stigmatic lobes with pollen grains in flower not visited by bees.

Figure 6: Main floral visitors of *Frailea gracillima*. (A) *Anthrenoides micans*. (B) *Dialictus sp.*

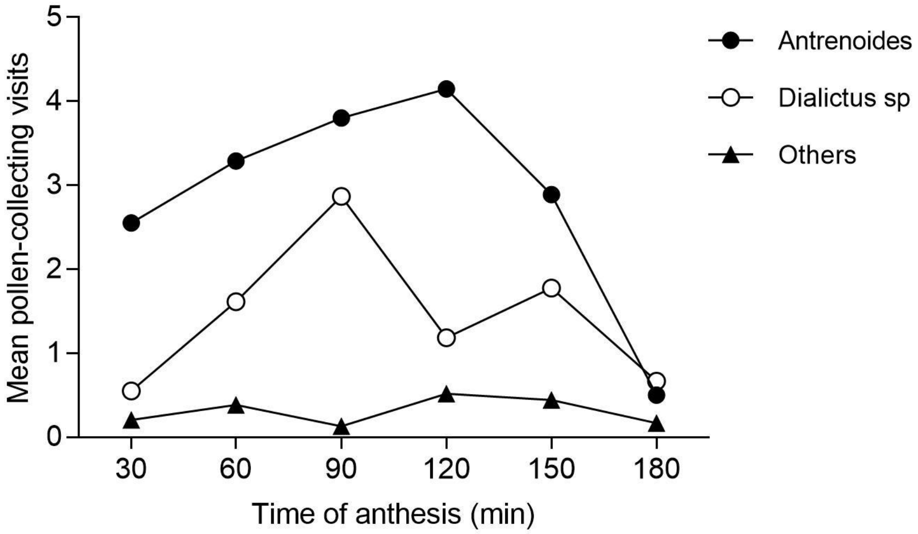

Figure 7: Frequency of visits by females to *Frailea gracillima* flowers throughout anthesis. Time of anthesis was represented on the Y axis through the minutes that elapsed after the opening of the flower (time 0) and flower closure after 150 ± 13 min. We represent an anthesis up to 180 minutes to characterize all floral visitors until the complete petal closure. The X axis represents the average number of visits by female bees received on a flower. Total number of visits recorded was 365 in 15 flowers. Male visits were excluded.

Figure 8: Bee positioned between the two bundles of stamens to collect pollen. *Anthrenoides micans* preferably collects pollen from the stamens attached to the style.

CONSIDERAÇÕES FINAIS

A observação detalhada das espécies em habitat natural é essencial para a construção do conhecimento sobre as interações entre plantas e polinizadores. A imersão em campo durante o desenvolvimento desta tese revelou aspectos únicos das interações, como a sincronia temporal da atividade das espécies envolvidas, funcionamento floral e comportamento de forrageio das abelhas nas flores complexas dos cactos. Devido ao fato das flores de cactos serem efêmeras, muitas vezes com períodos curtos de floração, estudos minuciosos sobre interações em habitat natural são escassos.

Estudamos detalhadamente duas espécies de cactáceas melitófilas pertencentes a dois gêneros. *Parodia*, um gênero que já era conhecido por manter relações com abelhas especialistas e *Frailea*, um gênero considerado majoritariamente cleistógamo que nunca foi estudado na perspectiva reprodutiva em habitat natural.

Em relação a *Parodia neohorstii*, que floresce em apenas alguns dias no ano, observamos que as abelhas sincronizaram a atividade de voo com abertura das flores e as polinizaram na primeira hora da antese. A eficiência na polinização é essencial para a reprodução *de P. neohorstii*, uma vez que a espécie é auto incompatível. O desencontro fenológico registrado entre o início da floração de *P. neohorstii* e a atividade de seus polinizadores efetivos foi uma oportunidade de quantificar qual é o real impacto da ausência das abelhas oligoléticas na reprodução da espécie. A drástica redução na produção de frutos e sementes na ausência de *Arhysosage cactorum* indica que *P. neohorstii* é dependente de polinizadores específicos para se reproduzir e caso o desencontro temporal entre as espécies se torne mais frequente, podem ocorrer prejuízos na reprodução dessa cactácea em habitat natural.

Um fator chave do funcionamento floral de *P. neohorstii* é o movimento tigmonástico dos estames. Este movimento age como um mecanismo de particionamento de pólen, restringindo a quantidade de recurso coletado pelos visitantes florais, uma vez que as flores oferecem todo o pólen de uma única vez. Estes movimentos, que são induzidos pelos polinizadores efetivos durante a coleta de pólen, atua como gatilho para a senescência floral. Após consecutivos movimentos de estames durante a antese, induzidos de forma experimental, as flores senesceram mesmo na ausência de polinização. Isso indica que o movimento dos estames pode agir como um sinalizador da presença dos polinizadores efetivos, e, consequentemente, da dispersão polínica. A ausência de movimentos pode indicar um ambiente com baixa disponibilidade de polinizadores, fator que induz a extensão da longevidade floral e colabora para o aumento das chances de exportação de polínica. Esses movimentos já foram reportados em várias espécies de cactáceas, mas até o momento não foram descritas funções relacionadas com a indução da senescência floral.

Parodia neohorstii é uma espécie criticamente ameaçada de extinção e em declínio populacional. O diagnóstico reprodutivo em habitat natural revelou que a espécie está produzindo frutos e sementes com muita eficiência, o que pode contribuir para a manutenção das populações a longo prazo. Porém, a espécie está sendo impactada negativamente pela criação de animais na área de ocorrência. Os danos físicos causados pelo consumo dos cactos por caprinos e pelo pisoteio por bovinos, geraram altas taxas de mortalidade nas populações estudadas. Esse fator pode impactar fortemente na viabilidade das populações em habitat natural a médio e longo prazo e contribuir para o declínio populacional. O impacto gerado pelos animais de criação nas populações de *P. neohorstii* pode ser semelhante ou até pior em populações de cactos simpátricos, como espécies dos gêneros *Parodia, Frailea, Gymnocalycium* e *Echinopsis*, que muitas vezes possuem espinhos mais macios e menos numerosos, o que aumenta a vulnerabilidade das espécies aos herbívoros. Uma ação para a conservação das cactáceas da região seria a restrição do acesso de animais nas áreas de ocorrência, principalmente em afloramentos rochosos, onde a oferta de alimentos para animais de criação é baixa e a restrição não prejudicaria na qualidade da pastagem disponível para os animais.

Em relação a *Frailea gracillima*, observamos que a cleistogamia não ocorreu em habitat natural durante o período de estudo e as flores apresentam características que favorecem a polinização cruzada. A grande quantidade de pólen co-específico depositado no estigma por abelhas contribuiu para a formação de frutos e sementes. Observamos que *F. gracillima* pode promover autopolinização autônoma tardia, possibilitada após o movimento dos estames e estigma ao longo da antese. Esse fator é importante, pois contribui para a produção de frutos e sementes mesmo em cenários de baixa densidade de polinizadores, o que não ocorre na espécie simpátrica *P. neohorstii*.

As espécies de cactáceas estudadas não produzem néctar. Por isso, a manutenção dos polinizadores no ambiente depende de outras espécies vegetais para fornecimento desse recurso. Dessa maneira, a conservação da comunidade vegetal do entorno é essencial para a qualidade reprodutiva dos cactos da região, uma vez que as mesmas espécies de abelha são encontradas em flores de diversas espécies de cactos melitófilos simpátricos.

Em síntese, este trabalho gerou conhecimento sobre aspectos da história natural de duas espécies de cactáceas. Contribuímos com a descrição de novos mecanismos de funcionamento floral em ambas as espécies estudadas, além de diagnosticar que a formação de frutos e sementes está ocorrendo com sucesso em habitat natural. Em relação a *Parodia neohorstii*, identificamos duas principais ameaças à espécie: 1) mudanças fenológicas podem alterar a interação com polinizadores e causar impactos negativos na reprodução em habitat natural; 2) a mortalidade de cactos é provocada principalmente pelos impactos gerados por animais de criação.

Estes resultados podem contribuir com ações direcionadas para a conservação, tanto das espécies foco deste estudo, quanto para outras cactáceas da região das Guaritas.