Universidade Federal de Uberlândia
Instituto de Biologia
Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais

Aspectos reprodutivos de orquídeas em ambientes de Vereda do Cerrado

João Custódio Fernandes Cardoso

2021

João Custódio Fernandes Cardoso

Aspectos reprodutivos de orquídeas em ambientes de Vereda do Cerrado

Tese apresentada à Universidade Federal de Uberlândia, como parte das exigências para obtenção do título de Doutor em Ecologia e Conservação de Recursos Naturais".

Orientador

Prof. Dr. Paulo Eugênio Oliveira

Co-orientador

Prof. Dr. Steve Dene Johnson

UBERLÂNDIA,

Agosto - 2021

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

C268
2021

Cardoso, João Custódio Fernandes, 1989-
Aspectos reprodutivos de orquídeas em ambientes de Vereda do Cerrado [recurso eletrônico] / João Custódio Fernandes Cardoso. - 2021.

Orientador: Paulo Eugênio Oliveira.
Coorientador: Steven Dene Johnson.
Tese (Doutorado) - Universidade Federal de Uberlândia, Pós-graduação em Ecologia e Conservação de Recursos Naturais.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.te.2021.395
Inclui bibliografia.
Inclui ilustrações.

1. Ecologia. I. Oliveira, Paulo Eugênio,1959-, (Orient.). II. Johnson, Steven Dene,1967-, (Coorient.). III. Universidade Federal de Uberlândia. Pós-graduação em Ecologia e Conservação de Recursos Naturais. IV. Título.

CDU: 574

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

Gizele Cristine Nunes do Couto - CRB6/2091

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais
Av. Pará, 1720, Bloco 2D, Sala 26 - Bairro Umuarama, Uberlândia-MG, CEP 38405-320
Telefone: (34) 3225-8641 - www.ppgeco.ib.ufu.br - ecologia@umuarama.ufu.br

ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Ecologia e Conservação de Recursos Naturais				
Defesa de:	Tese, número 71, COPEC				
Data:	vinte e sete de agosto de dois mil e vinte e um	Hora de início:	14:00	Hora de encerramento:	19:00
Matrícula do Discente:	11713ECR001				
Nome do Discente:	João Custódio Fernandes Cardoso				
Título do Trabalho:	Aspectos reprodutivos de orquídeas em ambientes de Vereda do Cerrado				
Área de concentração:	Ecologia				
Linha de pesquisa:	Ecologia vegetal				
Projeto de Pesquisa de vinculação:	Alternativas reprodutivas, interações bióticas e diversidade funcional no Cerrado				

Reuniu-se por webconferência, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ecologia e Conservação de Recursos Naturais, assim composta pelos doutores: João Marcelo Robazzi Bignelli Valente Aguiar - USP, Mariana Oliveira Duarte - UFMG, Rafaela Cabral Marinho - UFU, Raphael Matias da Silva - UnB, Paulo Eugênio Alves Macedo de Oliveira - UFU, orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Paulo Eugênio Alves Macedo de Oliveira, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao discente a palavra para a exposição do seu trabalho. A duração da apresentação do discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

Aprovado(a).

Esta defesa faz parte dos requisitos necessários à obtenção do título de Doutor.

O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Paulo Eugênio Alves Macedo de Oliveira**, **Membro de Comissão**, em 27/08/2021, às 19:09, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Rafaela Cabral Marinho**, **Usuário Externo**, em 30/08/2021, às 15:36, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Raphael Matias da Silva**, **Usuário Externo**, em 30/08/2021, às 18:39, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Mariana Oliveira Duarte**, **Usuário Externo**, em 08/09/2021, às 13:16, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **João Marcelo Robazzi Bignelli Valente Aguiar**, **Usuário Externo**, em 08/09/2021, às 16:28, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

A autenticidade deste documento pode ser conferida no site https://www.sei.ufu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **2939744** e o código CRC **7CBFB51C**.

Referência: Processo nº 23117.050121/2021-23 SEI nº 2939744

Índice

Resumo

As Veredas são ambientes únicos do bioma Cerrado, sendo responsáveis por abrigar grande diversidade e prover importantes serviços ecossistêmicos. Esses ambientes são particularmente diversos em Orchidaceae. Nessa tese, nós estudamos a biologia reprodutiva de orquídeas ameaçadas que ocorrem em Veredas com o intuito de demonstrar a diversidade de processos e o potencial de geração de conhecimento que esses ambientes abrigam. *Cyrtopodium hatschbachii* é uma espécie primariamente polinizada por abelhas, mas que apresenta taxas de frutificação baixas e até ausentes em alguns anos. Entretanto, nós demonstramos no primeiro capítulo como a herbivoria e a chuva, dois fatores que são detrimentais em separado, podem juntos facilitar a autogamia em uma planta e promover segurança reprodutiva. *Phragmipedium vittatum* é uma orquídea sapatinho que, para se reproduzir, aprisiona os polinizadores em seu interior. No segundo capítulo, nós demonstramos as várias estratégias que essas flores adotam que permitem o sucesso da polinização, desde a captura do polinizador, passando pelos movimentos intraflorais que eles são forçados a fazer, até a transferência de pólen e sua saída da flor. Para tanto, *P. vittatum* usa um sistema de mimetismo de afídeos que engana fêmeas grávidas de dípteros Syrphidae durante a entrada e saída da flor, já que elas estão a procura de sítios para oviposição onde suas larvas predadoras de afídeos irão se alimentar. Ainda estudando *P. vittatum*, encontramos que esta espécie trata-se de um modelo único no mundo pois apresenta variação intrapopulacional na ressupinação floral. Este processo trata-se do giro de aproximadamente 180º das flores, o que as deixa com o aspecto de estarem de cabeça para baixo. No terceiro capítulo, nós encontramos que aproximadamente 10% das flores da população não ressupinam. Nós testamos experimentalmente o papel da ressupinação no processo de reprodução das flores e encontramos que apenas flores com o labelo voltado para baixo apresentam sucesso reprodutivo masculino e feminino quando comparadas a flores com labelo posicionado para cima e para o lado. Esses resultados são importantes pois corroboram que a evolução da ressupinação provavelmente esteja relacionada a polinização, sendo que o labelo funciona como uma plataforma de pouso ou armadilha e a coluna voltada para baixo promove a deposição precisa de pólen. Por fim, no quarto capítulo demonstramos que a probabilidade das flores não ressupinarem está negativamente relacionada ao peso das mesmas. Essa é a primeira vez que se é demonstrado que o processo de ressupinação floral é dependente do peso. Em suma, esses resultados mostram a diversidade de diferentes histórias que esses organismos à beira do desaparecimento podem nos contar, bem como o potencial de geração de conhecimento. Assim, a conservação dessas espécies ameaçadas e dos seus respectivos ambientes é de extrema relevância para a manutenção de tais processos e interações, que certamente estão associados a um ambiente equilibrado e provedor de numerosos serviços ecossistêmicos.

Palavras-chave: Orchidaceae, Polinização por Engano, Ressupinação, Vereda.

Abstract

The Veredas wetlands are unique environments in the Cerrado biome, harboring high diversity and providing important ecosystem services. These environments are particularly diverse in Orchidaceae. In this thesis, we study the reproductive biology of endangered orchids that occur in Veredas wetlands to demonstrate the diversity of processes and the potential for generating knowledge that these environments provide. *Cyrtopodium hatschbachii* is a species primarily pollinated by bees, but with low fruit set rates, which may even be null in some years. However, we demonstrate in the first chapter how herbivory and rainfall, two factors that are detrimental when considered separately, can together facilitate autogamy and promote reproductive assurance in this species. *Phragmipedium vittatum* is a lady's slippery orchid that traps pollinators in order to reproduce. In the second chapter, we demonstrate the various strategies that these flowers adopt to enable successful pollination, from pollinator capture, through the intrafloral movements they are forced to make, until pollen transfer during their exit from the flower. To do so, *P. vittatum* uses an aphid mimicry system that deceives gravid females of Syrphidae dipterans during flower entry and exit, as they are looking for oviposition sites where their aphidophagous larvae will feed on. Using also *P. vittatum* as model, we found that it is a unique species in the world as it presents intrapopulation variation in floral resupination. This process refers to the twist of approximately 180º that occurs in some flowers before or during anthesis, which makes them upside down. In the third chapter, we show that approximately 10% of the flowers in the population do not resupinate. We experimentally tested the role of resupination in the process of reproduction and found that only flowers with the lip facing downward have male and female reproductive success, when compared to flowers with the lip positioned upward and sideward. These results are important because they corroborate that the evolution of resupination is related to pollination success, with the lip acting as a landing platform or trap and the column facing downward promoting precise pollen deposition. Finally, in the fourth chapter, we demonstrate that the probability of flowers failing to resupinate is negatively related to their weight. This is the first time that flower resupination has been demonstrated as being a weight dependent process. In short, these results show the diversity of life histories that these organisms threatened by extinction can tell us, as well as their potential for generating detailed knowledge. Thus, the conservation of these species and their respective environments is extremely important for the maintenance of such processes and interactions, which are certainly associated with an equilibrated environment that provides several ecosystem services.

Keywords: Orchidaceae, Pollination by Deception, Resupination, Vereda Wetlands.

Introdução Geral

Savanas tropicais são ambientes de elevada diversidade. A savana mais biodiversa do mundo trata-se do Cerrado brasileiro. Localizado na porção central do Brasil, é o segundo maior bioma do país (e da América do Sul), ocupando cerca de 22 % do território nacional (Ribeiro & Walter 2008). O Cerrado é um considerado um hot spot, isto é, um bioma com elevada diversidade mas ao mesmo tempo com uma porção significativa já tendo sida devastada. Isso faz do bioma uma região prioritária para conservação. Uma das características que está associada à diversidade de espécies é a diversidade de formações vegetais que as espécies podem ocupar. Ao contrário de outras savanas do mundo, o cerrado apresenta uma ampla gama de paisagens fisionômicas que vão desde formações campestres com predomínio de gramíneas, passando por formações tipicamente savânicas com presença de arbustos co-ocorrendo com gramíneas, até mesmo formações florestais com predomínio de espécies arbóreas que formam um dossel contínuo (Ribeiro & Walter 2008).

O Cerrado apresenta uma marcada sazonalidade climática. Isso faz com que existam duas estações bem definidas: uma chuvosa (de Outubro a Março) e uma seca (Abril a Setembro) (Alvares et al 2013). Essa restrição da disponibilidade hídrica faz com que várias espécies apresentem traços adaptativos modelados por tal condição. Como a paisagem do cerrado apresenta um aspecto menos úmido durante uma parte do ano, algumas pessoas não associam que alguns grupos de organismos possam ocorrer em tais formações. Por exemplo, apesar de as orquídeas serem popularmente associadas a formações florestais, elas não são associadas a formações mais abertas, que estão mais suscetíveis a variação de umidade e maior exposição solar, como é o caso das formações campestres e savânicas. Entretanto, o Cerrado apresenta uma grande diversidade de orquídeas nesses ambientes (Batista & Bianchetti 2003). De maneira similar às outras espécies que ocorrem em tais formações, as orquídeas também apresentam adaptações que permitem a persistência em tais ambientes. Isso inclui o hábito terrestre, muitas vezes com órgãos de reserva localizados no subterrâneo, floração e reprodução no período chuvoso acompanhando a sazonalidade e resiliência ao fogo.

Entretanto, um ambiente especial onde a diversidade de orquídeas é particularmente alta trata-se das Veredas do bioma Cerrado (Batista & Bianchetti 2003). Esses locais apresentam vegetação graminosa-arbustiva típica, geralmente com a presença da palmeira buriti (Araújo et al. 2002). Uma característica preponderante trata-se dos solos desses ambientes, que são permanentemente úmidos (hidromórficos). Isso acontece porque é nas veredas que ocorre a surgência do lençol freático, geralmente em locais com topografia característica de vales encaixados (Boaventura 2007). Por apresentarem permanente disponibilidade hídrica, as veredas são consideradas como os "oásis" ou o "berço das águas" do Cerrado. Elas são de crucial importância na manutenção de mananciais e provisionamento de águas que alimentam córregos e rios que correm no interior do cerrado e para todos os biomas adjacentes. Pela sua localização em uma área de planalto, o Cerrado é considerado a "caixa d'água" fornecendo água para o consumo da maior parte dos brasileiros. É nas Veredas que nasce a maior parte dessa água. Além disso, as Veredas apresentam uma importância intrínseca na formação de corredores ecológicos, fornecimento de água para plantas e animais e como habitat para diversas espécies adaptadas às condições específicas de solo hidromórfico (Boaventura 2007). É por isso que elas são consideradas APP, sendo que sua exploração é proibida por lei (Brasil 2012).

As Veredas são ambientes particularmente ricos em espécies da família Orchidaceae. Por exemplo, em um levantamento realizado no Distrito Federal, um terço de todas as espécies de orquídeas aparecem nessas formações úmidas (Batista &

Bianchetti 2003). Até o momento não se sabe exatamente o porquê de tamanha diversidade de orquídeas nesses ambientes, mas aparentemente isso pode estar relacionado à maior disponibilidade hídrica e maior incidência luminosa devido a falta de um dossel lenhoso contínuo. As orquídeas já são, por si só, organismos icônicos, fascinantes e misteriosos, com uma enorme diversidade de formas, cores e histórias de vida. Por serem um grupo tão carismático e intrigante, as orquídeas têm atraído a atenção de diversos tipos de pessoas, desde o público comum até célebres cientistas como o caso de Charles Darwin que dedicou dois volumes de um livro inteiramente escrito sobre elas (Darwin, 1862; 1877). A beleza das flores de orquídeas bem como os sistemas de polinização especializados estão entre os principais fatores que despertam interesse nesse grupo.

No caso das orquídeas de Veredas, apesar de se saber muito pouco ainda, muitas delas são ainda mais especiais. Isso se deve não somente ao fato de serem orquídeas ou apresentarem características florais e vegetativas específicas, mas infelizmente por serem ameaçadas de extinção. Por exemplo, esse é o caso das espécies *Cyrtopodium hatschbachii* e *Phragmipedium vittatum*. A primeira trata-se de uma espécie com flores de um rosa único e centro amarelado, com inflorescências altas que podem chegar a quase 2 m de altura. A segunda trata-se de uma orquídea sapatinho de princesa que recebe esse nome pela morfologia do seu labelo. Essa espécie é representante do único gênero de orquídeas sapatinho encontrado no Cerrado, sendo bastante rara desde muitas décadas atrás (Menezes, 1989). A fragmentação e destruição dos habitats bem como a coleta excessiva dessas espécies levaram a sua inclusão na lista de espécies ameaçadas.

Tendo em mente o potencial das orquídeas de cerrado e especialmente as de Veredas, essa tese de doutorado trata-se de uma coletânea de estudos que usa orquídeas como modelo de estudo para demonstrar como espécies de ambientes de Vereda e a beira da extinção guardam histórias únicas e apresentam diversos aspectos que podem ser estudados numa perspectiva mais ampla, gerando conhecimento em diversas áreas, podendo ser expandido para além das fronteiras do Cerrado *per se*. Para tanto, usamos como espécies modelo nesse trabalho *C. hatschbachii* e *P. vittatum*, tipicamente de Veredas.

Muitas orquídeas apresentam limitação de polinização, como é o caso de *C. hatschbachii*, que apresenta apenas em média 1 % de formação de frutos por visitas de abelhas. Assim, quaisquer frutos adicionais formados por outros mecanismos como, por exemplo a autopolinização, podem ser importantes para a geração de sementes na população, funcionando como segurança reprodutiva. No primeiro capítulo demonstramos como a florivoria seguida de chuva, dois fatores que são detrimentais em separado, podem promover o sucesso reprodutivo por autogamia em *C. hatschbachii*. Gafanhotos às vezes consomem as flores incompletamente, removendo a capa da polínia intacta. Isso facilita que essa polínia se hidrate, fique mais pesada e entre no estigma depois de um evento de chuva. Nós confirmamos isso em campo e experimentalmente e discutimos que esses eventos de autopolinização são importantes na formação de sementes viáveis numa população que tem limitação na polinização cruzada, que pode inclusive ser ausente em determinados anos.

Algumas espécies de plantas adotam a estratégia de aprisionar os polinizadores. Estas armadilhas envolvem várias adaptações que permitem desde a captura, passando pela remoção e deposição de pólen até a saída do polinizador. Essa estratégia de armadilha é adotada por *P. vittatum*, que apresenta o labelo em forma de bolsa. No segundo capítulo, descrevemos o mecanismo de armadilha e as várias adaptações morfológicas que permitem que o sistema funcione efetivamente. Nós demonstramos que a planta adota o sistema de mimetismo de afídeos, imitando locais de oviposição que enganam fêmeas grávidas de dípteros Syrphidae, cujas larvas se alimentam de afídeos.

Um importante traço floral que é basal e amplamente distribuído na família Orchidaceae é a ressupinação. Essa se trata do giro de aproximadamente 180º da flor, geralmente na fase de botão, o que deixa as flores de cabeça para baixo. Nós observamos que *P. vittatum* trata-se de um modelo único em Orchidaceae onde as flores usualmente resupinam, mas algumas flores na mesma população apresentam falha no processo, permanecendo imobilizadas nas brácteas. No terceiro capítulo, nós testamos o papel da ressupinação no sucesso da polinização. Através da manipulação de flores em diferentes posições, nós demonstramos que flores que não resupinam apropriadamente não aprisionam efetivamente os polinizadores, e portanto não exportam nem recebem políneas. Esses resultados demonstram como o processo de ressupinação arranja as flores numa posição apropriada que permite o sucesso da polinização.

No quarto capítulo, nós testamos quais são os fatores que são responsáveis pela ressupinação floral em *P. vittatum*. Através de experimentos manipulativos, nós mostramos a importância do peso da flor no processo de ressupinação. Assim, aquelas flores que não resupinam na população são as flores mais leves cujo peso não é suficiente para completar o giro. A influência de auxinas na ressupinação floral já é conhecida, mas esse trata-se do primeiro caso que descreve a influência do peso no processo. Além da maioria das orquídeas, a ressupinação é um mecanismo que ocorre em diversas outras famílias. Assim, nossos resultados podem ser estendidos para tantos outros sistemas, o que coloca o peso, até então negligenciado, como um importante traço na orientação floral.

Em suma, ao final dessa tese, além de discutir a importância intrínseca das espécies, nós demonstramos que espécies de Veredas, em especial as orquídeas, são importantes no equilíbrio das interações no ecossistema, muitas delas únicas. Além disso, mostramos que essas espécies são importantes na geração de conhecimento em diversas áreas, sendo passíveis de manipulações experimentais que complementam as observações naturalísticas. Assim, a conservação dos ambientes pristinos e emblemáticos de Vereda viabiliza a manutenção de mecanismos especiais como os demonstrados aqui, permitindo a conservação do ambiente em equilíbrio e mantendo os importantes serviços ecossistêmicos que somente as Veredas oferecem no Cerrado.

Rererências

Alvares CA, Stape JL, Sentelhas PC, et al (2013) Köppen's climate classification map for Brazil. Meteorologische Zeitschrift 22:711–728. https://doi.org/10.1127/0941-2948/2013/0507

Batista, J. A. N., & Bianchetti, L. D. B. (2003). Lista atualizada das Orchidaceae do Distrito Federal. *Acta botanica brasílica*,17(2), 183-201. https://doi.org/10.1590/S0102-33062003000200003

Boaventura RS (2007) Vereda: Berço das Águas. Ecodinâmica, Belo Horizonte.

Brazil (2012) Native Vegetation Protection Law (Federal law n° 12.651 from May 25th 201)2. Casa Civil, Brasília, DF.

Darwin C. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing. London: J. Murray.

Darwin C. 1877. The various contrivances by which orchids are fertilized by insects, ed. 2nd. John Murray, London. https://doi.org/10.5962/bhl.title.57232

Menezes, L. C. (1989). *Phragmipedium vittatum* (Vell.) Rolfe: an orchid in the process of extinction. The Orchid digest (USA).

Ribeiro JF, Walter BMT (2008) As principais fitofisionomias do bioma Cerrado. Cerrado: ecologia e flora.

Capítulo 1

Florivory can facilitate rain-assisted autogamy in a deceptive tropical orchid

Abstract

Florivores and rainfall generally have negative impacts on plant fecundity. However, in some cases they can mediate fruit set. Some plants face severe pollen-limited fecundity and any additional fruit set, even if from self-pollination, can be advantageous. This is the case in some tropical deceptive orchids, such as the threatened *Cyrtopodium hatschbachii*. Here we test the hypothesis that florivory of the anther cap would facilitate rain-assisted autogamy in this species. In the field, we followed flowers in which the anther cap was removed by the orthopteran *Stenopola* sp. and found cases where pollinia self-deposited after rainfall and in one case this resulted in swelling of the column typical of fruit development. This event comprised 33% of all fruit set in the population in 2019. We then experimentally varied anther cap removal and rainfall in a factorial design and found increased fruit set in the group with cap removal (simulated florivory) followed by rain. The water absorption by pollinia makes them heavier, causing the stipe to bend. The droplet of water on the stigma then shrinks and pulls the pollinia back onto the stigma, causing self-pollination. Seeds from self-pollination have considerable viability and may allow population persistence, given that bee-mediated cross-pollination is uncertain and even absent in some years. Our study provides a unique example of how two unrelated factors (*i.e.* florivory and rain) that are detrimental alone may together promote fruit-set.

Keywords: florivory, rain-assisted self-pollination, reproductive assurance

Introduction

Multiple biotic and abiotic factors can impact the pollination process and consequently fecundity of plants. The fitness effects of biotic interactions can vary according to context. For example, florivores (*i.e.* flower herbivores) will usually have a negative effect on plant fecundity by consuming perianth parts (diminishing attractiveness of flowers to pollinators and removing photosynthate structures) or reproductive parts (removing the potential gametes for seed production) (McCall and Irwin 2006). However, there can be “exceptions to the rule” when florivores have positive effects on fitness by facilitating autogamy (Hillier et al. 2018) or acting as a vector of pollen grains (Tan and Tan 2018). It has been suggested that the earliest pollination mutualisms would have involved vectoring of pollen by mandibulate insect florivores feeding on reproductive tissues of plants (Labandeira and Currano 2013). Here we examine a complex interaction between florivores and rainfall that results in increased seed production in an orchid with severely pollen-limited fecundity.

Many tropical epidendroid orchids do not offer floral rewards and experience very low levels of pollen removal and deposition, leading to pollen-limitation of fecundity (Tremblay et al. 2005). As an adaptive response to the low incidence of pollinator visitation, flowers of tropical epidendroid orchids are often extremely long-lived, a feature that has been important for their popularity as horticultural subjects. However, flower longevity can increase the risk of florivory as well as damage from abiotic environmental factors such as rainfall.

The effects of rainfall on the pollination process are generally considered to be negative. Rain can cause nectar dilution, pollen degradation (through osmotic shock) and disrupt pollinator foraging patterns and sensory signals (Lawson and Rands 2019). However, rainfall can also play a role mediating self-pollination (Hagerup 1950). Rain-assisted autogamy has been shown to occur in several orchid species (Catling, 1990). Autogamy is widespread in the Orchidaceae (Catling, 1990) and its frequent evolution may reflect the low degree of herkogamy (*i.e.* anther-stigma separation) in this family. Rain-assisted autogamy in orchids can be achieved in several ways. For instance, in *Cranichis candida*, the viscidium remains in the rostellum, and the raindrops promote self-pollination by dislodging the pollinarium or fragments of the pollinia (Pansarin et al. 2018). In *Liparis loeselii* (Catling, 1980) and *L. kumokiri* (Suetsugu, 2019), the anther cap rises during flower lifespan due to tissue degeneration. This leaves pollen masses exposed and water droplets that shrink by evaporation pull back the pollinia onto the stigma. In *Oeceoclades maculata*, rain removes the anther cap and bends the stipe downwards, bringing the exposed pollinia in contact with the stigma (Aguiar et al. 2012; González-Díaz and Ackerman 1988). The mechanical force of raindrops also removes the anther cap of *Acampe rigida* and causes pollinia to be ejected upwards and, pulled back by the stipe, to fall directly into the stigmatic cavity (Fan et al. 2012). Finally, in the genus *Cyrtopodium*, water droplets accumulate on the stigma and their evaporation draws the pollinaria onto the stigma (Pansarin et al. 2008). This mechanism was described for three species including *C. polyphyllum* (Pansarin et al. 2008), *C. paludicolum* and *C. hatschbachii* (Maciel et al. 2020). For the latter species it was shown that out of 2,528 flowers surveyed, 1.2% developed fruits as a result of bee visits while 0.16% developed fruits through rain-assisted self-pollination (Maciel et al. 2020). Thus rain-assisted autogamy accounted for 13% of all fruit formation in the species, though the viability of selfed seeds was not assessed by Maciel et al (2020). Rain-assisted self-pollination in *Cyrtopodium* differs from the other mentioned examples because the pollinia does not break up and the anther cap typically remains in place (Maciel et al. 2020). What if the anther cap is removed as in the other described systems? Which mechanisms could cause anther cap removal and would this increase the chances of autogamy?

During our studies of rain-assisted fruit production on *C. hatschbachii*, we observed that the long-lived flowers often suffer damage from florivores, particularly from the grasshopper *Stenopola* sp. (Orthoptera, Acrididae, Leptysminae) (Fig. 1a). This insect sometimes destroys the column, but in other cases it eats only the anther cap. We hypothesised that flowers with pollinia exposed to incomplete florivory through removal of the anther cap by grasshoppers would be more likely to mechanically self-pollinate when exposed to rainfall. To investigate this, we tracked flowers in the field. To confirm our prediction, we experimentally tested if the combination of florivory and rainfall would have a positive effect on fruit set. We varied anther cap removal and rainfall in a factorial design, expecting increased fruit set in the group with both anther cap removal and rain. We also tested whether seeds arising from selfing contain viable embryos.

Methods

Studied species and sites

Cyrtopodium hatschbachii occurs at Veredas, a swampy habitat formation in the Brazilian Cerrado savanna biome. Plants used in the greenhouse were transplanted from a population occurring in the Clube Caça e Pesca Itororó de Uberlândia (CCPIU) and then returned to this same location after the experiment. All other data were collected in a population occurring at the margins of the MGC-455 road in Uberlândia municipality, Minas Gerais state, Brazil.

Fig 1 (a) *Stenopola* sp. feeding on reproductive structures of a *Cyrtopodium hatschbachii* flower. (B) Intacted *C. hatschbachii* column showing anther cap (Ca), viscidium (Vi), rostellum (Ro) and the stigma (St). (C) Anther cap manually removed showing the pollinia. (D) Column after rain simulation at time of 0 min. (E) After 20 min showing the water absorption and swelling of pollinia. (F) After 40 min showing the pollinia bending downwards and formation of a water droplet linking it with stigma. (G-H) After rain the droplet starts to shrink and pulls back the pollinia into the stigma. (I) Swollen column after 24h showing that self-fertilization has occurred.

Photos in Figs b–i were taken from the same flower, with non-reproductive whorls removed to improve visualization. Scale bar: 0.5 cm.

Procedures

To test our hypothesis under natural conditions, we censused flowers in the field during September 2018 (n = 100 flowers/14 individuals) and 2019 (n = 203 flowers/22 individuals) classifying then as: (*i*) without florivory, (*ii*) florivory of the column (including the pollinarium), (*iii*) florivory of the pollinarium (including pollinia), (*iv*) florivory resulting in anther cap/anther cap removal. We also recorded the number of pollinia removed (male success) and deposition (female success) by legitimate bee pollinators. Flowers with anther cap removal were followed in 2018 (n = 5) and 2019 (n = 6) to assess if further rain events could lead to self-pollination under natural conditions.

We estimated seed viability of fruits from hand self- (n = 6), hand cross- (n = 8) and open-pollinations (n = 7) using the tetrazolium test by submitting seeds from fresh fruits to 1% 2,3,5 triphenyl tetrazolium chloride solution, wherein developed embryos are stained red and rudimentary ones remain unstained (*sensu* Grabe 1970). We analysed approximately 200 seeds per fruit and classified them as viable (stained) or unviable.

To experimentally test our hypothesis, we took nine *C. hatschbachii* individuals (n = 396 flowers) to the greenhouse. As flowers opened (acropetally), they were assigned to one of the following treatments: (*i*) "Control": flowers were kept intact (n = 90; Fig. 2 a, b); (*ii*) "florivory": anther cap was removed without further manipulation (n = 109); (*iii*) "rain": rainfall was simulated on the flower (n = 100); and (*iv*) "florivory + rain": treatments *ii* and *iii* were applied in sequence (n = 97). According to our hypothesis, we expected that the highest number of fruits would be formed under the "florivory + rain" treatment, followed by "rain" alone, and no fruit formation in the other treatments. We simulated florivory of the anther cap by gently removing it with forceps, without causing any damage to pollinia. We performed artificial rainfall using a pump pressure water sprayer. To simulate natural rainfall as appropriately as possible, we considered several variables. The amount and frequency of water on flowers were calculated based on mean monthly precipitation of the local geographical region (*sensu* Queiroz and Assunção 2013). We took into consideration the flowering period (August – November) and the area of the flower (3.5 cm of diameter). We calculated that every flower would receive 12.33 ml of water. We also calculated the mean angle of rainfall in the flowering period and found it to be 20.25° (*sensu* Assunção et al. 2011). We used a pressure water sprayer (Guarany® PCP-1P-04240000) loaded with distilled water to simulate rain on flowers. As each shoot of the sprayer deposited approximately 1 ml of water on the flower, we performed one shoot every 5 min during 1 h (Fig. 1 b–i). Water was sprayed at 40 cm to ensure that the pollinia movement was not due to the mechanical force of water. We used plastic cones to prevent water falling onto other flowers. Based on the mean interval of rain events of the region for the flowering period (4.3 days; *sensu* Queiroz and Assunção 2013), we simulated rain in treatments *iii* and *iv* every 4 days for flowers that were in anthesis. For each treatment group, we recorded (*i*) if pollinia contacted the stigma and resulted in column swelling but not fruit set, (*ii*) if there was fruit set, and (*iii*) the flower lifespan.

To investigate how the pollinia respond to water, we took pollinia before (hereafter, dry) and after 30 min immersion in distilled water (hereafter, wet) (n = 10 flowers). We measured height and width of the two pollinia structure and height and width of the respective stigma using a digital calliper (Digimess® 100.256; 0.01 mm accuracy). Then we calculated the area of stigmas and dry and wet pollinia based on an ellipse and the volume of dry and wet pollinia based on a prolate spheroid. Dry and wet pollinia were also weighed to the nearest 0.001 mg using a microbalance (Sartorius® CPA2P).

Statistical analyses

We investigated differences in the proportion of viable seeds according to treatments by employing a GLM (Generalized Linear Model) with quasibinomial error distribution to account for overdispersion and logit link function. We created our dependent variable by combining the number of viable seeds relative to the number of unviable using the *cbind* function in the R base package (Crawley 2013; Zuur et al. 2009).

We investigated differences in the probability of (i) pollinia contacting the stigma (and column swelling) and (ii) fruit set according to the distinct treatments by running separate GLMMs (Generalized Linear Mixed-Effects Models) with binomial error distribution using the R-package *lme4* version 1.1-21 (Bates et al. 2019). Plant identity was treated as random effect. Flower lifespan differences were investigated through a GLMM incorporating a Gamma distribution with log link and plant identity as random effect. We performed post hoc analysis using Tukey's multiple comparison test in the package *emmeans* version 1.4.7. (Lenth et al. 2020). Differences between control and hydrated pollinia according to volume and weight were investigated through separate GLMMs with Gaussian distribution and identity link setting flower identity as random effect. We also investigated differences between the areas of stigmas and dry and hydrated pollinia using another Gaussian GLMM with flower identity as random effect. Post-hoc comparisons were conducted using Tukey's test. We accessed the significance of models by using the likelihood ratio test (*i.e.* comparing the null against the full model to attain p-values; Zuur et al. 2009). Analyses were conducted using R software version 3.6.1 (R Core Team 2019).

Results

We found that the 52.1% and 41.8% of flowers suffered florivory in 2018 and 2019 respectively. The most common damage affected the entire column, comprising

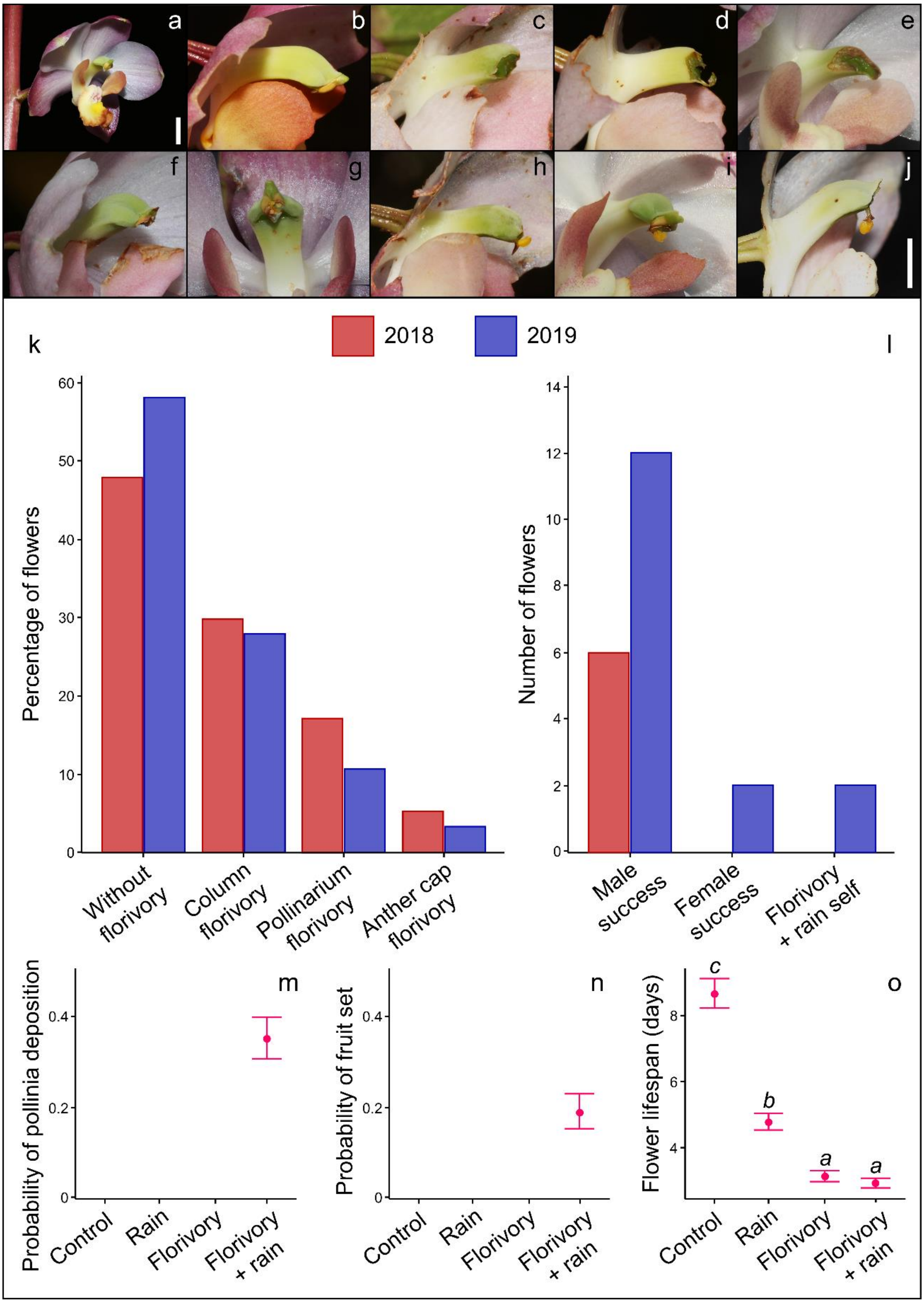


Fig 2 (a) Intact *Cyrtopodium hatschbachii* flower and (b) close up on a column without florivory. (c-j) examples of different florivory patterns: (c-e) florivory on the column (including pollinarium); (f, g) florivory on pollinarium. (h-j) florivory on anther cap which left pollinia exposed, (j) sometimes already oriented downwards. (k) Frequencies of flowers found in the field in 2018 and 2019 including those without florivory and with the distinct florivory patters. Frequencies within each year sum up to 100%. (l) Number of flowers from Fig k that had male and female (*i.e.* fruit set by bees) successes as well as self-pollinia deposition after florivory followed by rain under natural conditions. (m) Probability of pollinia self-deposition, (n) probability of fruit set and (o) flower lifespan according to the distinct treatment groups. Dots and line segments in Fig. m, n and o indicate back-transformed respectively marginal (model adjusted) means and standard errors. Different letters in Fig. o indicate significant differences at 0.05 leve

29.8% and 28.0% of the total number of flowers in 2018 and 2019, respectively (Fig. 2c–e, k). This type of florivory involved complete removal of pollinaria and damage to the stigma. The second most common outcome was florivory of pollinaria (17.0% in 2018 and 10.6% in 2019), which prevented male success (Fig. 2f–g, k). Florivory that affected just the anther cap involved 5.3% and 3.2% of all flowers in 2018 and 2019, respectively (Fig. 2h–k). In 2018, the flowers we surveyed apparently did not experience rain and none of those with anther cap florivory started developing fruits. In this same year, no fruit set resulted from bee visits while pollinia removal occurred in only 6.4% of flowers, all of them in flowers without florivory (Fig. 2l). In 2019, however, rainfall did occur and two out of six flowers with anther cap removal had self pollinia deposited onto their stigmas (Fig. 2l), and afterwards one of them showed swelling of the ovary typical of fruit development. There were two additional fruits (1.1% of flowers in the population) that arose from bee visits in 2019 and 12 flowers (6.3%) had pollinaria removed (Fig. 2l). We did not find significant differences among treatments in seed viability ($\chi^2 = 3.51$; $p = 0.173$). Viability of seeds in selfed fruits was high (mean ± SD: 52.7% ± 16.3), and similar to that in hand cross-pollinated (68.8% ± 23.5) and open-pollinated fruits (74.1% ± 16.8).

In our experiment that artificially assessed the effects of florivory and rain, we found significant effects of treatment type on floral longevity ($\chi^2 = 457.57$; $p < 0.001$; Fig. 2o). Control flowers had the highest lifespan (8.7 ± 2.4 days) followed by those under "rain" (4.8 ± 1.5), "florivory" (3.2 ± 1.2) and "florivory+rain" (2.9 ± 0.9), which were not significantly different from each other. "Florivory+rain" was the only treatment group wherein pollinia contacted the stigma and the ovary swelled indicating fruit-set potential (Fig. 1b–i), occurring in 35% of a total of 97 flowers ($\chi^2 = 106.27$; $p < 0.001$; Fig. 2m). Fruit formation was observed only in this treatment, occurring in 19% of flowers ($\chi^2 = 53.38$; $p < 0.001$; Fig. 2n). When we artificially removed the anther cap (as in Fig. 1b, c; Fig. 2h–j), pollinia absorbed water and swelled (Fig. 1d, e). Wet pollinia (3.4 ± 0.8 mm^3) were, on average, 61.8% larger in volume when compared to dry pollinia (1.3 ± 0.5) ($\chi^2 = 34.6$; $p < 0.001$). In addition to volume, the wet pollinia (2.09 ± 0.20 mg) were 69.4% heavier than dry pollinia (0.64 ± 0.13) ($\chi^2 = 61.6$; $p < 0.001$). The downward bending of the wet pollinia is likely related to the increase in their weight (Fig. 1e, f). After rainfall, a water droplet remains and links the pollinia with the stigmatic surface (Fig. 1f). When the droplet shrank due to evaporation, the pollinia was drawn towards the stigma, by-passing the rostellum and promoting self-pollination (Fig. 1g–i). This was possible because even though bulkier, the wet pollinia still fit inside the stigma. The area of the stigma (21.6 ± 2.1 mm^2) was respectively 43.1% and 73.1% larger than that of wet (12.3 ± 1.6) and dry pollinia (5.8 ± 1.4) ($\chi^2 = 89.26$; $p < 0.001$).

Discussion

Previous studies have suggested that orthopteran florivores can lead to fruit set even in self-incompatible plants with generalist morphology due to their movement in the flowers while feeding on powdery (granular) pollen (see Tan and Tan 2018). In fact, orthopterans may be legitimate pollinators in such cases where they effectively promote crossing, as in the orchid Angraecum cadetii (Micheneau et al. 2010), highlighting the negative-positive continuum of interactions between plants and florivores. In the bee-specialized orchid *C. hatschbachii*, opportunistic feeding by Orthoptera appear to have multiple effects. From the results reported here, we infer that florivory in combination with rainfall can lead to fruit set in *C. hatschbachii,* even although the overall effect of florivory is probably negative for the reproduction of this species. We show that florivory can completely impair female and/or male success when it affects the stigma or pollinarium, or reduce flower lifespan when it affects the anther cap. However, cap removal still preserves pollinia and stigmas, and although the florivore itself does not directly deposit pollinia onto stigmas, it increases the possibility of rain-assisted self-pollination.

Rain has complex effects for *C. hatschbachii*, reducing flowers lifespan and therefore the likelihood of future bee visits, but also plays a role in the process of autogamy. Since rain reduces foraging activity of pollinators and therefore the visitation chances (Hagerup 1950; Lawson and Rands 2019), the possibility of rain-assisted self-pollination provides mating system flexibility. This occurs because flowers are available to pollinators during rainless periods while they also have the chance of selfing during the rainy periods when conditions are unfavorable for crossing (*e.g.* Fan et al. 2012). When rain reaches *C. hatschbachii* pollinia in flowers not without an anther cap, the chances of self-deposition of pollinia are high (35%), and most of these flowers will successfully develop fruits. The formation of a water droplet and the selfing process is similar in flowers that still have the anther cap (*sensu* Maciel et al. 2020), but the difference is that the pollinia bends down much more easily without anther cap support, thus facilitating final self-deposition.

The mechanics of the pollinia movement is possible due to the sophisticated orchid pollinarium, comprised of the pollinia and accessory structures. The viscidium, the sticky and viscous structure which adheres the pollinia to the pollinators' body (Johnson and Edwards 2000), is diffuse and hydrophilic in *Cyrtopodium* (Mosquera-Mosquera et al. 2019). The viscidium becomes amalgamated with the stigma, serving as support for the pollinia as they bend down. The tegular stipe (Mosquera-Mosquera et al. 2019) keeps the pollinia hanging on the viscidium. Finally, the hard pollinia type of *C. hatschbachii* (Mosquera-Mosquera et al. 2019) has elastoviscin, a sticky gumlike substance (Schill and Wolter 1986), which provides cohesion and allows it to get wet without liquefying or breaking apart. These traits, together with the low degree of herkogamy, make the swinging movement of the pollinia and hence self-pollination possible.

It is known that deceptive orchids tend to have lower fruit set rates than do rewarding ones. Tremblay et al. (2005) calculated the average of fruit set for several orchid species and found that deceptive species have only half the success of rewarding species (20.7% *vs.* 37.1%). However, some deceptive tropical species, such as *C. hatschbachii*, show extremely low rates of fruit formation. In terms of bee-mediated fruit formation, Maciel et al. (2020) found rates of 1.21% in 2011 and 1.23% in 2012, while we recorded rates of 0% in 2018 and 1.06% in 2019. This highlights the uncertainty of fruit set for this species and how it is variable over time. Thus, any additional fruit is advantageous, even when arising from autogamy mediated by florivory and rain. Although most orchids are self-compatible, seed viability under self pollination is, on average, typically reduced (ca. 40.8%) when compared to cross (ca. 64.8%) (Tremblay et al. 2005; see also Jersáková et al. 2006). However, we found similar seed viability between self-and cross-pollinated fruits. The reasonably high seed viability in selfed fruits (52.7%) indicates that more than half of the seeds from a selfed fruit still have viable embryos that potentially can germinate and constitute seedlings.

One of the most important benefits of self-fertilisation is that it largely guarantees zygote formation. Such reproductive assurance is specially valuable in populations in which plants mostly or entirely fail to export and receive pollen, as in cases where little or no pollinator service is available (Cutter et al. 2019). Rain-assisted self-pollination may be a mechanism of reproductive assurance in these cases (Hagerup 1950; Catling, 1990; Fan et al. 2012). Although rare, florivory of the anther cap followed by rain-assisted self-pollination accounted for one third of all fruit set in the *C. hatschbachii* population in 2019. It has been suggested that orchids are specialized for episodic pollination events when it comes to fruit formation. Instead of maximizing pollinator attraction, they maximize seed production from every eventual fruit that is produced (Pérez-Hérnandez et al. 2011). Our results confirm that rare and episodic events of selfing may make an important contribution to seed production dynamics and eventually seedling recruitment in orchid populations. This is especially important when the size of the fruit is considered. One strategy that may compensate such lower fruit set rates of deceptive orchids is the production of fruits with higher seed numbers when compared to the rewarding counterparts (Sonkoly et al. 2016). In agreement, *Cyrtopodium hatschbachii* fruits are very large, probably having tens or hundreds of thousands of seeds. Thus, the considerable seed viability of large selfed fruits may allow population persistence as bee-mediated cross-pollination is uncertain and even absent in some years in this threatened plant species. This study thus provides a unique example of how two unrelated factors (*i.e.* florivory and rain) that are detrimental alone may together facilitate fruit-set in a plant species.

Acknowledgements

We are grateful to Hamilton Kikuti for allowing us to use the greenhouse, to Kátia Matiotti for the identification of the florivore, to Ana Silvia F. P. Moreira for lending us the microbalance, to Uiara C. Rezende for assistance in the greenhouse and Bruno Mamede and Vitor Mamede for assistance in the field.

References

Aguiar JMRBV, Pansarin LM, Ackerman JD, Pansarin ER (2012) Biotic versus abiotic pollination in *Oeceoclades maculata* (Lindl.) Lindl. (Orchidaceae). Plant Spec Biol 27:86-95. https://doi.org/10.1111/j.1442-1984.2011.00330.x

Assunção HF, Scopel I, Moragas WM, Fátima Z (2014) Uso de pluviômetros vetoriais para estimar a energia cinética das chuvas. Available at: http://www.simehgo.sectec.go.gov.br/downloads/publicacoes/artigos/004.pdf. Accessed 06 August 2014

Bates D, Maechler M, Bolker B, Walker S, Christensen RHB, Singmann H, Dai B, Scheipl F, Grothendieck G, Green P (2019) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-21. https://CRAN.R-project.org/package=lme4

Catling PM (1980) Rain-assisted autogamy in *Liparis loeselii* (L.) LC rich. (Orchidaceae). B Torrey Bot Club 107:525-529. https://doi.org/10.2307/2484083

Catling PM (1990) Auto-pollination in the Orchidaceae. In: Arditti J (ed) Orchid biology: reviews and perspectives. Timber Press, Oregon, 121–158.

Crawley MJ (2013) The R book. Chichester: John Wiley & Sons, Chichester

Cutter AD (2019) Reproductive transitions in plants and animals: selfing syndrome, sexual selection and speciation. New Phytol 224:1080-1094. https://doi.org/10.1111/nph.16075

Fan XL, Barrett SCH, Lin H, Chen LL, Zhou X, Gao JY (2012) Rain pollination provides reproductive assurance in a deceptive orchid. Ann Bot 110:953-958. https://doi.org/10.1093/aob/mcs165

González-Díaz N, Ackerman JD (1988) Pollination, fruit set, and seed production in the orchid, *Oeceoclades maculata*. Lindleyana 3:150-155.

Grabe DF (1970) Tetrazolium testing handbook for agricultural seeds. Handbook on seed testing, Association of Official Seed Analysts.

Hagerup O (1950) Rain-pollination. I kommission hos E. Munksgaard, Copenhagen

Hillier NK, Evans E, Evans RC (2018) Novel insect florivory strategy initiates autogamy in unopened allogamous flowers. Sci Rep 8:17077. https://doi.org/10.1038/s41598-018-35191-z

Jersáková J, Johnson SD, Kindlmann P (2006) Mechanisms and evolution of deceptive pollination in orchids. Biol Rev 81:219-235. https://doi.org/10.1017/S1464793105006986

Johnson SD, Edwards TJ (2000) The structure and function of orchid pollinaria. Plant Syst Evol 222:243-269. https://doi.org/10.1007/BF00984105

Labandeira CC, Currano ED (2013) The fossil record of plant-insect dynamics. Annu Rev Earth Pl Sc 41:287-311. https://doi.org/10.1146/annurev-earth-050212-124139

Lawson DA, Rands SA (2019) The effects of rainfall on plant–pollinator interactions. Arthropod-Plant Inte 13:561-569. https://doi.org/10.1007/s11829-019-09686-z

Lenth R, Singmann H, Love J, Buerkner P, Herve M (2020) emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.4.7. https://cran.r-project.org/package=emmeans

Maciel AA, Cardoso JC, Oliveira PE (2020) On the low reproductive success of two *Cyrtopodium* species (Orchidaceae: Cyrtopodiinae): The relative roles of biotic and abiotic pollination. Plant Spec Biol 35:49-58. https://doi.org/10.1111/1442-1984.12260

McCall AC, Irwin RE (2006) Florivory: the intersection of pollination and herbivory. Ecol Lett 9:1351-1365. https://doi.org/10.1111/j.1461-0248.2006.00975.x

Micheneau C, Fournel J, Warren BH, Hugel S, Gauvin-Bialecki A, Pailler T, Strasberg D, Chase MW (2010). Orthoptera, a new order of pollinator. Ann Bot 105:355-364. https://doi.org/10.1093/aob/mcp299

Mosquera-Mosquera HR, Valencia-Barrera RM, Acedo C (2019) Variation and evolutionary transformation of some characters of the pollinarium and pistil in Epidendroideae (Orchidaceae). Plant Syst Evol 305:353–374. https://doi.org/10.1007/s00606-019-01575-5

Pansarin ER, Bergamo PJ, Ferreira-Caliman MJ (2018) Pollinator-independent orchid attracts biotic pollinators due the production of lipoidal substances. Plant Biol 20:182-190. https://doi.org/10.1111/plb.12650

Pansarin LM, Pansarin ER, Sazima M (2008) Facultative autogamy in *Cyrtopodium polyphyllum* (Orchidaceae) through a rain-assisted pollination mechanism. Aust J Bot 56:363-367. https://doi.org/10.1071/BT07135

Pérez-Hérnandez H, Damon A, Valle-Mora J, Sanchez-Guillen D (2011) Orchid pollination: specialization in chance? Bot J Linn Soc 165:251–266. https://doi.org/10.1111/j.1095-8339.2010.01109.x

Queiroz AT, Assunção WL (2013) Análise geoestatística das precipitações na Fazenda Letreiro (Uberlândia-MG). Braz Geogr J 4:448-476.

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Schill R, Wolter M (1986) On the presence of elastoviscin in all subfamilies of the Orchidaceae and the homology to pollenkitt. Nord J Bot 6:321–324. https://doi.org/10.1111/j.1756-1051.1986.tb00885.x

Sonkoly J, Vojtkó AE, Tökölyi J, Török P, Sramkó G, Illyés Z, Molnár AV (2016) Higher seed number compensates for lower fruit set in deceptive orchids. J Ecol 104:343-351. https://doi.org/10.1111/1365-2745.12511

Suetsugu K (2019) Rain-triggered self-pollination in *Liparis kumokiri*, an orchid that blooms during the rainy reason. Ecology 100:e02683. https://doi.org/10.1002/ecy.2683

Tan MK, Tan HT (2018) A gentle floriphilic katydid *Phaneroptera brevis* can help with the pollination of *Bidens pilosa*. Ecology 99:2125-2127. https://doi.org/10.1002/ecy.2369

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: A spasmodic journey to diversification. Biol J Linn Soc 84:1-54. https://doi.org/10.1111/j.1095-8312.2004.00400.x

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) Mixed effects models and extensions in ecology with R. Springer, New York. https://doi.org/10.1007/978-0-387-87458-6

Capítulo 2

The lady's "slippery" orchid: Flowers of a Cypripedioideae reveal adaptations of semi-trap flowers

Abstract

Trap flowers are fascinating cases of adaptation. Some are called semi-traps as they force the pollinator to move actively through a pre-determined path, manipulating its movements in a way that culminates in pollen transfer as it leaves through a secondary opening. We used the lady's slipper orchid *Phragmipedium vittatum* as model to investigate the micro-morphological traits employed by semi-trap flowers that allow their complex pollination, an issue still poorly understood. We showed that the flower is rewardless, non-spontaneous selfer, and dependent on biotic pollination, performed by females of two hoverfly species. We divided the pollination mechanism in three phases, allowing to better depict which traits work at each moment. In the first phase (attraction and trapping), dark and high elevated aphid-like spots attract the attention of hoverflies to a slipping zone. This region has downward projecting papillate cells and mucilage secretion that promote slipperiness, causing pollinator to fall into the labellum. In the second phase (intrafloral movements), pollinator must perform a specific rout. As it goes up, a lateral constriction of the labellum funnels the passage to the central region. It holds on upward oriented hairs progressively longer in size that aid its grip. Inner aphid-like spots signalize the rout. As pollinator climbs, it passes the stigma depositing pollen and later by one of the articulated anthers where pollen smears are placed onto its back. In the last phase (exit), pollinator leaves by one of the lateral tight holes by holding on hairs projecting from the petals. Eggs found on both inner and outer aphid-like spots confirm an oviposition-site mimicry, indicating that the same exploitation mechanism is also used after insect trapping. We provide new highlights into the sophisticated micro-morphological traits used by trap flowers, allowing pollinator attraction and guidance and surface slipperiness and adhesiveness, promoting precise pollen transfer.

Keywords: trap flowers, floral mimicry, floral traits, Cypripedioideae, Syrphidae.

Introduction

"The labellum thus acts like one of those conical traps with the edges turned inwards"
Darwin (1862) when referring to a lady's slipper orchid

Plants and pollinators carry out a mutualistic relationship where flowers get pollen transport whilst pollinators gain resource (Ollerton, 2021). Some plants are dishonest, bearing their rewardless flowers that allow exploiting pollinator's service without rewarding them (Johnson and Schiestl 2016; Ollerton, 2021). However, some flowers exploit pollinators in a further level of cheating. This is the case of trap flowers, that imprison pollinators to acquire and export pollen, causing them to spend time and energy, and often not rewarding or even killing them (Faegri and Van der Pijl 1979; Endress, 1996; Richards, 1997; Johnson and Schiestl 2016; *e.g.*, Vogel and Martens 2000). The trapping strategy was first reported by Sprengel in his famous book published in 1793 (Faegri and Van der Pijl 1979) and are among the most sophisticated and fascinating mechanisms that evolved under the pressure for effective pollination (Bröderbauer et al. 2012). The key innovation of trap flowers is the architecture, commonly a chamber wherein pollinators enter actively or passively, remaining for a shorter or longer time, and where the insects contact the reproductive parts hidden inside (Faegri and Van der Pijl 1979; Endress 1996; Bröderbauer et al. 2012; Johnson and Schiestl 2016). Although the trapping strategy across several groups is related to similar traits, it is homoplasic (Endress 1996; Johnson and Schiestl 2016). For instance, only within Araceae, trap flowers evolved independently at least ten times (Bröderbauer et al. 2013). In addition to this later, trap flowers are widely distributed in several angiosperm families including Annonaceae, Apocynaceae, Aristolochiaceae, Calycanthaceae, Hydnoraceae, Malvaceae, Nymphaeaceae, Orchidaceae, Rafflesiaceae, Solanaceae and Taccaceae (Faegri and Van der Pijl 1979, Endress 1996; Richards 1997; Johnson and Schiestl 2016).

To attract pollinators into the trap, flowers use a complex combination of colors and scents, some of them even using thermogenesis that potentiates the emission of compounds (Faegri and Van der Pijl 1979, Richards 1997; Johnson and Schiestl 2016). However, none of these traits would work without the trap *per se*. Trap flowers are often considered as having bizarre shapes (Richards 1997). Such morphology is the responsible for the capture, manipulation, and release of pollinators, ensuring pollen reception and exportation. When compared to their pollinators, trap flowers are large. For instance, some of the largest flowers in the world such as *Aristolochia* and *Rafflesia* are trap flowers (Endress 1996). However, the reproductive structures *per se* are relatively small and most of the size is formed by the perianth (in general) modified into trap (Endress 1996). In addition to size, this structure has sophisticated traits that ensure a series of processes, from capture until exit of pollinators.

Some trap flowers are called "perfect traps" as they retain pollinators for longer and well determined periods, and usually the same entrance is also the exit (Faegri and Van der Pijl 1979). For instance, they occur commonly in the Annonaceae, Apocynaceae, Aristolochiaceae, and Araceae, which have protogynous trap flowers/inflorescences that first receive exogenous pollen onto stigmas when pollinators arrive and latter coat these with pollen before releasing them (Endress 1996; Richards 1997; Bröderbauer et al. 2012; Faegri and Van der Pijl 1979; Johnson and Schiestl 2016). In general, pollinators remain in the chamber for one or more days until the plant switches the sex phases and have their escape usually facilitated by the wilting and relaxing of the trap structures (Vogel 1965). Thus, another aspect of perfect traps is that pollinators have a passive behavior as their movement after trapped is ethologically irrelevant (Faegri and Van der Pijl 1979).

Other trap flowers are so-called semi-traps (or imperfect traps), as they do not impose pollinator imprisonment for a predetermined time interval, but rather force it to be more active and to take specific pre-determined movements (Faegri and Van der Pijl 1979; Johnson and Schiestl 2016). Most semi-traps conduct the pollinator through a specific route that will culminate in pollen deposition and then removal as it manages to leave the flower using an exit formed by another opening (Johnson and Schiestl 2016). Thus, such semi-traps involve specific narrow regions that restrict pollinator size, direction, and positioning, allowing precise and accurate pollen transfer.

Describing in detail, the pollination cycle of a semi-trap flower can be divided into three predetermined phases (Fig 1). In the first one, pollinator must enter the flower. For this, several traits work together attracting its attention including visual, chemical, and tactile traits. After it lands, it must be trapped into the chamber and this happens because the organ where pollinator lands turns inside, or more commonly because it simply falls (Faegri and Van der Pijl, 1979). For such kettle (pitfall) traps to work, the entry surface may be slippery (Poppinga et al. 2010; Johnson and Schiestl 2016). When inside the flower, the pollinator is not able to get out by the same entry hole because of the slipperiness of this region. After trapped, the second phase consists in guiding the pollinator through a path that leads to the reproductive organs and them to the exit. In the relatively big chambers of semi-trap flowers, pollinator can potentially go to many directions and position the body in many ways. Thus, it may be guided through an intricate internal arrangement of organs in such a way that ensures precise and accurate pollen transference. To avoid selfing, pollen deposition usually takes place first followed by pollen removal. Finally, the third phase consists in pollinator leaving the flowers. Although it appears simple, it involves tight holes where the pollinator must squeeze itself and may even die if does not leave (*e.g.*, Edens-Meier et al. 2011). To ensure pollen flow, semi-trap flowers constantly interact with their pollinators during the whole cycle, exploiting them specifically on each of the phase. To do so, a series of complex flower traits may have evolved under the pressure to increase fecundity mediated by pollinator morphology and their innate sensorial systems.

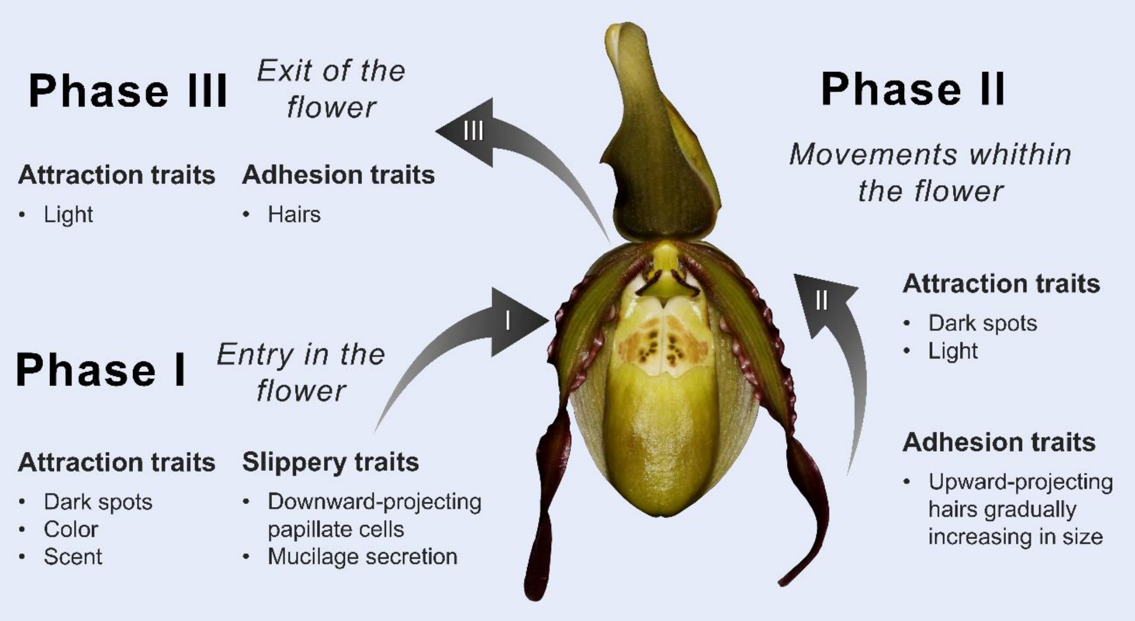


Figure 1. Phases of a trap-flower pollination having *P. vittatum* as example highlighting the respective traits that mediate pollination.

Semi-trap flowers employing the three-phase strategy are common in some families, such as the Orchidaceae, where they are present in different and unrelated groups. However, in no other group it is so widespread as in the Cypripedioideae subfamily. In fact, the modified pouch-like labellum is a synapomorphic trait of the group (Dressler 1981). It was lost only in a single species, the lipless *Phragmipedium lindenii* that has a third/middle anther that promotes selfing (Pemberton 2013). Although some other few cases of autonomous selfing have been described, the labellum plays a fundamental role trapping pollinators and assuring cross pollination in most species (Pemberton 2013; Edens-Meier et al. 2014). These orchids are primarily rewardless (but see Jiang et al. 2020), employing different kinds of mimicry (Pemberton 2013; Edens-

Meier et al. 2014). Although some knowledge has been gathered in the reproductive biology and the identity of pollinators, the proximal mechanisms that boost the pollinator-mediated pollen flow remain elusive. These include specially the morphological traits that allow the pollination cycle in the flower, including all three phases. For instance, we still do not know which micro-morphological mechanisms cause slipperiness and what guides pollinators within the flower and in their way out.

As a first step towards filling these knowledge gaps, we investigated the reproductive biology and the complex and well-articulated pollination mechanism of the rare and vulnerable *Phragmipedium vittatum*. We used this model species to address the following questions: what is its mating system and how is the plant dependent on the pollinators?; what is the effectiveness of this pollination mechanism?; and which are the micro-morphological adaptations that allow the mechanics of the pollination to work? We used field experimentation and observations and applied anatomy and histochemistry techniques that revealed some previously unknow traits related to pollinator trapping, prisoning, guiding of its movement within the flower and exit, providing new insights in the functional mechanics of semi-trap flowers.

Materials and Methods

Study species and site

Phragmipedium vittatum (Vell.) Rolfe (Cypripedioideae) is a paludal herb that occurs mainly in Veredas (occasionally on forests), plant formations of the Brazilian Cerrado biome, characterized by a dense herb-subshrub dominant layer, with scattered small trees and frequently (but not obligatorily) the buriti palm (*Mauritia flexuosa* L. f.) (Fig 1, Fig 2, Fig4a) (Araújo et al. 2002). It is a rare lady's slipper species in vulnerable state of extinction (Dias and Smidt 2020). We studied a population located on a private farm in the Goiás state, Brazil, during December 2017 – January 2021. The region has a seasonal climate (AW in the Köppen-Geiger classification), with a rainy summer and a dry winter (Alvares et al. 2013).

Figure 2. *Phragmipedium vittatum* on a vereda swamp.

Histological and histochemical analysis

We visually inspected opened flowers searching for any floral reward. We investigated the presence of secretory tissues immersing fresh flowers in 0.1 % ($^{w}/_{v}$) aqueous neutral red for 20 min (Vogel 1962, time adapted). To detect mucilage location, we immersed the fresh flowers in ruthenium red for 5 min (Gregory and Baas 1989, time adapted). We submitted longitudinal handmade cuts from flower samples to histochemical tests to detect chemical compounds related to pollination process. To investigate the presence of nectar, tests were carried out for reducing sugars (glucose, fructose) with Fehling reagent (Purvis et al. 1964), and starch grains with Lugol (Johansen

1940). We used Periodic acid-Schiff's (PAS) to test for the presence of total insoluble carbohydrates (Johansen 1940), Sudan red for total lipids (Sass 1951), Bromofenol blue for total proteins (Johansen 1940) and NADI reagent for terpenoids (David and Carde 1964). For all histochemical tests, appropriate controls were run simultaneously. Light microscopy observations were carried out using a coupled Uphoto system (Leica® ICC50HD).

For histological studies we made free-hand sections of sepals, labella, and other petals, clarified in sodium hypochlorite (Johansen 1940), and stained with Safranin and Astra blue (Gerlach 1984). Flowers were also submitted to scanning electron microscopy (SEM). The alcohol-fixed parts were dehydrated in an ethanol series, critical point-dried (using Leica® CPD 300) and them mounted on aluminium stubs with conductive adhesive. Samples were coated with gold in a sputter coater (Leica® EM SCD050) and photographed using a scanning electron microscope (Zeiss® EVO MA10) at 5 kV.

Mating system

The mating system (*sensu* Cardoso et al. 2018) was investigated in December 2020 by submitting newly opened flowers of previously bagged buds from different individuals to one the following hand-pollination treatments (*sensu* Kearns and Inouye 1993): 1) cross-pollination: the pollen smears of one anther were inserted in the stigma of other flower (n = 17); 2) self-pollination: the pollen smears of one anther were inserted into the stigma of the same flower (n = 13); 3) spontaneous self-pollination: buds were only bagged, without any manipulation (n = 21); 4) emasculation: the pollen smears of both anthers were removed, without posterior treatment (n = 17). We used "pollen smears" instead of pollinia throughout the manuscript because, technically, there is no pollinarium in the Cypripedioideae since pollen is loosely held together and the pollen mass is not attached to a viscidium (Dressler 1981). We also investigated the reproductive success of flowers from open pollination by inspecting fruit formation on 72 flowers from 22 individuals. The self-incompatibility index was determined by the ratio between the percentages of self- and cross-pollinated fruits, assuming that *P. vittatum* is self-incompatible if the obtained value is lower than 0.2 (ISI *sensu* Zapata and Arroyo 1978). Reproductive efficacy index was investigated by dividing percentages of open pollination by those of manual cross-pollination, in which values below 0.2 indicate pollen limitation (RE *sensu* Zapata and Arroyo 1978).

Seed analysis

We took ripe fruits from the different treatments (8 self-pollinated, 10 cross-pollinated and 9 open-pollinated) just before the capsule opening process (April 2017). Fruits were placed in separated vials until they fully opened. We submitted 500 fresh seeds per fruit to the tetrazolium test to investigate seed viability (*sensu* Grabe 1970). All the seeds of a fruit were separated (including those used in viability analysis and those remaining in the capsule), then immersed in a 50 ml solution containing $^4/_5$ ethanol 70 % and $^1/_5$ glicerine. This solution provides a homogeneous distribution of the seeds after mixed. Three aliquots of 0.25 ml were taken per fruit using a volumetric pipette and the number of seeds counted in microscope (mean value extracted). Afterwards, the proportion to the total volume was made to estimate the total number of seeds per fruit.

Pollinator observation

During 2018 and 2019, we performed 56 observation hours between 8 - 12h and 14 - 18h (totaling seven days). Observations were conducted by the same person, at approximately 1.5 m from a given patch containing approximately five open flowers. We them recorded pollinator visits, their frequency, behavior and if they were carrying pollen smears. Due to the low frequency of pollinator visits found through the direct observation method, in 2021, we conducted an experiment to assess flower visitation rates on a larger number of flowers. For two days, we stuffed cotton into both exiting holes of previously bagged plants, which prevented that any trapped pollinator would scape by the exiting holes. We unbagged flowers at 6AM and investigated the presence of any pollinator recording its identity every three hours until 18PM. Although some flowers detached throughout the day, we reached the final of the first day with 47 flowers and the second day with 42.

Pollinator effectiveness

To estimate the effectiveness of pollinators on male and female successes, we inspected flowers during 2016 - 2017 (n = 306 flowers), 2017 - 2018 (n = 161), 2018 - 2019 (n = 301) and 2020 - 2021 (n = 67) flowering seasons. We selected old flowers that were near to detach the stem or that were already detached. The pollen removal (male reproductive success) was inferred as one or two anther pollen removal. The presence/absence of female reproductive success was inferred by inspecting the deposition of pollen smears on stigma. In addition, we also calculated the reproductive efficacy index based on female success for the aforementioned flowering seasons by dividing pollen deposition percentages by those of manual cross-pollination (adapted from Zapata and Arroyo 1978).

Statistical analysis

To determine the mating system of *P. vittatum*, we compared the proportion of fruit set among those treatments that successfully set at least one fruit (emasculation and control excluded) through a GLM (generalized linear model) with binomial error distribution and logit link function. As response variable, we took the number of flowers that developed into fruits relative to the number of flowers that did not per individual using the *cbind* function in R software base package (Crawley 2013; Zuur et al. 2009). We used this same procedure to investigate differences related to seed viability according to treatments that formed fruits: we took the number of viable seeds relative to the number of unviable per fruit (*i.e.*, the proportion of viability) as dependent variable and fitted a binomial GLM with logit link. Differences in the number of seeds among treatment levels were investigated by fitting a GLM with gaussian distribution and identity link.

We compared if the presence of reproductive success on flowers differed according to sex (male *vs.* female), flowering season and the interaction term by fitting a binomial GLMM (generalized linear mixed-effects model) having flower identity as random effect. For the presence of male reproductive success, we grouped flowers with one and two anther pollen removal.

GLMs were built in R base package while the GLMM in the R-package *lme4* version 1.1.23 (Bates et al. 2015). Models' significance was tested using type II tests using the R-package *car* version 3.0.10 (Fox and Weisberg 2019). Whenever we had a significant predictor with more than two levels, *post hoc* analyses were performed using the Tukey multiple comparison test in the R-package *multcomp* version 1.4.13 (Hothorn et al. 2008). All analyses were carried out in R version 4.0.2 (R Core Team 2020).

Results

General observations

Phragmipedium vittatum flower once a year, between October until May, but peaking in December - February. Flowers open acropetally, with a mean number of 6.86

(± 2.22 SD) flowers per individual and a mean inflorescence height of 74.79 cm (± 16.87) (n = 100). Virgin/bagged flowers lasted an average of 9.46 days (± 0.86) (n = 26). After approximately six days, flowers become old and later detach from the stem. After senescence and detachment of a flower, another opens, and usually a single flower is opened at a time per inflorescence. Average flower size, measured as the longitudinal size of the labellum, was 3.87 cm (± 1.69; n = 507). Labellum color ranges from greenish, through yellowish until brownish, with wavy lateral petals bordered by dark reddish brown at the base and fully colored at the tip. Flowers were rewardless and odor to human nose was not found.

Mating system and seed analysis

No fruits were formed after spontaneous self-pollination or emasculation. *Phragmipedium vittatum* was self-compatible, presenting an ISI of 0.96 (< 0.2). However, there were differences among the treatments that set fruits ($\chi^2 = 27.56$, df = 2, $p < 0.001$), with open-pollination showing smaller fruit set proportions when compared to cross- and self-pollinated flowers, which were not significantly different from each other (Table 1). We did not find pollen limitation in 2021 since the population had a reproductive efficacy index of 0.36 (< 0.2).

Table 1. Results concerning the three treatments that set fruits in *Phragmipedium vittatum*. Fruit formation indicates number of fruits developed per number of flowers observed (percentage in parenthesis). Number of seeds and seed viability proportion indicate mean ± SD values. Distinct letters indicate statistically clear differences at 0.05 level.

	Self-pollination	Cross-pollination	Open-pollination
Fruit formation	11/13 (84.62 %)^b	15/17 (88.24 %)^b	23/73 (31.51 %)^a
Seed number	50825 ± 17212^a	74423 ± 13445^b	61687 ± 15283^{a,b}
Seed viability	0.12 ± 0.12^a	0.32 ± 0.20^b	0.61 ± 0.11^c

Seed number estimates varied widely, ranging between 25650 and 101460. We found significant differences in the number of seeds across pollination treatments (χ^2 = 10.46, p = 0.005; Table 1). Cross-pollinated fruits had, on average, 54 % more seeds than self-pollinated fruits. However, there were not differences between open-pollination and the two other hand-pollination treatments. We also found significant differences in seed viability (χ^2 = 2252.3, df = 2, p < 0.001; Table 1). All groups were different from each other. On average, the number of viable seeds from cross-pollination fruits was approximately twice that of self-pollination, and the number of viable seeds of open-pollination was around twice that of cross-pollination.

Pollinators and their interplay with floral morphology

During our systematic pollinator observation, we found that *P. vittatum* was pollinated by two species of female hoverflies (Diptera: Syrphidae): *Allograpta exotica* (Wiedemann, 1830) (Fig 3a-j) and *Dioprosopa clavata* (Fabricius, 1794) (Fig 3k-n). In our direct observation, we found 20 approaches from *A. exotica*. They only hovered in front of the flower seven times (35 %) and landed in one of the whorls 13 times (65 %), never falling into the pouch. Three of these flies (15 %) had pollen smears attached to their back. Concerning *D. clavata*, we found three approaches. In one of them, it only hovered while in the other two they did fall and were trapped within the pouch. None of them had pollen smears attached.

Later, during our exiting hole blocking experiment, we found a total of 22 individuals trapped. 13.64 % of these were captured between 6AM – 9AM, 54.54 % between 9AM – 12PM, 22.73 % between 12PM – 15PM and 9.09 % between 15PM – 18PM. Except for one *Allograpta exotica* found between the 9AM – 12PM period, all remaining individuals were *D. clavata* and pollen smears were present on the back of only one of these.

We found that as pollinators walk on flowers, they make abdomen movements typical to oviposition behavior. *Allograpta exotica* usually first hovers in front of flowers, sometimes landing on sepals and lateral petals (Fig 3a). Then, it lands on the labellum border and starts to move through the opening hole towards the region of the infolded lobes (Fig 3b-e). When compared to *D. clavata* behavior, it generally lands quicker and directly on this area.

This region where the two infolded lobes of labellum are welded forms a vertical slipping zone with the presence of characteristic dark spots. Through SEM technique it was possible to identify that this region has downward projecting papillate cells displayed in rows, forming a ridged micropattern (Fig 4b,c). In the lower portions where labellum folds, cells become convex-shaped and arranged in a homogeneous pattern (Fig 4d). Anatomically, it is possible to have a different view and notice the shape differences in the epidermal cells of the slipping zone, forming a gradient relief between the dark spots and the other cells surrounding them (Fig 4g). Such dark spots are formed by cells that group together and besides having different coloration, are taller than the surrounding cells and digitiform, forming high relief punctuations (Fig 4e-g). At this same frontal region, we found a positive reaction with the neutral red, indicating high metabolic activity (Fig 4l). We also found a reaction with ruthenium red, indicating the presence of mucilage (Fig 4m). We then confirm that mucilage is produced and secreted by the epidermis cells as signalized by the positive reaction with Periodic acid-Schiff's (Fig 4n). As the pollinator reaches this slippery region, probably attracted by the dark spots, it loses hold and falls into the labellum pouch (Fig 3e,f). Both the orientation of downward projecting papillate cells of epidermis and mucilage secretion probably increase slipperiness in a way that the animal cannot hold on and slide down.

Figure 3. Pollination process of *Phragmipedium vittatum*. (a-d) *Allograpta exotica* walking through the flower until it (e) reaches the slipping zone and (f) falls in the labbelum pouch. (g) *Allograpta exotica* trying to get out by the pouch entrance and (h-i) effectively leaving by one of the exit holes (pollen removed by a previous pollinator visit). (j) *Allograpta exotica* arriving on a flower carrying pollen smears on the dorsum. (k) *Dioprosopa clavata* trying to leave by one of

the exiting holes grabbing the hairs from the petal. (l-n) *Dioprosopa clavata* leaving the exiting hole with pollen smears attached to its dorsum. Arrows indicate pollinators. Scale bars = 1 cm.

The basal part of the labellum constitutes a slipper-shaped pouch (Fig 4a). The posterior part of the entrance hole margin is constituted by the infolded lobes which are formed by a smaller portion of the parenchyma that gradually thickens (≈ 22 cell layers) towards the anterior region where the outside of the labellum folds into the pouch forming a thicker tissue (that forms a hollow space between the two portions of the labellum) (Fig 4h). Both outer and inner (arrow) surfaces are covered by the same uniseriate and simple epidermis (Fig 4h). Internally it forms a thin and slippery surface that prevents the hoverfly to get out, causing it to fall repeatedly when trying to escape by the entrance hole (Fig 3g; Fig 5f).

The interior of the pouch is covered by tectorial trichomes, except in the front wall near to the entrance. The bottom where pollinator falls has shorter trichomes bearing 3 to 5 cells (Fig 4a, i). The posterior wall of the pouch has upward oriented trichomes (Fig 3a) that gradually increase in number and size (6 up to 12 cells approximately) from the bottom to the top (Fig 4a, j). The trichomes have a thick cuticle that stained with Sudan red (Fig 4o). In order to get their way-out, pollinators need to climb through this posterior portion of the labellum. The progressive increment in the size of these hairs, their upward orientation and stickiness provided by the cuticle probably helps the pollinator to cling. This labellum posterior zone also has some dark spots similar to those of the slipping zone (Fig 4a) that gradually increase their density and apparently form a path that guides the pollinator together with the hairs. The posterior region of the pouch also has a lateral constriction that funnels the passage to the central region (where bigger hairs and spots are located) (Fig 4a). When reaching the top of the labellum, pollinator must squeeze itself between the posterior wall and the stigma located below the staminode in a way that pollen smears present on their back remain adhered on the minute papillae of the stigmatic surface. The staminode that is positioned at the tip of the column has a flattened shape that blocks the base of the labellum and creates two basal lateral apertures. The anthers are also laterally positioned, just before the exiting of these holes. When pollinator is almost getting out by one of the holes, pollen is deposited onto its back by the respective (right or left) anther that act like an articulation mediated by the unidirectional movement. The exit hole is covered by long and abundant hairs from the lateral petals that are grabbed by the pollinator and help it to push its way out (Fig 3h,k,l). After leaving, some pollinators immediately fly away while others may still spend some time on the inflorescence cleaning themselves. Then, if the hoverfly is deceived again and gets trapped by another flower, it will perform cross pollination (Fig 3j).

Considering our anatomical and histochemical analyses, we did not find any elaiophores (*i.e.*, specialized oil secretory glands) or nectaries in histological sections. Neither of the tissues reacted positively with the Fehling reagent, Lugol, Bromophenol blue nor NADI reagent.

We found that syrphid flies laid their eggs on several external flower parts including in the petals, staminode and labellum (Fig. 5a-i). In this latter, eggs were found in the external structure of the pouch, around the entrance and in the infolded lobes where the dark spots are located. We also found eggs laid internally on the labellum posterior zone where dark spots were also present (Fig. 5j-k). Such egg-laying pattern suggests that flowers probably employ oviposition-site mimicry. We never found any aphids on flowers.

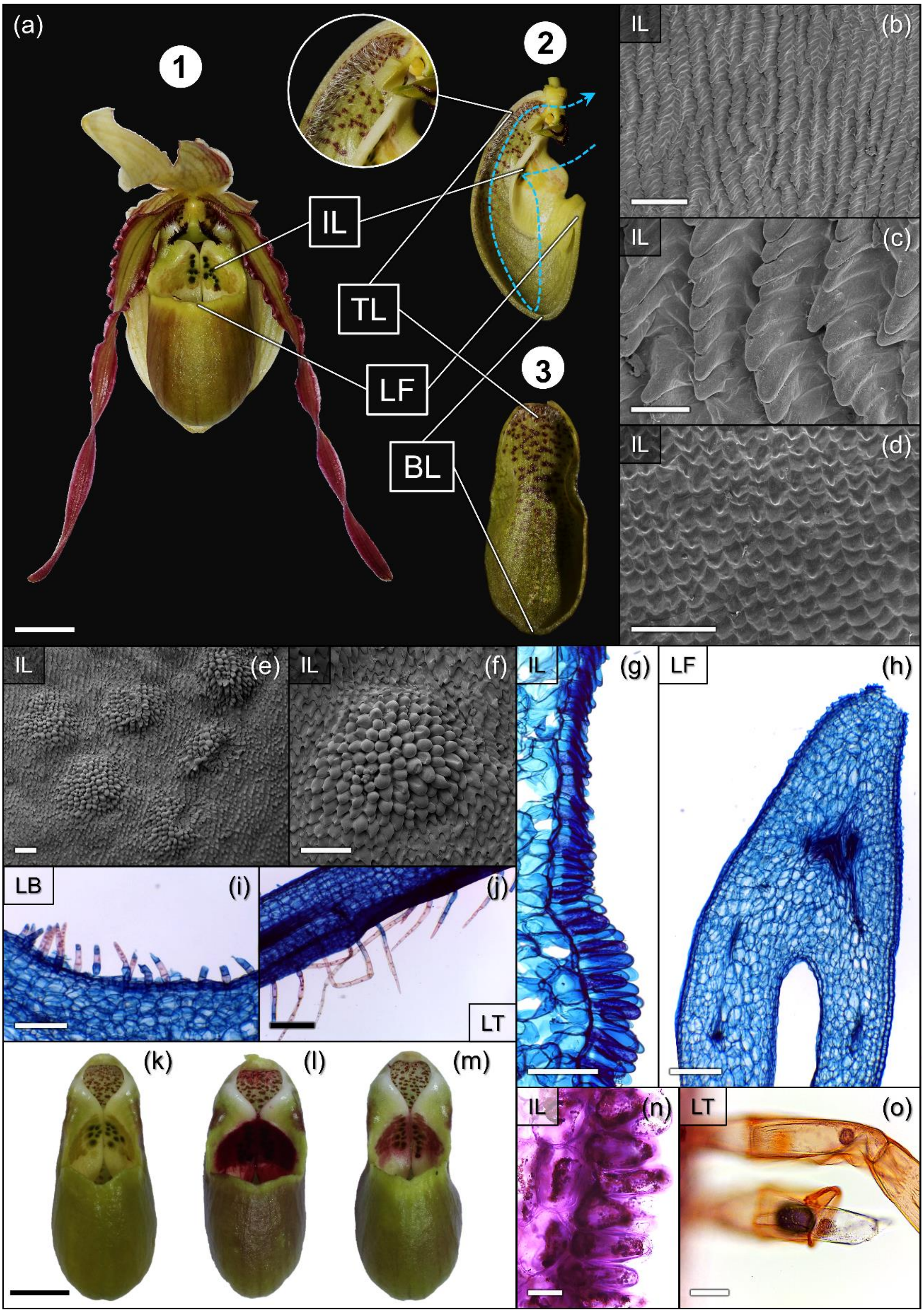


Figure 4. Floral morphology of *Phragmipedium vittatum*. (a) Frontal view of a flower (1), lateral (2) and frontal (3) views of the labellum sectioned. The blue dotted line in 2 indicates the route traced by the pollinators. IL: Infolded Lobes; LF: Labellum Front; LT: Labellum Top; LB: Labellum Base. (b, c) SEM in the IL in the slipping zone of the IL with different zoomings. (d)

Transition between the slipping zone the lower part of the IL. (e, f) SEM in the dark spots in the IL with different zoomings. (g) Anatomy of a spot in the IL. (h-j) Anatomy of LF, LB and LT, respectively. (k) Normal labellum followed by (l) neutral red and (m) ruthenium red treated labella. (n) Periodic acid-Schiff's positive reaction on the IL. (o) Sudan red positive reaction on the trichomes of the TL. Scale bars: a, k = 1 cm; b, d, e, f, h, i, j = 200 µm; c, g, l, m = 50 µm.

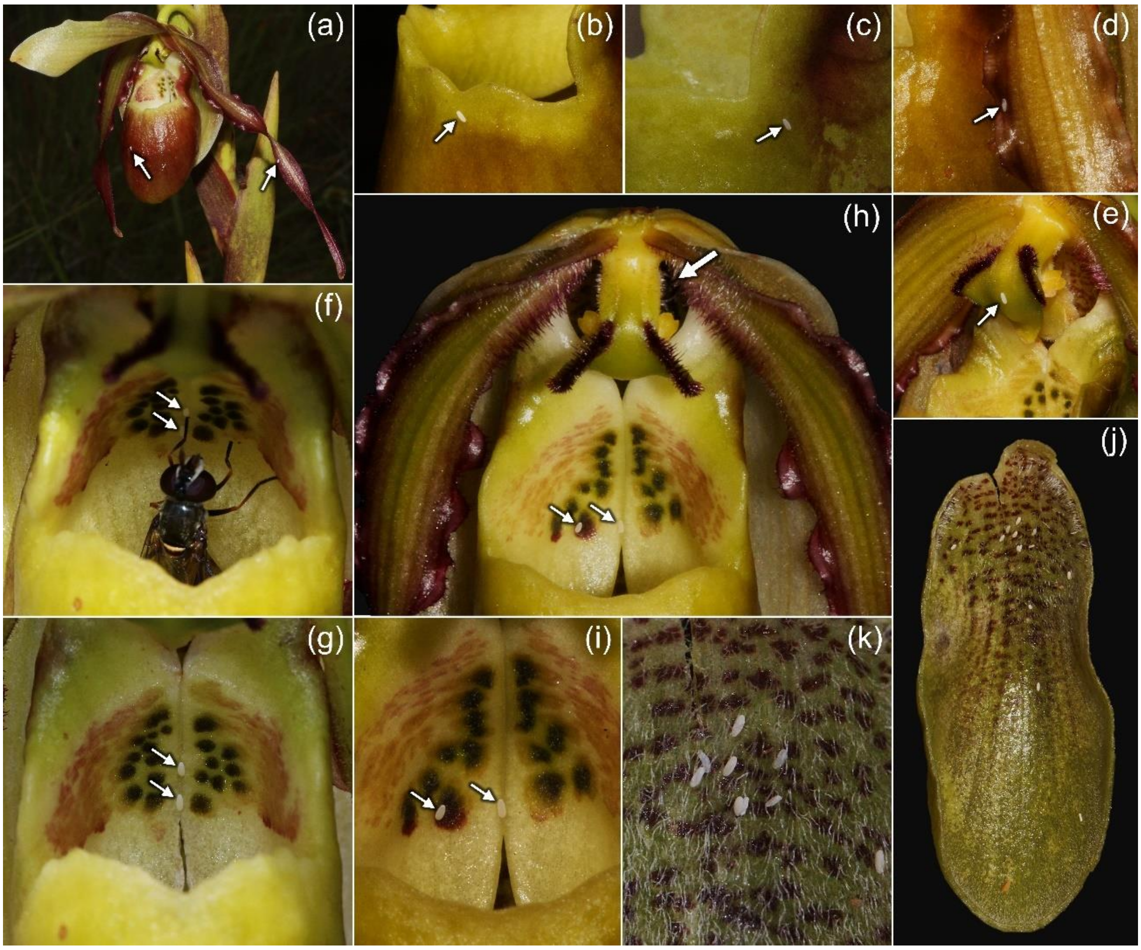


Figure 5. Egg laying patterns by syrphid flies in *Phragmipedium vittatum* (indicated by arrows). (a) Eggs seem from distance on a flower. Eggs layed in the (b-c) labellum, (d) petals and (e) the staminode. (f) *Dioprosopa clavata* trying to escape after falling on the pouch and (g) falling back again after deposited the eggs in the spots. (h) Eggs laid in the spots with detail of the pollinator inside seen in right exit hole (bigger white arrow). (i) zooming of those same eggs. (j, k) eggs laid in the inner part of the flower on the dark spots in different zoomings.

Pollinator effectiveness

We found significant differences in reproductive success according to sex ($\chi^2 = 415.52$, df = 1, $p < 0.001$, Table 2), with male success (38.44 %) being more than twice as large than the female (16.05 %). Considering only male success, one anther pollen removal (30.42 %) occurred more frequently in flowers than two anther removal (8.02

%) (Table 2). Pollen smears removal from two anthers indicates that some flowers were visited by hoverflies at least twice. There were no differences among the distinct flowering seasons ($\chi^2 = 0.98$, df = 3, p = 0.81) or the interaction term (sex*season: $\chi^2 = 1.99$, df = 3, p = 0.58). Considering the RE index across flowering seasons, we found that it ranged around the 0.2 cutoff value, being larger once and smaller three times (Table 2). Average RE index was 0.18 suggesting some pollination limitation.

Table 2. Total number of *Phragmipedium vittatum* flowers surveyed, percentages of male and female successes of and the reproductive efficacy (RE) index across four flowering seasons.

Year	Flowers	♂ Success			♀ Success	RE
		One anther	Two anthers	Total		
2016	306	32.03 %	8.17 %	40.20 %	15.03 %	0.17
2017	161	31.06 %	5.59 %	36.65 %	20.50 %	0.23
2018	301	27.24 %	6.31 %	33.55 %	15.28 %	0.17
2020	67	35.82 %	20.90 %	56.72 %	13.43 %	0.15

Discussion

Flower traits in semi-trap flowers are thought to work effectively attracting pollinators, keeping then imprisoned for a while and then guiding their exit through a specific path where pollen transference occurs along the way (Faegri and Van der Pijl 1979; Johnson and Schiestl 2016). Here, using a lady slipper as model system, we depict the several traits a semi-trap employs that promote pollinator attraction (when outside and inside the flower), trapping, and guiding of their specific intrafloral movements until the exit. First, we investigated the reproductive biology and pollination dynamics of *P. vittatum*, showing that it is a rewardless, non-spontaneous selfer, which depends on biotic pollination performed by syrphids. Then, we demonstrated that *P. vittatum* pollination is based on a complex semi-trap system involving specific characteristics that come into play in each one of the three phases of the pollination cycle, ensuring pollen transference.

We also demonstrate that the system works based on oviposition-site mimicry mechanism, in which the plant uses aphid-like cues to deceive gravid female hoverflies looking for oviposition sites to supply their aphidophagous larvae, being deceived into serving as pollinators. Below, we discuss in detail our findings and their implications.

Mating system and reproductive success

We show that *P. vittatum* is a rewardless, self-compatible, non-apomictic and non-spontaneous selfer species. Artificial self- and cross pollination successes were both high and similar, demonstrating that there is no self-incompatibility mechanism influencing fruit set. However, we found smaller values of seed viability in artificially selfed fruits when compared to both artificial cross and open pollination, suggesting inbreeding depression. Furthermore, seed numbers after selfing were lower when compared to cross pollination. Thus, autogamy apparently brings deleterious effects, and our results highlight the importance of pollinator mediated crossing in seed quality and quantity.

Although some Cypripedioideae are autogamous, including some *Phragmipedium* (*e.g.*, *P. reticulatum*, *P. lindenii*, *P. boisserianum and P. longifolium*) (Pemberton 2013; Edens-Meier et al. 2014; Morales 2017), *P. vittatum* is dependent on biotic pollination. Studies in *Phragmipedium* reproductive biology are scarce, but the existent evidence shows that fruiting success varies with *P. besseae* having only 4.3 % (Edens-Meier et al. 2014), *P. longifolium* having 12 % (Morales 2017), *P. pearcei* having 50 %, and the autonomous selfers (*P. reticulatum* and *P. lindenii*) showing 100 % (Edens-Meier et al. 2014). Our studied *P. vittatum* set 31.51 % exclusively by pollinator visits, having intermediate values when compared to its congeners.

Along the four flowering seasons, pollen smears removal from one anther occurred 30.42 % while removal from two anthers occurred only in 8.02 %, indicating repeated visits to a flower are possible but only occasional. In addition, pollen smears deposition rate is much smaller than removal, indicating that probably most flies do not visit flowers twice (*i.e.*, removing and not depositing the pollen). Looking at pollen removal and deposition over time show that it may vary across flowering seasons. Although we had a higher RE index when we surveyed fruits (0.36), the RE values based on pollen smears deposition using a larger sample size and a wider period showed lower values and indicated pollination limitation (< 0.2 RE) in three out of four years. This highlights that the flowers do not reach their maximum fruit set potential due to pollinator limitation, which agrees with the smaller fruit set proportions that deceptive flowers usually have when compared to the rewarding ones (Tremblay et al. 2005).

Phase I: attraction and trapping

As in other flowers, pollinator attraction is the first step of pollination also in semi-trap flowers (Fig. 1). The usage of trapping devices is a key trait associated to several oviposition-site mimicry systems (Bröderbauer et al. 2013; Johnson and Schiestl 2016). Hoverfly pollination in some slipper orchids apparently takes place by oviposition-site mimicry. Most syrphidae gravid females are specialized in laying their eggs on aphid colonies, on which predatory larvae feed (Schneider 1969; Rojo et al. 2003; Almohamad et al. 2009). During their searching behavior, syrphid females use several cues including visual, olfactory, gustatory, and tactile (Almohamad et al. 2009). The spots on *P. vittatum* labellum seems to mimic aphid agglomerations and the respective cues they emit, exploiting the preferences of the gravid females. Such strategy has been observed in the hoverfly pollinated *Cypripedium lentiginosum* (Zhongjian et al. 2008), *Paphiopedilum* and other *Phragmipedium* (Pemberton 2013; Edens-Meier et al. 2014). Interestingly, non-hoverfly pollinated *Cypripedium* and *Paphiopedilum* usually do not bear the aphid-like spots. The visual resemblance and the behavior of pollinators are among the main indication of oviposition-site mimicry, with egg-laying providing hard evidence that females have been effectively duped by flowers (Urru et al. 2011; Johnson and Schiestl 2016). Here we show that *P. vittatum* aphid mimicry system seems so effective that female hoverflies lay their eggs in several flower parts, including on the dark spots. Female aphids may lay eggs directly on aphids, but also close to the colonies (Almohamad et al. 2009), which may explain oviposition in other parts of the flower such as on the external part of labellum, petal, and staminode.

In *Phragmipedium*, the slipping zone consists in the flat surface formed by the infolded lobes of the labellum where the spots are located, seemingly mimicking aphids. While in *Paphiopedilum* spots seem to be elevated and shiny warts, they visually did not look elevated in *Phragmipedium*. However, here we show that in addition to the color change, there is an increase in the relief of the aphid-like spots that is distinguishable under magnification. Since syrphid females can use the shape of aphids as a visual cue (Almohamad et al. 2009), such relief may be noticed by hoverflies when they come closer and be important in attraction. In addition, syrphid females prefer to oviposit on colonies with higher number and density of aphids (Almohamad et al. 2009; Nelson et al. 2012). Thus, the occurrence of several spots simulating an agglomeration pattern may also be an important attractant, which mimics a suitable food supply for larvae and elicit their landing and oviposition behaviors. In addition, the vertical surface of the slippery zone can be important since some female hoverflies prefer vertical over horizontal structures of plants (Almohamad et al. 2009 and references therein). Color is also an important cue. The yellowish tones of several color parts of *P. vittatum* may function as attractant, since hoverflies have an innate preference for yellow colors of flowers (*e.g.*, Neimann et al. 2018; Rodríguez-Gasol et al. 2019). Some darker structures of *P. vittatum*, such as the descending petals, may also be an important attractant because some female hoverflies prefer plants with darker than lighter strips (Almohamad et al. 2009 and references therein). Finally, aphids' honeydew is a potential attractant as syrphid females use it as a food source (Schneider 1969) and also as gustatory cue, triggering their oviposition behavior (Almohamad et al. 2009; *e.g.*, Budenberg and Powell 1992). Thus, as mucilage constitutes a hydrogel (Røn et al. 2016), it is likely that its presence in the slipping zone also plays a role in pollinator attraction, probably mimicking aphid honeydew. Other possible roles of mucilage involving slipperiness are discussed in the next topic.

Aphid mimicry is an interesting mechanism known only for two orchid lineages: in some members of the Cypripedioideae subfamily and in others of the genus *Epipactis* (Epidendroideae). Egg laying on external parts of the labellum were also reported in the congener *Phragmipedium longifolium* (Morales 2017). Aphid mimicry has been described in some hoverfly pollinated species from the sister genus *Paphiopedilum*, with some cases involving oviposition, such as in *P. rothschildianum* (Atwood 1994), *P. callosum* (Johnson and Schiestl 2016), and in *P. dianthum* (Shi et al. 2007). Although several factors are responsible by triggering oviposition behavior in female hoverflies, olfactory cues are among the most important (Almohamad et al. 2009; Stökl et al. 2011), probably being the factor triggering egg-laying behavior on both *Paphiopedilum* (Bänziger et al. 2012) and *Phragmipedium.* In agreement, a study with another lady's slipper, *Cypripedium subtropicum*, showed that flowers mimic aphid alarm pheromones which are important volatile components in the attraction of hoverflies, although no eggs were found on these flowers (Jiang et al. 2020). This species has hairy tufts at the slippery zone that both visually resemble an aphid colony covered with honeydew and rewards hoverflies, characterizing a reward mimicry mechanism and being the only rewarding lady's slipper described so far.

Some *Epipactis* (Epidendroideae) have black callus-like swellings on the labellum hypochile that are thought to imitated aphids and induce egg laying (Ivri and Dafni, 1977; Jin et al. 2014; Srimuang et al. 2018). Stökl et al. (2011) showed that E. *veratrifolia* flowers emit a mixture of volatile compounds similar to those alarm pheromones released by several aphid species. In agreement, they also showed via electrophysiological experiments that such compounds are detected by the antennae of hoverflies. Finally, both headspace samples of the scent released by flowers and a synthetic mixture of four compounds (produced by the flowers) increased the egg-laying by hoverflies. As a specific aphid alarm pheromone is not released, it is probably that flowers employ a generalized aphid mimicry system. This is in agreement with the oviposition behavior of hoverflies, that usually lay eggs on several aphid species. For instance, *Allograpta exotica*, one of *P. vittatum* pollinators, is known to exploit at least 18 different species of aphids (Rojo et al. 2003).

After insect attraction, trap flowers must effectively imprison them, in order to proceed with the pollination process (Fig. 1). Generally, insects have two attachment devices on their forelegs: claws allow them to cling on rough surfaces and adhesive pads to smooth surfaces (Beutel and Gorb 2001). Falling depends on nullifying the adhesive devices of insects. Kettle trap flowers and carnivorous plants have several micro-structural strategies that induce slipperiness, including traits (and their combinations) such as epidermal cell curvatures, cuticular folding, idioblastic elements and three-dimensional epicuticular wax crystals (Poppinga et al. 2010). Our study provides new insights into the floral micro morphology characteristics that contribute to pollinator prisoning within the pouch. An important trait is the presence of downward projecting papillate cells of epidermis. This is the most common slippery strategy among trap flowers and carnivorous plants, having evolved independently several times (Poppinga et al. 2010). They boost slipperiness by preventing claw anchorage via hooking due to their inappropriate cell shape (Poppinga et al. 2010). This type of sculptural surface has also been found in unrelated groups including in trap flowers from Apocynaceae (*Ceropegia*), Araceae (*Arisarum*, *Arum*, *Biarum*, *Colocasia*, *Pinellia*) and Aristolochiaceae (*Aristolochia*) families as well as in carnivorous plants from Cephalotaceae (*Cephalotus*) and Sarraceniaceae (*Darlingtonia*, *Sarracenia*) (Poppinga et al. 2010). However, as far as we know, this is the first time it is reported in Orchidaceae.

Another important trait we describe is the mucilage secretion in the slipping zone. Surfaces with a liquid film are thought to promote anti-adhesiveness by preventing the attachment of adhesive pads leading to a "aquaplaning" effect. For instance, some *Nepenthes* carnivorous plants have radial ridges of smooth and overlapping epidermal cells with hydrophilic surface chemistry, which makes the surface wettable (Bohn and Federle 2004). Under natural conditions, liquid films are formed by nectar secretion, rain, and condensation. However, the surface is not slippery for insects when is dry, indicating that the peristome slipperiness is weather-dependent (Bauer et al. 2008). Contrary to this unpredictable activation of *Nepenthes* pitcher traps, the mucilage secretion apparently provides a permanent wet surface in *P. vittatum*. This viscoelastic and polyssaccaride-rich hydrogel has lubricating and slippery properties (Røn et al. 2016), which may be important in promoting slipperiness via aquaplaning. In addition, it is known that papillate cells play an important role in surface wettability, turning surfaces superhydrophilic because water naturally spreads throughout the area (Koch et al. 2008). Thus, the papillate cells already mentioned can also improve mucilage permanence on surface. Furthermore, the ridged micropattern formed by the papillated cells in *P. vittatum* may also improve wettability functioning as water channels. In the *Nepenthes* example, the peristome ridges accumulate extrafloral nectar, which renders wettability and make the surface slippery for insects (Bauer et al. 2008). As far as we know, we provide the first report of mucilage as a potentially slippery strategy in insect trapping plants. As mucilage avoids tissue dissection, other secondary effects may involve the maintenance of cell turgor and shape, especially the elevated region of aphid like spots and the maintenance of a typical shiny/wetted surface that resembles aphid exudate accumulation.

In *Cypripedium calceolus*, there are tabular epidermal cells with idioblasts that are thought to offer no possibility for claw interlocking (Poppinga et al. 2010) in addition to undetermined fatty liquids (Daumann 1968) hypothesized to contaminate the adhesive pads of insects (Poppinga et al. 2010). In *Paphiopedilum*, Besi et al. 2021 showed the presence of trichomes in the staminode (the slippery region in this genus) of three species (*P. barbatum, P. callosum* and *P. niveum*). Despite these punctual observations, knowledge about slipperiness traits in orchids and, more specifically in the Cypripedioideae, is scarce. Here we provide new evidence on which traits mediate pollinator falling via anti-adhesive micro-structural strategies. The combination of several anthi-adesive strategies is thought to increase slipperiness (Poppinga et al. 2010). Thus, downward projecting papillate cells and mucilage secretion on the vertical surface of the labellum lobes are adaptations that can boost pollinators' fall into the pouch via disruption of both claw interlocking and adhesive pads, respectively.

Phase II: intrafloral movements and pollination

After trapped, insects may perform a specific and pre-determined rout within the trap-flower structure (Fig. 1). Although the hoverflies try, they cannot get out from the pouch by where they got in because they keep slippering back in the surface that labellum folds inward. This region has convex-shaped cells that are also thought to prevent claw interlocking (Poppinga et al. 2010). After imprisoned, how do they know they have to go up? A possible explanation is that light entrance on the top of the flower makes this region brighter, attracting the attention of the insect. Since flies are positively phototropic, illumination is used by several semi-trap flowers to guide the pollinators in the intrafloral space to reach reproductive organs and the exit. This may be achieved by window panes, colorless and translucent regions surrounded by areas with darker pigmentation, where light from the outside can easily enter (Dafni 1984; Endress 1996; Faegri and Van der Pijl 1979). Window panes have been reported in other orchid semi-trap flowers (Lehnebach et al. 2005), including in the *Cypripedium* lady's slippers (Sugiura et al. 2001; Szlachetko et al., 2020). In *P. vittatum*, although it does not occur clearly at the top of the flower, it is possible to notice areas with less pigmentation in the middle of the labellum where the infolded lobes fuse, extending until the areas surrounding the exit holes. In agreement, these regions coincide with the path pollinators go though, probably serving as a lighting guide.

It is known that hairs may signalize routes of exit (Faegri and Van der Pijl, 1979). Hairs in the posterior part of the labellum seems common in the Cypripedioideae, being reported in other *Phragmipedium* (Pemberton 2013), *Cypripedium* (Sugiura et al. 2001; Szlachetko et al. 2020), *Paphiopedilum* (Shi et al. 2009) and *Selenipedium* (Cribb and Schiuteman 2015; Szlachetko and Kolanowska, 2016). Darwin (1862) hypothesized that the extrafloral hairs probably secreted nectar as reward. In *P. vittatum* we observed that these structures are non-secretory. Such hairs progressively increase in size as the pollinator manages to go up, probably serving as a "stairway" for insects to hold on, aiding their grip by grabbing the longer trichomes during climbing. Hair downward orientation has been suggested to improve slipperiness (Richards 1997; Johnson and Schiestl 2016). On the contrary, the upward hair orientation as we found here may reduce slipperiness and allow the pollinator to hold on more firmly. In addition, the cover by lipids found in these hairs probably make them resistant and non-slip. This allows the mechanical support to insects climb and reduce the water loss by cells, keeping them turgid.

The effectiveness of the dark spots found into the labellum has never been reported and suggest that flowers still use the aphid colony mimicry exploiting the pollinator sensory system even after prisoning. The eggs found on this region corroborate this, indicating the usage of the same oviposition-site mimicry mechanism to pollinator get in and get out of the pouch. Such inner spotting may also be noticed in other lady's slipper orchids pollinated by hoverflies (*e.g.*, Ren et al. 2011; Jiang et al. 2020) and even by bees, such as *Cypripedium reginae* which has pink-magenta spotting in the labellum interior (Edens-Meier et al. 2011). Although in bee and other insect pollinated species the mimicry mechanism might be different (*i.e.*, generalized food mimicry; Pemberton 2013; Edens-Meier et al. 2014), our direct evidence with egg laying suggests that internal spotting is important in guidance, and may probably function in other systems as well.

The labellum lateral constriction creates a central rout where hairs and spots are concentrated, leading to reproductive organs. When the hoverfly reaches a certain height, it is pressed against the flattened and papillose stigma depositing any pollen smears located on its back. Pollinator continues its climbing and further ahead pollen is deposited by one of the articulated (right or left) anthers while it comes across the respective exit hole. In oviposition-site mimicry systems, insects tend to move around haphazardly looking for oviposition places, which may not lead to efficient pollen transfer (Johnson and Schiestl 2016). Thus, the usage of traps, chambers and narrow exit passages allow plants to manipulate pollinators into a precise position for pollen placement (Johnson and Schiestl 2016). In agreement, the set of features reported here such as less pigmented regions that allow light passage, upward oriented hairs progressively longer, inner aphid-like spotting, and labellum lateral constriction forces the pollinator to walk narrow intrafloral paths. Thus, although *P. vittatum* semi-trap flowers are relatively large compared to their pollinators. this leads to a precise pollen deposition onto the thorax back.

Phase III: flower exit

After pollen deposition by one of the anthers, pollinator must keep following its course (Fig. 1). At this point, as it is closer to the exit, light entering by the exit holes becomes more intense, guiding their way-out carrying pollen smears. Another trait that is related to pollination biomechanics is the presence of hairs coming from the lateral petals surrounding the holes. Such petals' hairs projecting towards the exit opening holes have also been reported in *Cypripedium* (Sugiura et al. 2001; Szlachetko et al. 2020), *Paphiopedilum* (Shi et al. 2009) and *Selenipedium* (Szlachetko and Kolanowska, 2016). We observed that, as pollinator holds these hairs, they also may increase anchorage and get out through the tight hole. In fact, we found that some hoverflies may get stuck in the exit hole and even die (Cardoso, personal observation), indicating that the absence of such hairs would make this more common. After finally leaving, pollinators carry pollen smears onto their back and will cross pollinate flowers if they are deceived again by a different flower where the whole three-phase cycle starts again.

Conclusions

In this study, we used *P. vittatum* as model system to better understand the different microstructures that allow a semi-trap flower to exploit their pollinators. In the Cypripedioideae subfamily, the usage of semi-traps is basal and widespread. Although they may be pollinated by a wide range of pollinators including flies (from

Sarcophagidae, Drosophilidae and Syrphidae families), polyletic bees, bumblebee queens and parasitoid wasps (Pemberton 2013; Edens-Meier et al. 2014), the several mechanisms shown here may be extended to the other members of the lineage. First, we show that this lady's slipper is rewardless and dependent on biotic pollination to obtain seed quality and quantity. *Phragmipedium vittatum* employs oviposition-site mimicry as flowers have the aphid-like spots that deceive gravid female hoverflies with aphidophagous larvae. By dividing the pollination mechanism into three phases, we could depict better which potential anatomical and histochemical traits allow the system functioning on each period. During the phase I, pollinator is attracted to the slipping zone of the labellum, which has high elevated spots that resemble aphids in color, morphology, and arrangement. When reaching this region, they lose their foothold because downward projecting papillate cells and mucilage secretion probably promote slipperiness. After falling into the pouch, phase II takes place where pollinators are guided to perform specific intrafloral movements, going up towards the reproductive parts. Upward oriented hairs progressively longer aid their grip and create a path together with the lateral constriction of the labellum. Less pigmented regions on the labellum probably allow light entrance guiding pollinators and the inner aphid-like spotting are also used as visual cue. Pollination occurs after they pass the stigma depositing pollen. Pollen exportation occurs after passing one of the lateral anthers that comes next. In the phase three they exit the flower through a narrow hole helped by the petal hairs that the pollinators use to hold on. Importantly, here we demonstrate that the same mimicry mechanism (resembling an aphid colony) is used to exploit the female hoverflies outside and inside the flower (*i.e.*, trapping and then guiding them within the flower), which is confirming by their egg-laying behavior on both. Our results shed light into the sophisticated micro-morphological adaptations that semi-traps have that allow them to exploit and manipulate their pollinators.

Acknowledgements

We are grateful to R. T. Trevizan by her help with scanning electron microscopy analyses, to D. C. Oliveira by providing all the structure for carrying out anatomy and histochemistry tests, and M. N. Morales by the precious identification of pollinators. We thank A. P. S. Caetano, J. P. B. Alves and C. E. P. Nunes for several discussions about the flower micromorphology and the mimicry mechanism. JCFC and UCR are grateful to CAPES (*Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior*) – Finance Code 001 – for their PhD grants. SDJ is grateful to the South African Research Chairs Programme (NRF grant 46372). PEO is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for his research grant and financial support.

References

Almohamad, R., Verheggen, F., & Haubruge, É. (2009). Searching and oviposition behavior of aphidophagous hoverflies (Diptera: Syrphidae): a review. Biotechnologie, Agronomie, Société et Environnement, 13(3), 467-481.

Alvares, C. A., Stape, J. L., Sentelhas, P. C., Moraes, G., Leonardo, J., & Sparovek, G. (2013). Köppen's climate classification map for Brazil. Meteorologische Zeitschrift, 22, 711–728. https://doi.org/10.1127/0941-2948/2013/0507

Araújo, G. M., Barbosa, A. A. A., Arantes, A. A., & Amaral, A. F. (2002). Composição florística de veredas no Município de Uberlândia, MG. Revista Brasileira de Botânica, 25, 475–493. https://doi.org/10.1590/S0100-84042002012000012

Atwood, J.T. 1984. Pollination of *Paphiopedilum rothschildianum*: brood-site imitation. Natl. Geogr. Res. 1: 247-254.

Bänziger, H., Pumikong, S., & Srimuang, K. O. (2012). The missing link: bee pollination in wild lady slipper orchids *Paphiopedilum thaianum* and *P. niveum* (Orchidaceae) in Thailand. Mitteilungen der Schweizerischen Entomologischen Gesellschaft, 85(1), 1.

Bates D, Maechler M, Bolker B, Walker S (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. https://doi.org/10.18637/jss.v067.i01

Bauer, U., Bohn, H. F., & Federle, W. (2008). Harmless nectar source or deadly trap: *Nepenthes* pitchers are activated by rain, condensation and nectar. Proceedings of the Royal Society B: Biological Sciences, 275(1632), 259-265. https://doi.org/10.1098/rspb.2007.1402

Besi, E. E., Jia, L. S., Mustafa, M., Yong, C. S., & Go, R. (2021). Comparative floral surface micromorphology helps to discriminate between species of *Paphiopedilum* (Orchidaceae: Cypripedioideae) from Peninsular Malaysia. Lankesteriana, 17-31. https://doi.org/10.15517/lank.v21i1.46714

Beutel, R. G., & Gorb, S. N. (2001). Ultrastructure of attachment specializations of hexapods (Arthropoda): evolutionary patterns inferred from a revised ordinal phylogeny. Journal of Zoological Systematics and Evolutionary Research, 39(4), 177-207. https://doi.org/10.1046/j.1439-0469.2001.00155.x

Bohn, H. F., & Federle, W. (2004). Insect aquaplaning: *Nepenthes* pitcher plants capture prey with the peristome, a fully wettable water-lubricated anisotropic surface.

Proceedings of the National Academy of Sciences, 101(39), 14138-14143. https://doi.org/10.1073/pnas.0405885101

Bröderbauer, D., Diaz, A., & Weber, A. (2012). Reconstructing the origin and elaboration of insect-trapping inflorescences in the Araceae. American journal of botany, 99(10), 1666-1679. https://doi.org/10.3732/ajb.1200274

Budenberg, W. J., & Powell, W. (1992). The role of honeydew as an ovipositional stimulant for two species of syrphids. Entomologia Experimentalis et Applicata, 64(1), 57-61. https://doi.org/10.1111/j.1570-7458.1992.tb01594.x

Cardoso, J. C. F., Viana, M. L., Matias, R., Furtado, M. T., Caetano, A. P. D. S., Consolaro, H., & Brito, V. L. G. D. (2018). Towards a unified terminology for angiosperm reproductive systems. Acta Botanica Brasilica, 32(3), 329-348. https://doi.org/10.1590/0102-33062018abb0124

Core Team, R. (2020). R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing https://www.R-project.org/

Crawley, M. J. (2013). The R book. John Wiley & Sons.

Cribb, P., & Schiuteman, A. (2015). A new species of *Selenipedium* (Orchidaceae: Cypripedioideae) from Ecuador. Lankesteriana. https://doi.org/10.15517/lank.v15i3.21109

Dafni, A. (1984). Mimicry and deception in pollination. Annual review of ecology and systematics, 15(1), 259-278. https://doi.org/10.1146/annurev.es.15.110184.001355

Darwin, C.R. (1862) The Various Contrivances by which Orchids are Fertilised by Insects. John Murray, London.

Daumann, E. (1968). Zur Bestäubungsökologie von *Cypripedium calceolus* L. Österreichische Botanische Zeitschrift, 115(4), 434-446. https://doi.org/10.1007/BF01456538

David, R.; Carde, J.P. (1964). Coloration différentielle des inclusions lipidiques et terpéniques des pseudophylles du Pin maritime au moyen du réactif nadi. CR Acad Sci Paris, 258, 1338-1340.

Dias, M.C.; Smidt, E.C. 2020. *Phragmipedium* in Flora do Brasil 2020. Jardim Botânico do Rio de Janeiro. Available at: http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB32001. Accessed on May 27, 2021

Díaz Morales, M. (2017). Biología reproductiva y mecanismo de atracción de polinizadores de *Phragmipedium longifolium* (Orchidaceae: Cypripedioideae) en Costa Rica.

Dressler, R.L. (1981). The orchids: natural history and classification. Harvard University Press, Cambridge, Mass, USA

Edens-Meier, R., Arduser, M., Westhus, E., & Bernhardt, P. (2011). Pollination ecology of *Cypripedium reginae* Walter (Orchidaceae): size matters. Telopea, 13(1-2), 327-340. https://doi.org/10.7751/telopea20116024

Edens-Meier, R., Luo, Y. B., Pemberton, R., & Bernhardt, P. (2014). Pollination and floral evolution of slipper orchids (subfamily Cypripedioideae). Darwin's Orchids Then and Now. https://doi.org/10.7208/chicago/9780226173641.001.0001

Endress, P. K. (1996). Diversity and evolutionary biology of tropical flowers. Cambridge University Press.

Faegri, K., & Van der Pijl, L. (1979). A short history of the study of pollination ecology. The Principles of pollination ecology, 1-3. https://doi.org/10.1016/B978-0-08-023160-0.50005-0

Fox J, Weisberg S (2019). An {R} Companion to Applied Regression, Third Edition. Thousand Oaks CA: Sage. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Grabe, D. F. (1970). Tetrazolium testing handbook for agricultural seeds. Stillwater: Association of Official Seed Analysts.

Gregory, M., Baas, P. (1989). A survey of mucilage cells in vegetative organs of the dicotyledons. Israel Journal of Botany, 38(2-3), 125-174.

Hothorn T, Bretz F, Westfall P (2008). Simultaneous Inference in General Parametric Models. Biometrical Journal 50(3), 346-363. https://doi.org/10.1002/bimj.200810425

Ivri, Y., & Dafni, A. (1977). The pollination ecology of *Epipactis consimilis* Don (Orchidaceae) in Israel. New Phytologist, 79(1), 173-177. https://doi.org/10.1111/j.1469-8137.1977.tb02193.x

Jiang, H., Kong, J. J., Chen, H. C., Xiang, Z. Y., Zhang, W. P., Han, Z. D., ... & Lee, Y. I. (2020). *Cypripedium subtropicum* (Orchidaceae) employs aphid colony mimicry to attract hoverfly (Syrphidae) pollinators. New Phytologist, 227(4), 1213-1221. https://doi.org/10.1111/nph.16623

Jin, X. H., Ren, Z. X., Xu, S. Z., Wang, H., Li, D. Z., & Li, Z. Y. (2014). The evolution of floral deception in *Epipactis veratrifolia* (Orchidaceae): from indirect defense to pollination. BMC Plant Biology, 14(1), 1-10. https://doi.org/10.1186/1471-2229-14-63

Johansen, D.A. 1940. Plant Microtechnique. London, McGraw-Hill Book Company.

Johnson, S. D., & Schiestl F. P. (2016). Floral mimicry. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198732693.001.0001

Kearns, C. A., & Inouye, D. W. (1993). Techniques for pollination biologists. University press of Colorado.

Koch, K., Bhushan, B., & Barthlott, W. (2008). Diversity of structure, morphology and wetting of plant surfaces. Soft Matter, 4(10), 1943-1963. https://doi.org/10.1039/b804854a

Lehnebach, C. A., Robertson, A. W., & Hedderley, D. (2005). Pollination studies of four New Zealand terrestrial orchids and the implication for their conservation. New Zealand Journal of Botany, 43(2), 467-477. https://doi.org/10.1080/0028825X.2005.9512968

Neimann, A., An, L., & Lunau, K. (2018). The yellow specialist: Colour preferences and colour learning of the hoverfly *Eristalis tenax* (Diptera: Syrphidae). Entomologie Heute, 30, 27-44.

Nelson, E. H., Hogg, B. N., Mills, N. J., & Daane, K. M. (2012). Syrphid flies suppress lettuce aphids. BioControl, 57(6), 819-826. https://doi.org/10.1007/s10526-012-9457-z

Ollerton, J. (2021). Pollinators and Pollination: Nature and Society. Pelagic Publishing Ltd. https://doi.org/10.53061/JAOK9895

Pemberton, R. W. (2013). Pollination of slipper orchids (Cypripedioideae): a review. Lankesteriana International Journal on Orchidology, 13(1-2), 65-74. https://doi.org/10.15517/lank.v0i0.11539

Poppinga, S., Koch, K., Bohn, H. F., & Barthlott, W. (2010). Comparative and functional morphology of hierarchically structured anti-adhesive surfaces in carnivorous plants and kettle trap flowers. Functional Plant Biology, 37(10), 952-961. https://doi.org/10.1071/FP10061

Purvis, M. J., Collier D. C. & Walls D. (1964). Laboratory Techniques in Botany. Butterwoths, London.

Ren, Z. X., Li, D. Z., Bernhardt, P., & Wang, H. (2011). Flowers of *Cypripedium fargesii* (Orchidaceae) fool flat-footed flies (Platypezidae) by faking fungus-infected foliage. Proceedings of the National Academy of Sciences, 108(18), 7478-7480. https://doi.org/10.1073/pnas.1103384108

Richards, A. J. (1997). Plant breeding systems. Garland Science. https://doi.org/10.1007/978-1-4899-3043-9

Rodríguez-Gasol, N., Avilla, J., Alegre, S., & Alins, G. (2019). *Sphaerophoria rueppelli* adults change their foraging behavior after mating but maintain the same preferences to flower traits. BioControl, 64(2), 149-158. https://doi.org/10.1007/s10526-019-09928-2

Rojo, S., Gilbert, F., Marcos-García, M. A., Nieto, J. M., & Mier, M. P. (2003). A world review of predatory hoverflies (Diptera, Syrphidae: Syrphinae) and their prey (No. DC).

Røn, T., Rishikesan, S., Chronakis, I. S., & Lee, S. (2016). Slippery when sticky: Lubricating properties of thin films of *Taxus baccata* aril mucilage. Biointerphases, 11(1), 011010. https://doi.org/10.1116/1.4942208

Sass, J. E. (1951). Botanical Microtechnique, 2nd edn. The Iowa State College Press, Ames, IA. https://doi.org/10.5962/bhl.title.5706

Schneider, F. (1969). Bionomics and physiology of aphidophagous Syrphidae. Annual review of Entomology, 14(1), 103-124. https://doi.org/10.1146/annurev.en.14.010169.000535

Shi, J., Cheng, J., Luo, D., Shangguan F.Z., Luo,Y.B., 2007. Pollination syndromes predict brood-site deceptive pollination by female hoverflies in *Paphiopedilum dianthum* (Orchidaceae). Acta Phytotaxon. Sin. 45, 551-560. (In Chinese with English abstract) https://doi.org/10.1360/aps07025

Shi, J., Luo, Y. B., Bernhardt, P., Ran, J. C., Liu, Z. J., & Zhou, Q. (2009). Pollination by deceit in *Paphiopedilum barbigerum* (Orchidaceae): a staminode exploits the innate colour preferences of hoverflies (Syrphidae). Plant Biology, 11(1), 17-28. https://doi.org/10.1111/j.1438-8677.2008.00120.x

Srimuang, K. O., Bänziger, H., & Watthana, S. (2018). Pollination-system diversity in *Epipactis* (Orchidaceae): New insights from studies of *E. flava* in Thailand. Plant Systematics and Evolution, 304(8), 895-909. https://doi.org/10.1007/s00606-018-1520-5

Stökl, J., Brodmann, J., Dafni, A., Ayasse, M., & Hansson, B. S. (2011). Smells like aphids: orchid flowers mimic aphid alarm pheromones to attract hoverflies for pollination. Proceedings of the Royal Society B: Biological Sciences, 278(1709), 1216-1222. https://doi.org/10.1098/rspb.2010.1770

Sugiura, N., Fujie, T., Inoue, K., & Kitamura, K. (2001). Flowering phenology, pollination, and fruit set of *Cypripedium macranthos* var. *rebunense*, a threatened lady's slipper (Orchidaceae). Journal of Plant Research, 114(2), 171-178. https://doi.org/10.1007/PL00013980

Szlachetko, D. L., & Kolanowska, M. (2016). Notes on the Genus *Selenipedium* (Orchidaceae, Cypripedioideae) with Descriptions of New Taxa. Systematic Botany, 41(1), 142-159. https://doi.org/10.1600/036364416X690705

Szlachetko, D. L., Górniak, M., Kowalkowska, A. K., Kolanowska, M., Jurczak-Kurek, A., & Archila Morales, F. (2020). The natural history of the genus *Cypripedium* (Orchidaceae). Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 1-25. https://doi.org/10.1080/11263504.2020.1785963

Tremblay, R. L., Ackerman, J. D., Zimmerman, J. K., & Calvo, R. N. (2005). Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. Biological Journal of the Linnean Society, 84(1), 1-54. https://doi.org/10.1111/j.1095-8312.2004.00400.x

Urru, I., Stensmyr, M. C., & Hansson, B. S. (2011). Pollination by brood-site deception. Phytochemistry, 72(13), 1655-1666. https://doi.org/10.1016/j.phytochem.2011.02.014

Vogel, S. (1962). Duftdrüsen im Dienst der Bestäubung tiber Bau Und Funktion der Osmophoren. Akademie der Wissenschaftenund der Literatur, Mainz. Mathematisch-Naturwissenschaftliche Klasse, 10, 598-763.

Vogel S (1965) Kesselfallen-Blumen. Die Umschau in Wissenschaft und Technik 65, 12–17.

Vogel, S., & Martens, J. (2000). A survey of the function of the lethal kettle traps of *Arisaema* (Araceae), with records of pollinating fungus gnats from Nepal. Botanical Journal of the Linnean Society, 133(1), 61-100. https://doi.org/10.1111/j.1095-8339.2000.tb01537.x

Zapata, T. R., & Arroyo, M. T. K. (1978). Plant reproductive ecology of a secondary deciduous tropical forest in Venezuela. Biotropica, 221-230. https://doi.org/10.2307/2387907

Zhongjian, L., Lijun, C., Wenhui, R., Liqiang, L., & Yuting, Z. (2008). Correlation between numerical dynamics and reproductive behavior in *Cypripedium lentiginosum*. Acta Ecologica Sinica, 28(1), 111-121. https://doi.org/10.1016/S1872-2032(08)60021-9

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). Mixed effects models and extensions in ecology with R. Springer Science & Business Media. https://doi.org/10.1007/978-0-387-87458-6

Capítulo 3

Up around the flower bend: Disentangling flower resupination and its consequences to reproductive success

Abstract

Some flowers show drastic changes in orientation during anthesis. This is the case of resupination: the ≈ 180º twist of flowers, leaving them upside down. It is thought to arrange flowers in a proper position, which promotes pollinator landing in a specific direction plane, allowing precise pollen transference. Although resupination is widely distributed across 14 unrelated families, its adaptative value was never tested. To investigate resupination effects properly, it is necessary a special system with variable flower twisting. We found it in a rare lady's slipper orchid: *Phragmipedium vittatum*. During two flowering seasons, we surveyed 4984 flowers and found that approximately 10% of them were not properly resupinate, being either non- (upward, 0º - 60º) or half-resupinate (sideward, 60º - 120º). We conducted and experiment artificially twisting flowers into non- (≈ 0º), half- (≈ 90º) and resupinate (≈ 180º) positions. We used a wire to tie flowers in addition to a fourth control group with intact resupinate ones. Pollination is specialized, involving the hoverfly trapping into the pouch and sophisticated intrafloral movements that lead to the coupling of reproductive organs. We hypothesized that non- and half-resupinate flowers would not allow any of these and pollen transference would occur only in resupinate flowers. We corroborate this as resupinate treatment and control groups were the only with pollen smears removal (both with 75%) and deposition (respectively 13.89% and 10.71%). Although hoverflies were still attracted by non- and half-resupinate flowers, their orientation seems ineffective to trap pollinators. Artificially twisting resupinate flowers with pollinators inside into non- and half-resupinate positions quickly caused the insects to come out by the same entrance hole. As the plant is non-autonomous selfer, those flowers that do not resupinate will not set fruits. We demonstrate that resupination is related to precise pollen transference and its breakdown interrupts fruit set chances.

Keywords: Flower orientation, resupination, pollinator behavior, Orchidaceae.

Introduction

"Flower resupination — when the upper lip looks toward the ground, and the under lip toward heaven."

(Lee, 1774 *apud* Yam et al. 2009)

Angiosperms display a huge diversity of floral traits such as color, odor, shape and size, that are hypothesized to optimize individual reproduction and evolve in response to selection mediated by pollinators (Darwin, 1877; Stebbins, 1970; Harder & Johnson, 2009; Caruso et al. 2018). More recently, attention has been drawn to other traits such as flower orientation, which is the angular arrangement of a flower in relation to the ground (*e.g.*, vertical, horizontal or pendant). For instance, a vertical floral orientation allows the approach of pollinators from any random direction (Fenster et al. 2009). On the other hand, other orientations such as horizontal, pendant and semi-pendant promote the directionality of pollinator movement, leading to higher accuracy and precision of pollination through the correspondence of reproductive organs and the pollen placement in a specific area (Fenster et al. 2009). In addition to pollinator behavior, floral orientation may be further related to plant male and female reproductive successes (Wang et al 2014a), reproductive isolation between phylogenetic related species (Campbell et al. 2016), diversity of pollinators and their relative importance (Wang et al. 2014b), flower visual perception by pollinators (*e.g.*, polarization of light; Foster et al. 2014), and pollinator partitioning (Eisikowitch & Rotem 1987; Botes et al. 2009).

The floral orientations that promote pollinator unidirectionality are often related to zygomorphic floral symmetry (Neal et al. 1998; Ushimaru et al. 2009). This is traditionally recognized as a trait that forces the pollinator to occupy a specific direction plane when arriving in a flower, also increasing pollen deposition accuracy and precision.

In this sense, both zygomorphy and floral orientation are forms of phenotypic specialization that act synergistically optimizing pollination (Neal et al. 1998). Although floral orientation may change during a flower lifespan (*e.g.*, Eisikowitch & Rotem 1987), some zygomorphic flowers, may suffer even more drastic and specialized changes to achieve a certain orientation. This is the case of resupination, a mechanism that refers to the twist of ≈ 180º of flowers (Ames 1938; Arditti, 2003; Yam, et al. 2009) based on the torsion of pedicel, ovary, corolla or even the rachis (Ames 1938; Dworaczek & Claßen-Bockhoff, 2016 and references therein). After that, the flower that was directed upward becomes downward leaving it upside down.

Although it may sound unusual, resupination is a widespread phenomenon across angiosperms, evolving independently in 14 remotely related families (Dworaczek & Claßen-Bockhoff, 2016 and references therein). However, in no other family it is so common as in the Orchidaceae, the largest family of flowering plants (Christenhusz & Byng, 2016). In fact, it is a basal (Dressler, 1981) and dominant genetic trait in the family (Kim et al., 2010) and, despite some reversals, it is part of the bauplan of most orchid flowers.

The causal/proximal aspects of resupination in Orchidaceae are relatively well understood including the genetic dominance (Kim et al. 2010), the anatomical (Dines et al. 1994) and physiological components (Arditti, 2003; Yam, et al. 2009). However, its ultimate consequence, *i.e.*, on plant reproduction, has only been suggested since Darwin and not systematically tested. Studies under natural conditions addressing consequences in reproductive success as well as in the interaction with pollinators have never been done so far. Although remarkable among orchids and a classical issue on early literature (*e.g.*, Darwin 1877; Ames 1938; Hill, 1939), resupination remains largely unexplored when compared to other traits of the orchids (*e.g.*, column, pollinia, labellum, velamen). In addition, albeit several correlational and experimental studies highlight the importance of floral orientation, the specialized case of resupination has never being addressed. Some open questions include: (i) how the presence/absence of resupination influences the interaction with pollinators and consequently (2) the reproductive output.

Resupination lies on the idea that the reversal arranges zygomorphic flowers in a proper position that allows pollinator landing (Ames 1938 Darwin 1877; Van der Pijl & Dodson 1966; Mondragón-Palomino & Theißen, 2009). This arranges pollinator in a specific direction plane, allowing precise and accurate pollen transference. To test this in an appropriate way, we must have a special model with variable flower twist. Here, we find this suitable model as being a rare and vulnerable lady's slipper orchid: *Phragmipedium vittatum* (Cypripedioideae) (Fig. 1). This is a syrphid-fly pollinated species that employs oviposition-site mimicry (Cardoso et al. in prep). These hoverflies usually lay their eggs in aphid colonies in which aphidophagous larvae feeds on. Apparently, the pregnant females are deceived by flower morphology, which includes specialized aphid-mimicking spots located in a slippery zone of the pouch-like labellum. In this region, there is also mucilage accumulation, and the epidermis has downward pointing papillate cells (Cardoso et al, in prep). This makes the surface slippery, preventing the pollinator from holding on and causing it to slide down into the pouch. It can scape only after climbing the labellum back where pollen is deposited on stigma and them removed from anthers before the hoverfly leaves by one of the exiting holes. When inside, the posterior portion of the labellum interior has resistant hairs covered by cuticle that progressively increase in size as the pollinator climbs, probably using them as a "stairway" to hold on. There are also dark spots in this posterior region, suggesting that flowers use the same mimicry mechanism exploiting the pollinator sensory system in their way in and out of the flower. Egg-laying on both inner and outer surfaces of flowers provide a strong evidence of oviposition-site mimicry.

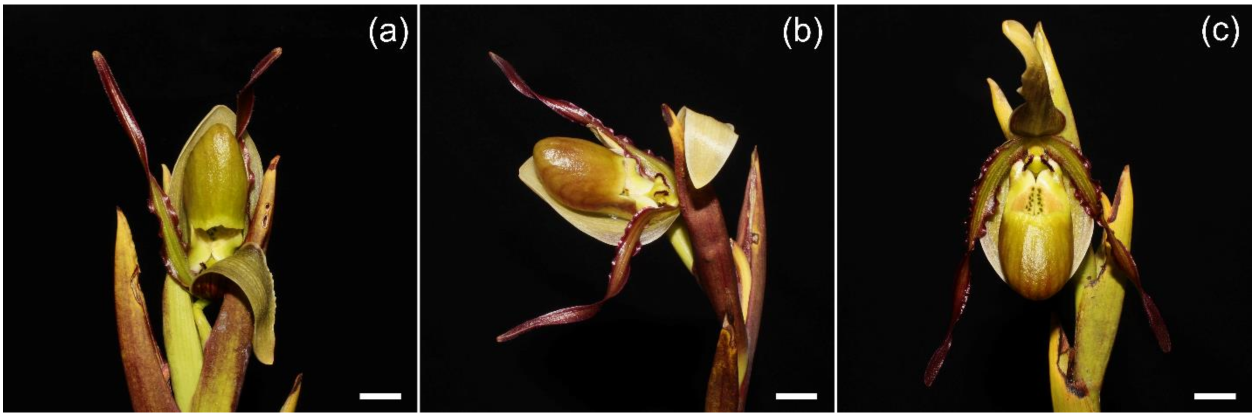


Fig. 1 *Phragmipedium vittatum* flowers on (a) non-resupinate, (b) half-resupinate and (c) normal/fully resupinate positions, respectively. Scale bar: 1cm.

Having such a sophisticated pollination system as example, here we characterize the different twist levels in the population and test how they may affect the reproduction of plants. We hypothesize that pollination is only possible because of resupination, that allows initial pollinator falling, trapping, and posterior pollen smears deposition and removal as it manages to escape the flower. The lack of proper resupination changes floral arrangement and disrupts the direction plane of arrival. In this sense, we predict that flowers that do not fully resupinate will not show either pollen deposition or removal.

Material and Methods

Study species and site

Phragmipedium vittatum (Vell.) Rolfe have herbaceous habit and occurs mainly in swampy regions of central Brazil (in the Cerrado biome). It is currently in a vulnerable state, threatened of extinction (Dias & Smidt 2020). Flowers open acropetally and last nine days, on average. After approximately six days, they start to become yellowish before detaching from the stem. After detachment, another flower opens and usually a single flower is opened each time per inflorescence (Cardoso et al., in prep). We studied a population that occurs at a private farm in the Goiás state. Climate of the region is seasonal with a rainy summer and a dry winter (Alvares et al., 2013). The studied population flowers once a year, between October and May, but peaking in December – February (Cardoso et al., in prep).

Procedures

We censused open flowers during 2016 - 2017 and 2018 - 2019 flowering considering their level of twist, grouping then in three categories: 1) non-resupinate: flowers positioned upward, between 0º - 60º (Fig. 1a; Fig. 2a); 2) half-resupinate: flowers positioned sideward, between 60º - 120º (Fig. 1b; Fig. 2b); 3) resupinate: flowers positioned downward, between 120º - 180º (Fig. 1c; Fig. 2c). Them, we investigated the role of resupination by conducting an experiment in which we artificially manipulated the orientation of flowers using plasticized white wires. During eight days in 2021, newly opened flowers from previously bagged buds were submitted to one of the following treatments: 1) non-resupination: flowers were tied on the bracts in an upside-down position with the tip of the labellum facing up (≈ 0º; n = 17); 2) half-resupination: flowers were tied in a lateral position with the tip of the labellum facing sideward (≈ 90º; n = 31); 3) resupination: flowers were tied in their normal position with the tip of the labellum facing downward (≈ 180º; n = 35); 4) control: flowers were only unbagged without the placement of the wire (≈ 180º; n = 28). After tied, the excess of wire was removed using pliers to reduce its display. The male and female successes (*i.e.*, pollen smears removal and deposition, respectively) were checked in the fifth day after treatment. In addition, to correct for confounding factors, we collected the height of flower on inflorescence, the number of the flower in inflorescence (from the botton to top) and the labellum length and width. We used these two latter to calculate the volume of the labellum based on the formula of a prolate ellipsoid ($^4/_3$*pi*($^{labellum\_height}/_2$)*($^{labellum\_width}/_2$)$^{\wedge 2}$). We used the labellum volume since it is a good proxy of flower size and weight (Cardoso et al., in prep).

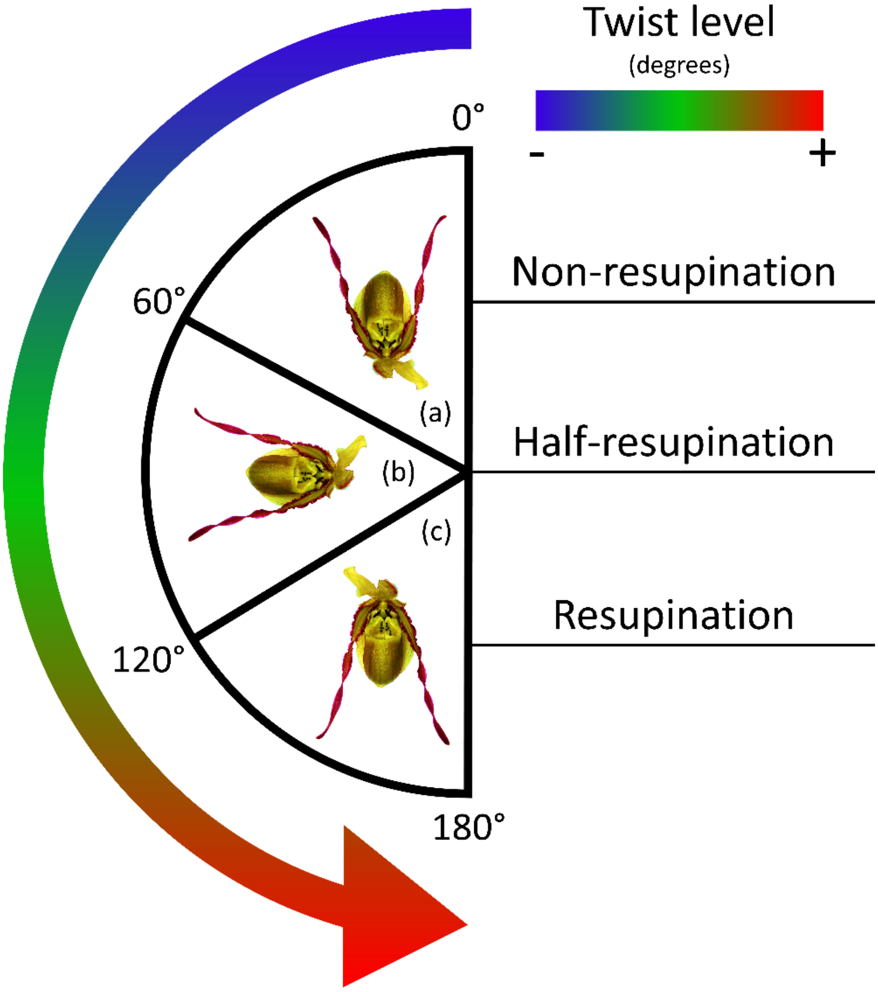


Fig. 2 Different patterns of flower orientation classifications in *Phragmipedium vittatum* along the continuum of degrees of the semi-circle. (a) Non-resupinate: between 0º - 60º; (b) half-resupinate: between 60º - 120º; (c) resupinate: between 120º - 180º.

We stuffed cotton into both exiting roles of flowers to prevent captured pollinators from leaving (see Cardoso et al., in prep). Later, when the *Dioprosopa clavata* pollinators were inside the pouch, we artificially simulate half- (n = 7) and non-resupination (n = 9) to investigate the role of flower orientation in allowing the pollinators to exit by the entrance hole. When the pollinator was at the bottom of the pouch, we manually twisted the flower and recorded if it left by the entrance hole and the time spent to leave.

Statistical analyses

For each year of survey, we performed pairwise chi-square goodness of fit tests to access if the frequency of flowers differed among the three category levels of twist (non-, half- and full resupination). Using the *p.adjust* function in R base package, we applied Bonferroni correction to the p-values of each year to reduce the chances of type I error.

We investigated differences among the treatment groups in the artificial twisting experiment by fitting a GLMM (generalized linear mixed model) with binomial distribution and logit link. We grouped data with male success (pollen smears removal from one or two anthers) as our response variable having the treatment group as fixed effect and flower heigh, number of the flower in the inflorescence and labellum volume as random effects. We used model adjusted values for plotting by back-transforming estimated marginal means and standard errors using the R-package RVAideMemoire version 0.9-77 (Hervé, 2020). We did not investigate effects on the female reproductive success because it comprised a smaller number of occurrences (see results).

We conducted the GLMM using the R-package *lme4* version 1.1.25 (Bates et al. 2019) and tested the significance through the likelihood ratio (LR) test (*i.e.*, by comparing models with the predictor variable against that without it to attain p-values; Zuur *et al.*, 2009). Post-hoc analyses were conducted using the Tukey adjusted contrasts in the R-package *emmeans* version 1.4.8. (Lenth 2020). Since the presence of only zeros in some groups did not allow a proper post-hoc comparison, we added a constant of +1 in all groups and re-ran the GLMM for conducting the contrasts. All analyses were carried out in R version 4.0.3 (R Core Team 2020).

Results

We observed that flowers did not fully resupinate because they remained trapped between the bract and the stem (Fig. 1a,b). We surveyed a total of 2901 flowers in 2016 - 2017 flowering and 2083 flowers in 2018 - 2019 (Fig. 3a). In the first flowering, fully resupinate flowers were the most common (92.31%), being significantly different from non- (4.17%) ($\chi^2 = 2335.9$; df = 1, $p < 0.001$) and half-resupinate (3.52%) ($\chi^2 = 2387$; df = 1, $p < 0.001$), with these latter not being different from each other ($\chi^2 = 1.62$; df = 1, $p = 0.61$). We found similar patterns for 2018 - 2019 flowering season, with resupinate flowers (89.63%) being significantly more abundant than both non- (5.09%) ($\chi^2 = 1571.8$; df = 1, $p < 0.001$) and half-resupinate ones (5.28%) ($\chi^2 = 1561.5$; df = 1, $p < 0.001$), which were not different from each other ($\chi^2 = 0.074$; df = 1, $p = 0.79$).

We found significant effects in our experiment comparing artificially twisted flowers with respect to pollen smears removal ($\chi^2 = 80.93$; df = 3, $p < 0.001$). Flowers tied on a normal/resupination position had 75.00% of pollen smears removal (Fig. 3b), with 50.00% being removal from one and 25.00% from two anthers. This group was not different from that of control flowers, that had the same frequency of pollen smears removal (75.00%; Fig. 3b), being 42.86% from one anther and 32.14% from two. This indicates that there was no effect of the wire on floral display and visitation rates. On the other hand, non-resupination and half-resupination groups did not have a single pollen smear removal. (Fig. 3b).

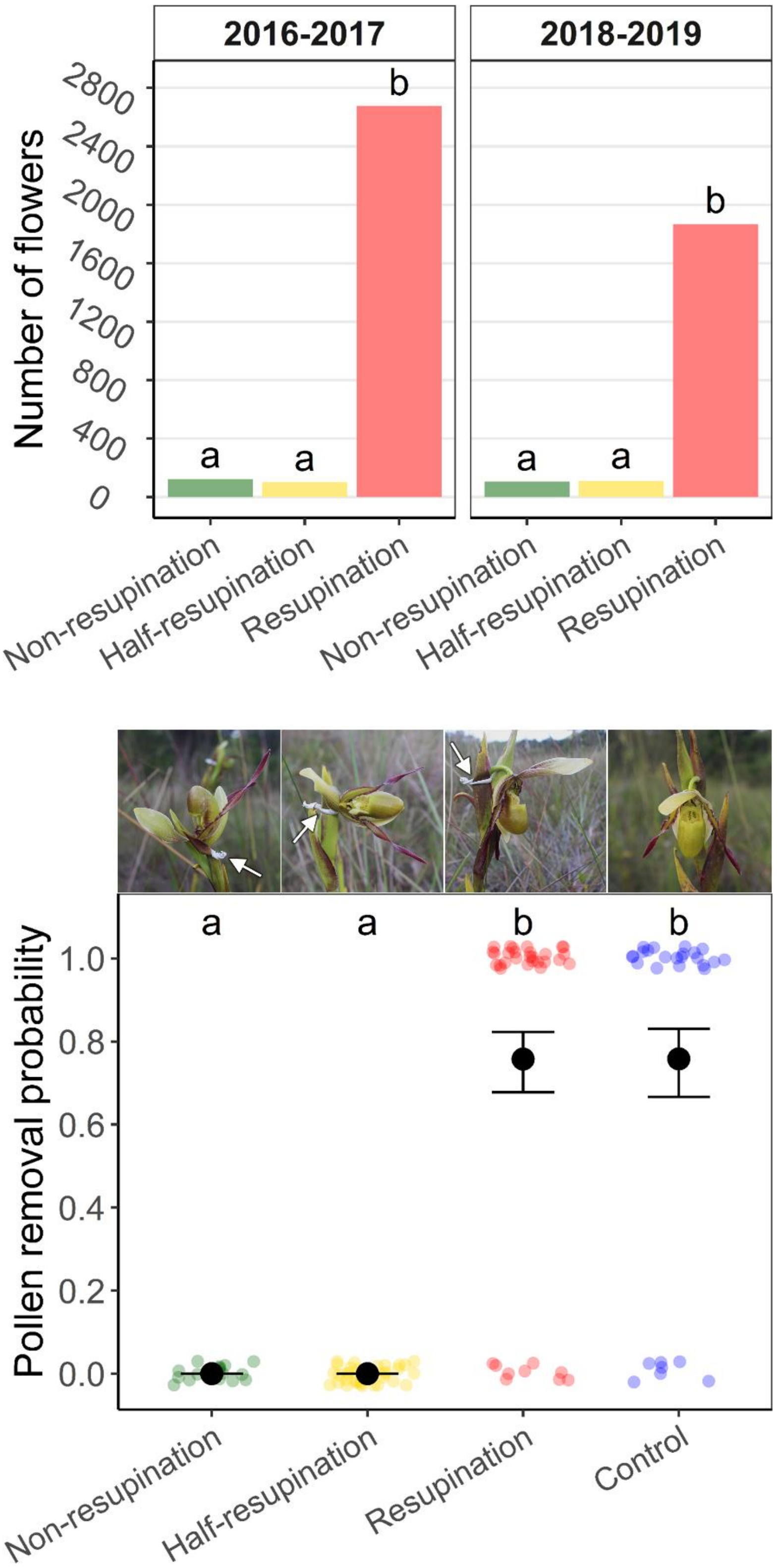


Fig. 3 (a) Frequency of resupination categories in 2016 - 2017 and 2018 - 2019 years. (b) Probability of pollen smears removal according to the different treatment levels. Black dots and line segments show back-transformed marginal (model adjusted) means and standard errors, respectively. Colored dots show observed data points. Different letters indicate significant differences at 0.05 level. White arrows show the wire tied on inflorescences, whenever present.

We found similar patterns related to pollen smears deposition. Flowers from the resupination group had 13.89% of deposition followed by those of control group that had 10.71%. Again, no female success was observed either in groups submitted to non- and half-resupination. We observed that although non- and half-resupinate flowers still attracted the attention of hoverflies, their orientation seems ineffective to trap them through gravitational attraction, since they only fell in the pouch in the downward oriented flowers.

Later, we confirm that the flower orientation is important in keeping the hoverflies trapped, preventing them from leaving through the pouch entrance. After artificial twist, all *D. clavata* individuals within flowers in which twist was simulated went out through the initial entrance hole. In half-resupination simulation, they went out on an average of only 6.67 sec (± 12.28 SD) while those under half resupination left in an average of 7 sec (± 5.89 SD).

Discussion

Phragmipedium vittatum has a highly phenotypically specialized pollination mechanism involving several adaptive traits that ensure pollinator falling, trapping, and climbing. These traits work together in the pollination biomechanics, allowing efficient deposition and exportation of pollen smears. However, such a sophisticated system only works because flowers are in a proper orientation. Here, we demonstrate that both pollen smears exportation and deposition only occur in flowers that are upside down. Without resupination, even though pollinators are attracted to the aphid-like spots at the slippery zone, flowers fail in trapping them. In addition, they can easily get out by the entrance hole of the labellum as we showed artificially twisting flowers with pollinators inside.

Our results highlight the importance of floral orientation and, more specifically resupination in the effective usage of pollinators.

Although the proportion of non-twisted flowers (non- + half-resupination) was lower than those with normal resupination (7.69% in 2016 and 10.37% in 2019), this may have important negative impacts in the overall reproductive output of the population. Since *P. vittatum* is xenogamous and non-autonomous selfer (Cardoso et al., in prep), those ~ 10% are faded to reproductive fail. This is important specially when we contrast with the reproductive biology patterns. Cardoso et al. (in prep) report lower fruit formations in natural pollination treatments (31.51%) when compared to manually cross (88.24%). In addition, they found that the mean of pollinia deposition during four years of sampling was only 16.06%, highlighting pollinator limitation in the long term. These results indicate that flowers do not reach their maximum reproductive potential. This is especially important at the individual level. For instance, an individual with 2 flowers having one failing to twist would lose half of its chances of pollinator visitation and reproduction. Orchids are r-strategists when it comes to the number of seeds set per fruit unity, investing in quantity to increase chances of effective dispersion and seedling establishment (Arditti & Ghani 2000). For instance, our studied species can have more than a hundred thousand seeds (maximum = 101,460) (Cardoso et al, in prep). Thus, as each single fruit can bear so many seeds, the reduction in fruit set by ontogenetic problems related to resupination cannot be overlooked. Such wastage of flowers available to pollination and the related fruit set reduction effects are especially important in the case of *P. vittatum*, a threatened species that lives in highly restrictive and competitive environments (swamps), which may further disrupt the equilibrium and maintenance of populations. Thus, our results highlight the importance of proper orientation through resupination in plant reproduction.

Zygomorphy in flowers is considered a derived and more phenotypically specialized trait related to the diversification of several groups (Neal et al. 1998; Sargent, 2004; Endress 2012). It is often related to the horizontal orientation of flowers, restricting the pollinator direction of approach to a single plane, and constraining its landing behavior to a determined site. (Neal et al. 1998; Ushimaru et al. 2009). In addition, the horizontal position of zygomorphic flowers may facilitate pollinator recognition and memorization of complex flower traits (Neal et al. 1998; Ushimaru et al. 2009). After recognition and consistent unidirectional landing, the clustering of stigmas and/or anthers found in several species contact the pollinators' body in a smaller area, reducing the variance and promoting higher deposition accuracy (Fenster et al 2009). Orchids function as an extreme of this, clustering pollen in pollinia or pollen masses that are placed in precise locations on pollinators (Dressler, 1981; Armbruster, 2014). Although this increases both accuracy and precision, a consistent pollinator approaching is required for the mechanism to work. Thus, as resupination reorients flowers horizontally, it is apparently related to other floral traits including zygomorphy, landing platform arrangement (*e.g.*, labellum, standard or other modified lip in other families) and clustering of reproductive organs.

Resupination is closely related to flower zygomorphy in several families. For instance, in the subfamily Apostasioideae, the first branching group of the Orchidaceae, resupination does not occur in the actinomorphic genus Apostasia, but is present in the zygomorphic Neuwiedia (Kocyan and Endress, 2001). In agreement, one of the most noticeable structure promoting zygomorphy in orchids is the labellum, that probably is the result of the differential expression of specific genes when compared to other whorls (Mondragón-Palomino & Theißen, 2009).

The relationship between labellum and pollination process has been assumed for a long time (Ames 1938; Darwin 1877; Van der Pijl & Dodson 1966). In the classic orchid bauplan, the labellum seems to be adapted to several specific pollinators (Mondragón-Palomino & Theißen, 2009) and although it can attract and reward pollinators, one of its major functions is that it serves as a landing platform in resupinate flowers (Ames 1938; Darwin 1877; Van der Pijl & Dodson 1966). As discussed, this promotes consistent pollinator directionality and pollen deposition. An important feature is that resupination in orchid flowers not only places the labellum lowermost but also rearranges the column uppermost, leaving anther and stigma in direct opposition to the labellum (Mondragón-Palomino & Theißen, 2009). When the insect enters the flower, pollen is deposited nototribically (at the topside of the pollinator). When entering a different flower, stigma is faced first, and pollen transference occurs successfully due to orchids low herkogamy.

Comparatively, non-resupinate flowers would have pollen deposited sternotribically (at the underside of the body). This would make pollinia more easily removed by the pollinator through cleaning behaviors and easier to fall due to gravity. Although the vast majority of orchids are adapted to nototribic pollination (Johnson & Edwards, 2000) and resupinate, a useful contrast is the pollination mechanics of species-rich orchid groups with non-resupinate flowers, in which pollinia usually is transported ranging on the proboscis or eyes of pollinators such as flies, butterflies and moths (*e.g.*, Johnson 2000). However, this bellow body deposition seems not effective in other pollinators. This would be the case of bees (but see some examples as Johnson & Steiner, 1994), one the most common group of orchid pollinators (Baguette et al. 2020), which would easily remove pollinia from proboscis and venter due to their grooming behaviors. In fact, the midline of the dorsal thorax is one of the safest sites for pollen deposition for being one of the areas that bees least clean pollen (Koch et al. 2017; Tong & Huang 2018). Furthermore, it was demonstrated that pollen-sacs and stigma of the apparently resupinate *Salvia pratensis* (Lamiaceae) contacted the same safe site of the bee pollinator, which was the dorsal thorax (Koch et al. 2017). The functional similarities are interesting as some resupinate *Salvia* use the upper lip as landing platform (Claßen-Bockhoff et al. 2004). Thus, resupination, landing platform creation and nototribic pollination probably have a correlated evolution, as sternotriby (bellow pollen deposition) would reduce the chances of effective cross-pollen transportation. These ideas may open new avenues of investigation.

Our study system with variable twist on flowers provides unique circumstances to test the role of resupination. Further approaches with this special system indicate that flower weight is the most important trait determining resupination success in this special species (Cardoso et. al, in prep), which suggests a selection on flower weight that ensures reproduction. Our results indicate that the breakdown of resupination affects the pollination dynamics of flowers with complex mechanisms of pollen transference adapted to specific pollinators. We provide important insights into the ecology and evolution of floral orientation, which may be expanded to the several other flowers from distinct angiosperm families that resupinate as an adaptive trait, thus enabling accurate pollen transference.

Acknowledgements

JCFC is grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for a PhD grant. SDJ is grateful to the South African Research Chairs Programme (NRF grant 46372). PEO is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for his research grant and financial support.

References

Alvares, C. A., Stape, J. L., Sentelhas, P. C., Gonçalves, J. D. M., & Sparovek, G. (2013). Köppen's climate classification map for Brazil. Meteorologische Zeitschrift, 22(6), 711-728. https://doi.org/10.1127/0941-2948/2013/0507

Ames, O. (1938). Resupination as a diagnostic character in the Orchidaceae with special reference to *Malaxis monophyllos*. Botanical Museum Leaflets, Harvard University, 6(8), 145-183. https://doi.org/10.5962/p.295120

Arditti, J. (2003). Resupination. Lankesteriana 7: 95-96.

Arditti, J., & Ghani, A. K. A. (2000). Numerical and physical properties of orchid seeds and their biological implications. New Phytologist, 145(3), 367-421. https://doi.org/10.1046/j.1469-8137.2000.00587.x

Armbruster, W. S. (2014). Floral specialization and angiosperm diversity: phenotypic divergence, fitness trade-offs and realized pollination accuracy. AoB Plants, 6. https://doi.org/10.1093/aobpla/plu003

Baguette, M., Bertrand, J. A., Stevens, V. M., & Schatz, B. (2020). Why are there so many bee-orchid species? Adaptive radiation by intra-specific competition for mnesic pollinators. Biological Reviews, 95(6), 1630-1663. https://doi.org/10.1111/brv.12633

Bates, D., M. Maechler, B. Bolker, S. Walker, R. H. B. Christensen, H. Singmann, B. Dai, F. Scheipl, G. Grothendieck, P. Green. 2019. lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-21. https://CRAN.R-project.org/package=lme4

Botes, C., Johnson, S. D., & Cowling, R. M. (2009). The birds and the bees: using selective exclusion to identify effective pollinators of African tree aloes. International Journal of Plant Sciences, 170(2), 151-156. https://doi.org/10.1086/595291

Campbell, D. R., Jürgens, A., & Johnson, S. D. (2016). Reproductive isolation between *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth foraging choices. New Phytologist, 210(1), 333-342. https://doi.org/10.1111/nph.13746

Caruso, C. M., Eisen, K. E., Martin, R. A., & Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. Evolution, 73(1), 4-14. https://doi.org/10.1111/evo.13639

Christenhusz, M. J., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. Phytotaxa, 261(3), 201-217. https://doi.org/10.11646/phytotaxa.261.3.1

Claßen-Bockhoff, R., Speck, T., Tweraser, E., Wester, P., Thimm, S., & Reith, M. (2004). The staminal lever mechanism in *Salvia* L. (Lamiaceae): a key innovation for adaptive radiation?. Organisms Diversity & Evolution, 4(3), 189-205. https://doi.org/10.1016/j.ode.2004.01.004

Darwin, C. (1877). On the various contrivances by which British and foreign orchids are fertilised by insects. John Murray. https://doi.org/10.5962/bhl.title.60877

Dias, M.C.; Smidt, E.C. 2020. *Phragmipedium* in Flora do Brasil 2020. Jardim Botânico do Rio de Janeiro. Available at: http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB32001. Accessed on May 27, 2021

Dines, T. D., & Bell, A. D. (1994). Differential cell enlargement and its possible implication for resupination in *Lemboglossum bictoniense* (Orchidaceae). Botanical journal of the Linnean Society, 114(1), 67-79. https://doi.org/10.1111/j.1095-8339.1994.tb01924.x

Dressler, R.L. (1981) Orchids: natural history and classification. Harvard University Press, Cambridge, MA.

Dworaczek, E., & Claßen-Bockhoff, R. (2016). 'False resupination' in the flower-pairs of *Thalia* (Marantaceae). Flora, 221, 65-74. https://doi.org/10.1016/j.flora.2015.08.007

Eisikowitch, D., & Rotem, R. (1987). Flower orientation and color change in *Quisqualis indica* and their possible role in pollinator partitioning. Botanical Gazette, 148(2), 175-179. https://doi.org/10.1086/337645

Endress, P. K. (2012). The immense diversity of floral monosymmetry and asymmetry across angiosperms. The Botanical Review, 78(4), 345-397. https://doi.org/10.1007/s12229-012-9106-3

Fenster, C. B., Armbruster, W. S., & Dudash, M. R. (2009). Specialization of flowers: is floral orientation an overlooked first step?. The New Phytologist, 183(3), 502-506. https://doi.org/10.1111/j.1469-8137.2009.02852.x

Foster, J. J., Sharkey, C. R., Gaworska, A. V., Roberts, N. W., Whitney, H. M., & Partridge, J. C. (2014). Bumblebees learn polarization patterns. Current Biology, 24(12), 1415-1420. https://doi.org/10.1016/j.cub.2014.05.007

Harder, L. D., & Johnson, S. D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytologist, 183(3), 530-545. https://doi.org/10.1111/j.1469-8137.2009.02914.x

Hervé, M. 2020. RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R package version 0.9-78. https://CRAN.R-project.org/package=RVAideMemoire

Hill, A. W. (1939). Resupination studies of flowers and leaves. Annals of Botany, 3(4), 871-887. https://doi.org/10.1093/oxfordjournals.aob.a085096

Johnson, S. D. (2000). Batesian mimicry in the non-rewarding orchid *Disa pulchra*, and its consequences for pollinator behaviour. Biological Journal of the Linnean Society, 71(1), 119-132. https://doi.org/10.1006/bijl.1999.0430

Johnson, S. D., & Edwards, T. J. (2000). The structure and function of orchid pollinaria. Plant Systematics and Evolution, 222(1), 243-269. https://doi.org/10.1007/BF00984105

Johnson, S. D., & Steiner, K. E. (1994). Pollination by megachilid bees and determinants of fruit-set in the Cape orchid *Disa tenuifolia*. Nordic Journal of Botany, 14(5), 481-485. https://doi.org/10.1111/j.1756-1051.1994.tb00637.x

Kim, S. Y., Endo, M., Yun, P. Y., & Kanno, A. (2010). Production of intraspecific hybrids between wild-type and petaloid-sepal cultivars in *Habenaria radiata*. Scientia horticulturae, 124(3), 415-418. https://doi.org/10.1016/j.scienta.2010.01.022

Koch, L., Lunau, K., & Wester, P. (2017). To be on the safe site–Ungroomed spots on the bee's body and their importance for pollination. PloS one, 12(9), e0182522. https://doi.org/10.1371/journal.pone.0182522

Kocyan, A., & Endress, P. K. (2001). Floral structure and development of *Apostasia* and *Neuwiedia* (Apostasioideae) and their relationships to other Orchidaceae. International Journal of Plant Sciences, 162(4), 847-867. https://doi.org/10.1086/320781

Lenth RV (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.5.3. https://CRAN.R-project.org/package=emmeans

Mondragón-Palomino, M., & Theißen, G. (2009). Why are orchid flowers so diverse? Reduction of evolutionary constraints by paralogues of class B floral homeotic genes. Annals of Botany, 104(3), 583-594. https://doi.org/10.1093/aob/mcn258

Neal, P. R., Dafni, A., & Giurfa, M. (1998). Floral symmetry and its role in plant-pollinator systems: terminology, distribution, and hypotheses. Annual Review of Ecology and Systematics, 29(1), 345-373. https://doi.org/10.1146/annurev.ecolsys.29.1.345

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Sargent, R. D. (2004). Floral symmetry affects speciation rates in angiosperms. Proceedings of the Royal Society of London. Series B: Biological Sciences, 271(1539), 603-608. https://doi.org/10.1098/rspb.2003.2644

Stebbins, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: Pollination mechanisms. Annual Review Ecology and Systematics 1:307–326 https://doi.org/10.1146/annurev.es.01.110170.001515

Tong, Z. Y., & Huang, S. Q. (2018). Safe sites of pollen placement: a conflict of interest between plants and bees?. Oecologia, 186(1), 163-171. https://doi.org/10.1007/s00442-017-3999-9

Ushimaru, A., Dohzono, I., Takami, Y., & Hyodo, F. (2009). Flower orientation enhances pollen transfer in bilaterally symmetrical flowers. Oecologia, 160(4), 667-674. https://doi.org/10.1007/s00442-009-1334-9

Van der Pijl, L, & C. H. Dodson. 1966. Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables, Fla.

Wang, H., Tie, S., Yu, D., Guo, Y. H., & Yang, C. F. (2014a). Change of floral orientation within an inflorescence affects pollinator behavior and pollination efficiency in a bee-pollinated plant, *Corydalis sheareri*. PloS one, 9(4), e95381. https://doi.org/10.1371/journal.pone.0095381

Wang, H., Xiao, C. L., Gituru, R. W., Xiong, Z., Yu, D., Guo, Y. H., & Yang, C. F. (2014b). Change of floral orientation affects pollinator diversity and their relative importance in an alpine plant with generalized pollination system, *Geranium refractum* (Geraniaceae). Plant ecology, 215(10), 1211-1219. https://doi.org/10.1007/s11258-014-0379-y

Yam, T. W., Arditti, J., & Cameron, K. M. (2009). “The orchids have been a splendid sport”—an alternative look at Charles Darwin's contribution to orchid biology. American Journal of Botany, 96(12), 2128-2154. https://doi.org/10.3732/ajb.0900122

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). Mixed effects models and extensions in ecology with R. New York, NY: Springer. https://doi.org/10.1007/978-0-387-87458-6

Capítulo 4

The flowers meet Newton: testing the role of gravitational pull in flower orientation

Abstract

Resupination refers to the twist of flowers of approximately 180º, leaving them upside-down. It is a widespread trait across angiosperms, but in no other family it is so common as in the Orchidaceae, where it has been shown as driven by auxins. This hormonal control puts resupination as a positive gravitropic phenomenon, which is an active mechanism due to directional growth. In this study, we demonstrate that gravity may influence resupination in another way: through gravitational pull. According to this idea, the weight of the flower may influence the twist, this time as a passive mechanism. We used a unique population of a lady's slipper orchid that has variable twist, with some flowers failing to resupinate. We hypothesized that heavier/more voluminous flowers are more likely successfully resupinate. We show that flowers that naturally do not resupinate are smaller and lighter. We conducted a manipulative experiment removing floral whorls and show that both resupination probability (*i.e.*, with more than 120º twist) and proportion of arc degrees twisted (from 0º – 180º) were determined by flower weight. We demonstrate that pre-anthesis buds gain considerable weight due to water uptake, which increases the influence of gravitational pull on flowers. However, we still noticed that the tip of the ovarium slightly bends due to gravitropism. Although this initial movement was not enough to complete the twist, it is important to move the center of mass of the zygomorphic flowers away from the inflorescence axis in a way that gravitational pull may act on the weight. Finally, we demonstrate that the presence of unusual long bracts is the reason why flowers get stuck, highlighting the importance of weight to flowers get rid of them. The role of weight as an essential trait arranging flowers on a proper position provides new insights into the working machinery that ensures efficient attraction and morphological fit of pollinators.

Keywords: Gravity, Orchidaceae, Resupination, Flower Orientation

Introduction

"In almost all the species, one of the petals, which is properly the upper one, is larger than the others and stands on the lower side of the flower, where it offers a landing-place for insects, having been carried round by the twisting of the ovarium."

Darwin,1877, The various contrivances by which orchids are fertilised by insects

This quote by Charles Darwin in his famous book about orchids shows that, for a long time, it has been known that orchids (and other flowers) do a weird thing: they twist and change orientation drastically, getting upside down! This is called resupination, a mechanism that was initially described by Linné in the 18th century (Linné, 1753). Classical definitions of resupination characterize it as a complete turn of flowers through rotation so that what was directed upwards becomes downwards (*sensu* Goebel, 1924). Such traditional definition brings the idea of a fixed 180° twist, which is used by some authors (*e.g.*, Hill, 1939; Dworaczek & Claßen-Bockhoff, 2016; Smyth, 2016). However, a more non-conservative definition (which we use herein), is that flower movement is such that leaves the flower properly upside down oriented by performing a 180° circuit or more or less than that, varying according to the initial position of flower or inflorescence (Ames 1938; Nyman et al. 1984; Arditti, 2003; Yam, et al. 2009). Resupination is associated to the torsion of pedicel, ovary or even corolla (Dworaczek & Claßen-Bockhoff, 2016 and references therein) or rachis (Ames 1938). It is a homoplastic trait, being scattered in 14 angiosperm families (Dworaczek & Claßen-Bockhoff, 2016 and references therein). However, in the monocots, it has been reported only in Heliconiaceae, Zingiberaceae and specially in Orchidaceae.

Unlike in any other family, there are numerous interesting peculiarities of the twist in orchids. For instance, the resupination presence may be related with sexual dimorphism in several *Catasetum* (Epidendroideae: Catasetinae) species that have staminate/resupinate and pistillate/non-resupinate flowers (Dressler, 1981; Romero et al 2009). In other species, although it looks like that they are not twisted, they actually performed hyper-resupination with a twist of 360º with the labellum returning to its original/uppermost position (Darwin 1877; Ames 1938; Hill, 1939; Dressler, 1981; Arditti, 2003; Yam, et al. 2009). In fact, some movements are complex and may occur at different planes as in *Angraecum eburneum* (Epidendroideae: Angraecinae) whose flowers twist themselves in so many directions and movements that together they sum 630º (Hill, 1939)!

In orchids, resupination is probably basal and occurs in most of species, having been lost or modified in many groups (Dressler, 1981). As it is widespread, is probably that resupination is inherited as a dominant character in orchids. Kim et al. (2010) corroborated this by crossing wild/resupinate and cultivar/non-resupinate types of *Habenaria radiata* (Orchidoideae: Habenariinae) and found that intraspecific hybrids had resupinate flowers. At early ontogenetic phases of orchids, the labellum (a modified petal) is adaxial, and becomes lowermost in the perianth prior to anthesis phase or soon after it (Ames 1938; Hill, 1939; Dressler, 1981; Nyman et al. 1984; Nyman et al. 1985; Arditti, 2003; Yam, et al. 2009).

The movements involve ontogenetic tissue modification. For instance, by comparing pedicels of the orchid *Rhynchostele bictoniensis* (Epidendroideae: Oncidiinae) prior and after resupination, Dines et al. 1994 demonstrated that specialized raphide-bearing cells in the cortex expand axially inducing the radial expansion of cortical parenchyma promoting the twist. The changes in cell reconfiguration are physiologically driven by auxins (Nair & Arditti 1991; Arditti, 2003; Yam, et al. 2009). Nyman et al. 1985 experimentally demonstrated that the removal of the entire gynostemium from buds, its tips (stigma and pollinia) or even pollinia solely prevents resupination of *Dendrobium* (Epidendroideae: Dendrobiinae) hybrids. Since the pollinia produces large amounts of indoleacetic acid, this suggested that the mechanism was related to auxins. This was latter demonstrated by artificial applications of indoleacetic and naphthaleneacetic acids to non-resupinate buds of another hybrid *(Aranda Kooi Choo*, Epidendroideae) whose gynostemium have been previously removed, causing the flowers to resupinate (Nair & Arditti 1991). Such hormonal control puts resupination as a positive gravitropic phenomenon. In agreement, working with the same hybrid, it was latter shown that the application of gravitropism-affecting chemicals such as morphactin and 2,3,5-triiodobenzoic acid (TIBA) prevent resupination (Nair & Arditti 1992).

By definition, gravitropism is the directed growth of a plant or plant organ in response to gravity (Kiss, 2000; Nakamura et al 2019). Gravitropism involves complex intracellular mechanisms with sequential mechanisms, since gravity sensing, gravity signaling and intercellular signal transmission until the asymmetric organ growth (see Nakamura et al 2019 and references therein). Thus, gravitropism may occur in nearly weightless conditions (*e.g.*, as in root tips) and is an active mechanism. However, are there other non-gravitropic mechanisms that may relate to resupination?

Contrary to gravitropism, gravitation is a passive mechanism and dependent on weight. According to Newton's law of universal gravitation, two body masses attract each other with a force that is directly proportional to the product of the two masses, and inversely proportional to the square of the distance separating them (Newton, 1687; Cohen & Whitman, 1999). As the mass of the earth and the distance from its center to the center of mass of flowers in a population can be considered constant, the weight of a flower is the single determinant of how much it will be influenced by the Earth's gravitational pull.

The role of flower weight has not been dissociated from gravitropism itself in flower resupination. The orientation of an organ may also be affected by its weight since heavier structures tend to suffer more influence from the gravitational pull. Flower weight might be especially important in orchids, that besides being frequently resupinate, have several species with showy and heavy flowers. For instance, some orchid flowers weight more than 100 g (Gerlach, 2011). An analogy with fruits can help us to understand how gravitational attraction is different from gravitropism. Fruits usually appear hanging on plants probably not because their pedicel grew directing down, but rather because fruits are heavy, and them they change their orientation during development according to the gravitational pull. Before anthesis, flower buds function as sinks of photoassimilates, nutrients and mainly water (Galen et al. 1999). Thus, considering the resupination of flowers, we hypothesize that in addition to the hormonal control already known, weight gain through water uptake by pre-anthesis buds may also play a role, in which the direction to twist is determined by the intrafloral weight differences provided by zygomorphy. In this case, the labellum, which is the heavier part of several orchids, would play the most important role. According to this idea, gravity would still act, but indirectly: first on the weight attraction towards earth because buds become heavier before anthesis and consequently in flower reorientation (and not only in flower orientation directly as an intracelular mechanism). Although some authors have briefly speculated that weight may play a role in flower resupination (Ames, 1938; Pfeifer, 1966; Kurzweil & Kocyan, 2002), it has never been tested so far.

The ideal way to investigate the role of weight in flower resupination properly is to have a special system in which the same population has both non-resupinate and resupinate flowers, allowing to access if weight determines the chances of resupination. Here, we found such model species as being *Phragmipedium vittatum* (Cypripedioideae subfamily), a lady's slipper orchid with variable twist on the same population (Cardoso et. al, in prep; Cardoso & Oliveira, in press; Fig. 1). Approximately 10 % of flowers are non-resupinate. They fail to complete the twist because they remain trapped between the bract and the stem (Fig. 1B; Fig. 4C,E). We characterize the flower twist of this species and conduct a series of observational and experimental essays. According to our hypothesis of weight influencing the chances of resupination, we predict that (i) the whorls from buds right after resupination are heavier than those before resupination due to weight increment via water uptake; (ii) lighter flowers have more changes of not performing their natural resupination movement because they do not have enough weight to get rid of bracts; and (iii) the experimental removal of heavier whorls would make flowers lighter and therefore increase the chances on non-resupination. In addition to the unprecedent test of weight on resupination, our results are important because they might shed light into the evolution of the character on the several angiosperm families and especially in the Orchidaceae, since our studied species is from the basal Cypripedioideae subfamily, offering insights to comparisons with the more derived groups.

Figure 1. *Phragmipedium vittatum* details. (A) Bract comparisons in relation to the flower for *P. vittatum* (right plane) and for two representants of the sister genus *Paphiopedilum* (*Paphiopedilum* cf. *delenatii* in the top left and *Paphiopedilum* sp. in the botton left). (B) *Phragmipedium vittatum* in the habitat showing a resupinate flower (in the left) next to a non-resupinate flower.

Material and Methods

Studied species

Phragmipedium vittatum (Vell.) Rolfe (Cypripedioideae) is a paludal herb that occurs mainly in the veredas of the Cerrado (the Neotropical savanna in central Brazil), characterized by by a dense herb-subshrub dominant layer, with scattered small trees and frequently the buriti palm (*Mauritia flexuosa* L. f.) (Araújo et al. 2002). It consists in a rare lady's slipper species in vulnerable state of extinction (Dias & Smidt, 2020). Our studied population is situated on a private farm in the Goiás state, Brazil. All procedures were carried out using plants under natural conditions between 2018 and 2020.

Resupination description

To characterize the twist along the semicircle and how it takes place along time, we followed 25 flowers measuring their angulation with a protractor. We selected flowers the day before they resupinate and followed them during five time periods for two days: 6AM, 9AM, 12AM, 15AM and 18AM.

We compared if the proportion of flower twist differ among the different time periods by fitting a GLMM (generalized linear mixed-effects model) using the R-package *glmmTMB* (Brooks et al. 2017) (as in all following GLMMs). We fitted a random slope of time within flower identity with correlated intercept to account for temporal pseudorreplication (Crawley, 2013). As response variable, we took the proportion of resupination as response variable ($^{\text{Resupinate arc degrees}}/_{180^{\circ}}$) and used the beta distribution (with logit link). The beta distribution allows values between 0 and 1 but not exactly equal to these. The twist of flowers varied from 10º (proportion = 0.05555…) until 180º (proportion = 1). To get around this methodological detail, we them redistributed data setting 0.05556 as minimum and 0.99999 as maximum using the R-package *scales*

(Wickham and Seidel, 2020). After this procedure, the relative distances between data points did not change but rather their scale.

We tested model significance through the likelihood ratio (LR) test by comparing the full against the null model (*i.e.*, without the fixed effect) to attain p values to the effect of the predictor variable (Zuur et al. 2009). *Post hoc* analysis was conducted by comparing the estimated marginal means using Tukey adjusted contrasts in the R-package *emmeans* version 1.5.0 (Lenth et al 2020) (as in all following *post hocs*).

Factors related to resupination

To investigate which factors are responsible for impairing normal resupination, we sampled flowers in anthesis phase. To classify them, we used a protractor and divided the 180° of potential twist into three categories (*sensu* Cardoso et al. in prep): 1) non-resupinate – flowers between 0° - 60°, positioned upwards (Fig. 4C); 2) half-resupinate – flowers between 60° - 120°, positioned sidewards (Fig. 4E); 3) resupinate – flowers between 120° - 180°, positioned downwards (Fig. 4A). From a total of 507 flowers surveyed, 71 were non-resupinate, 59 half-resupinate and 377 resupinate. On each flower, we measured its position on the inflorescence (from one to six), bract size, labellum height and labellum width. These later two were used to calculate labellum volume based on the formula of a prolate ellipsoid $(^{4}/_{3}*pi*(^{\text{labellum height}}/_{2})*(_{\text{labellum width}}/_{2})^{\wedge 2})$. Labellum volume was further used as a proxy to discuss the flower size and weight (see topic below).

We investigated the possible factors influencing flower non- and half-resupination by applying a multinomial logit model using the R-package *mlogit* (Croissant, 2020). We used position on the inflorescence, bract size and labellum volume as predictor variables (correlations: flower position *vs.* bract: 0.06; flower position *vs.* volume: 0.07; bract *vs.*

volume: 0.45). In the levels of the response variable, we used full resupination as reference level and non- and half-resupination as the two possible alternative outcomes.

Weights relationships and differences

To get a better understanding of the distribution of floral whorls weights, we took 106 newly opened flowers and used a microbalance (0.001g readability) to weight floral parts separately. These included the labellum, petals, sepal and synsepal (fusion of the two other sepals) (Fig. S1). Flowers were collected early in the morning to avoid any weight loss due to evaporation because of sunlight incidence. We also measured the labella height and width to investigate its relationship with weights.

To perform exploratory data analysis, we used the R-package *GGally* (Schloerke et al. 2021) and created a Pearson-based correlation matrix between the labellum volume and all the weight variables, including total weight. We them compared the weights of whorls (labellum, petals, sepal and synsepal) by fitting a GLMM adjusting a gamma distribution with log link and setting flower identity, flower number on the inflorescence and inflorescence height as random effects. We tested model significance through the LR and performed post-hoc analysis using Tukey adjusted contrasts.

Weights before and after resupination

To investigate if resupination process is related to the gain of weight due to water uptake, early in the morning, we collect pre-anthesis buds that fully resupinate (n = 15) and others that did not resupinate yet (n = 12). In addition to labellum volume, we collected data on the weight of whorls (labellum, petals, sepal and synsepal) and in this time we included the ovaria. We did so to show that the changes related to resupination are related to the gain in weight by flower whorls and not in the more basal floral part (the ovary).

Considering this data of buds before and after resupination, we compared the labellum volume, weights of whorls, flower total weight and ovarium weights through Welch's t tests or Mann–Whitney U tests when outliers were present (defined when data points fell out of upper and lower limits of boxplot whiskers). In both tests, we specified the alternative hypothesis of higher values on post- when compared to pre-resupinate data.

Floral parts removal experiment

We accessed the role of distinct floral parts and their respective weights on the resupination process by conducting an experiment removing them with forceps from pre-resupinate buds that would open in the following day. The pollen removal (group 1) constituted an important treatment to test the role of its auxin production and the influence on twist (Table S1). Since in the Cypripedioideae subfamily there is no pollinarium because pollen is loosely held together and the pollen smears are not attached to a viscidium (Dressler 1981), we used the term "pollen" instead of pollinia. Non-reproductive whorls were also removed including the petals, sepal, synsepal and labellum (groups 2 - 5; Table S1). We hypothesized that the heavier the whorl removed, the higher the chance of non-resupination, especially regarding the labellum, the heavier whorl and the most important promoting zygomorphy. We also removed the bract to confirm its role breaking the natural twist of flowers (group 6; Table S1). Since both bract and labellum are key to our questions, we removed these both to investigate if the remaining whorls are capable of resupinate without them (group 7; Table S1). To see the effect of labellum presence and if the flower twists only with its weight, we had a group with bract and whorls removal, but still leaving the labellum (group 8; Table S1). To see how the column behaves when free to resupinate without either bract or the non-reproductive whorls, we removed all these (bract + whorls) (group 9; Table S1). Another group consisted in the removal of all non-reproductive whorls, but this time leaving the bract to see its role in breaking resupination when the flowers are much less heavy (group 10; Table S1). We had a control group where buds were untreated (group 11; Table S1). A second control group comprised buds that were only opened with forceps at the junction between sepal and synsepal to check the effect of our manipulation in resupination (group 12; Table S1). Overall, 318 flowers were treated (see sample sizes in Table S1).

After assigned to one of the following treatment groups, flowers were them checked after two days when already in anthesis phase. Using a protractor, we recorded if flowers resupinate (twist $\geq 120^\circ$) or not and the amount of arc degrees of the semicircle they twisted (from 0° to 180°). We also recorded the flower number on the inflorescence and the height of flower from the ground to further correct for these factors.

We analyzed data from the experiment in two ways: considering resupination as categoric (*i.e.*, the probability – the presence of the complete twist) and as continuous (*i.e.*, the proportion – the arc degrees twisted). In the first, we fitted a GLMM adjusting a binomial distribution with logit link followed by LR test on the presence/absence of resupination. Treatment was treated as fixed effect and number of flower on the inflorescence and flower height as random effects. After finding a non-positive-definite Hessian matrix, we used the BFGS (Broyden–Fletcher–Goldfarb–Shanno) algorithm as alternative optimizer to improve the model convergence. In the second GLMM on continuous data, we fitted the same model structure, but in this time we adjusted a beta distribution (with logit link) having the proportion of resupination as response variable $({}^{\text{Resupinate arc degrees}}/_{180^\circ})$. Since the proportion of twist varied between 0 (0°) and 1 (180°), we them redistributed the data to be ≥ 0.00001 and ≤ 0.99999. In both GLMMs, we performed post-hoc using Tukey adjusted contrasts.

Flower weight vs. *resupination hypothesis*

We tested if the resupination mean probability and proportion across the treatment groups from the *"Floral parts removal experiment"* topic may be determined by flower weight. We took the groups: pollen removal, petals removal, sepal removal, synsepal removal, labellum removal, all whorls removal and control 2. We chose these groups because all of them where somehow manipulated (control 1 not included) and all of them were controlled by the presence of the adjacent bract. Them, we considered the type of floral whorls present on each group to calculate the mean flower weight of that respective group based on the weights found in "*Weights relationships and differences*" topic. For instance, the mean weight of the labellum removal group would be the difference between the mean flower total weight and the mean labellum weight.

We fitted a GLM (generalized linear model) followed by LR test having the mean probability of resupination as dependent variable and the mean flower weight as independent variable adjusting a beta distribution (with logit link). Since we had the presence of a zero value, we redistributed the data to be ≥ 0.00001 and ≤ 0.9333333 (*i.e.*, setting a new minimum and keeping the same maximum). We fitted another beta GLM having the mean proportion of the semicircle arc degrees twisted as dependent variable $({}^{\text{Resupinate arc degrees}}/_{180^{\circ}})$. As a goodness-of-fit measure of our model, we calculated the Efron's *pseudo-*R^2 using the R-package *performance* version 0.4.8 (Lüdecke et al. 2020). We extracted the model marginal effects for plotting using the R-package *ggeffects* version 0.16.0 (Lüdecke, 2018).

We visually checked the fit of the GLM and all other GLMMs (including those from "*Resupination description*", "*Weights relationships and differences*" and "*Floral parts removal experiment*" topics) using the histogram of residuals, and, in the R-package *DHARMa* (Hartig, 2020), the QQ plot of residuals and the plot of residual *vs.* predicted values after simulating residuals 250 times. Using the R-package *RVAideMemoire* version 0.9-78 (Hervé, 2020), we back-transformed the estimated marginal means and standard errors for plotting GLMMs adjusted values involving categorical variables. All analyses were carried out in R software version 4.1.0 (R Core Team 2021).

Results

Following pre-anthesis buds prior to resupination on the day before resupination, we found that their positions were not precisely facing up (first day μ = 27.57º) (Fig. 2 A). We observed that this might slightly vary throughout the day and was apparently occasioned by the tip of the inflorescence bending towards the sun. There were no differences among the time periods of the first day, and they were all different from any period of the second day. In the second day, flowers at 6AM (μ = 94.00º) had an angle of twist more than two times higher when compared to the previous period (first day/6PM μ = 30.40º). The 9AM (μ = 116.60º) and 12PM (μ = 122.80º) periods had significantly higher values and were not different from each other. Then, 15PM (μ = 140.40º) had higher proportional values of twist followed by 18PM (μ = 162.60º).

By visually assessing flowers progression individually, we may affirm that they resupinate in two different ways (Fig. 2 A). In the first, flowers perform most of their twist (getting rid from the bract) overnight and in the first hours of the dawn. This may be related to the water uptake by flowers, that is probably higher during the night and first hours of the morning without the heat of the sun. In the following periods, mainly in the

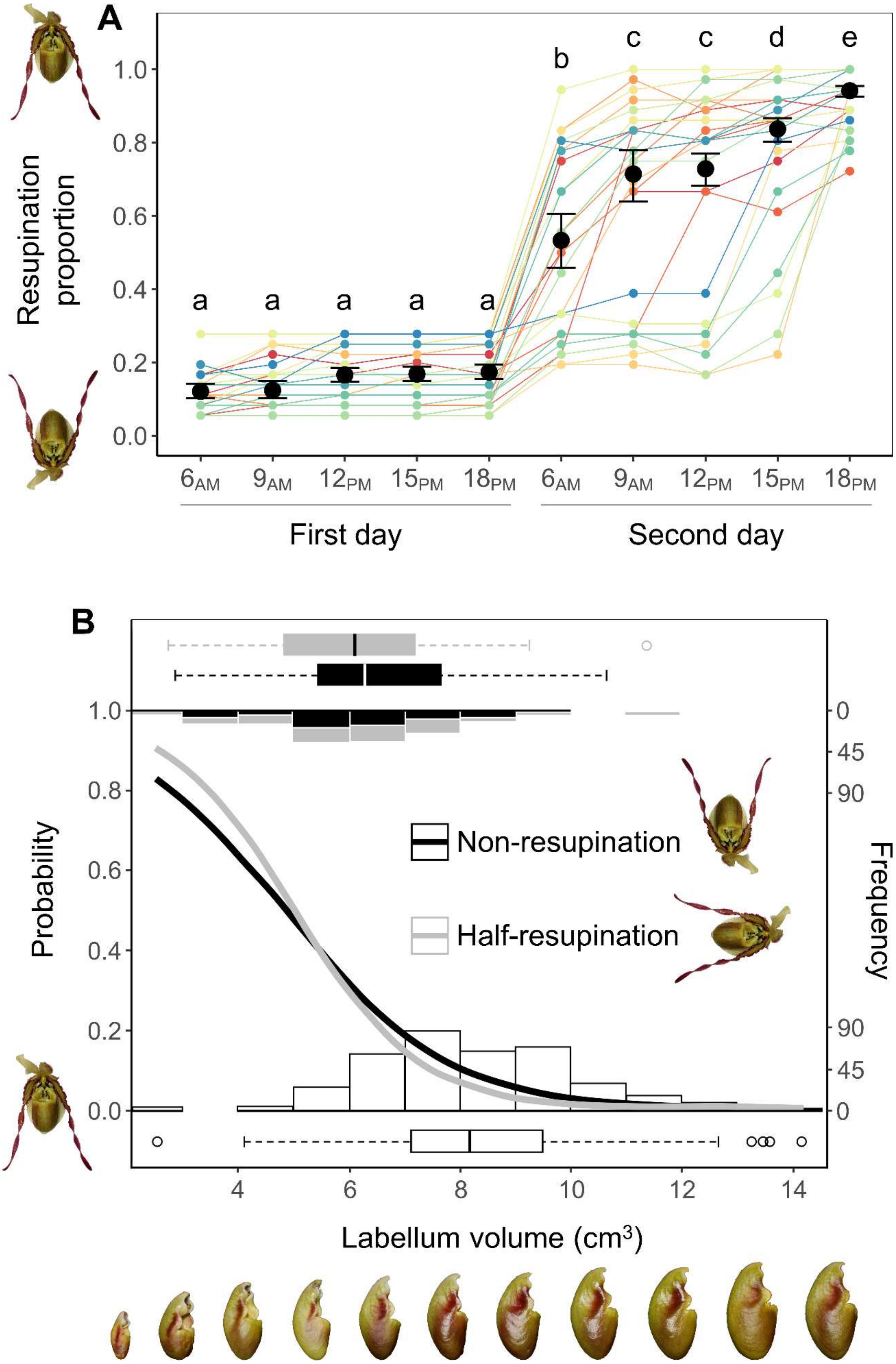


Figure 2. Description of the resupination process since the day before resupination (first day) until the day twist occurs (second day) along distinct periods of the day (A). Values in y axis show the proportion of arc degrees twisted, ranging from 0 (0º - flowers facing up) until 1 (180º

- flowers facing down). Distinct letters show significant differences at 0.05 level. Distinct colors of dots and lines refer to the control of flower identity. Probability of non-resupination (black) and half-resupination (gray) (1.0) of *Phragmipedium vittatum* flowers according to labellum volume having resupination (white) as reference level (0.0) (B). Labella at the bottom show the volume variation in the population. y1 shows the predicted probability and y2 shows the frequency on each group. Box plots and their median, quartiles and outliers show the distribution of each group.

afternoon, flowers started their anthesis (Fig. 2A). In the second group, flowers progressively increase their angulation throughout the day, and perform their main twist in the last time periods, thus getting rid from the bract. We observed that some of these flowers even started their anthesis as the day passed by. In the final hours and in the following days, flowers tend to reach their final position approaching 180º (Fig. 2A).

The increment of volume and weight in the flower buds was confirmed with the comparison between pre- and post-resupinate buds. The volume of labella in post-resupinate buds (5631.25 ± 674.90 mm^3) increased 71.16 % when compared to the pre-resupinate ones (3290.14 ± 663.67 mm^3) ($U = 0$; $p < 0.001$; Fig. 3A). We found an increase in the weight of whorls indicating water accumulation. Post-resupinate labella (0.99 ± 0.09) was 45.59 % heavier than pre-resupinate (0.68 ± 0.10) ($t = 8.20$, $p < 0.001$; Fig. 3B). Post-resupinate sepal (0.18 ± 0.03 g) was 20 % heavier than pre resupinate (0.15 ± 0.03 g) ($t = 2.26$; $p = 0.017$; Fig. 3C). Post-resupinate synsepal (0.40 ± 0.07 g) was 14.29 % heavier than pre resupinate (0.35 ± 0.07 g) ($U = 54$; $p = 0.042$; Fig. 3D). Post-resupinate petals (0.17 ± 0.03 g) were 41.67 % heavier than pre-resupinate ones (0.12 ± 0.02 g) ($U = 18$; $p < 0.001$; Fig. 3E). In agreement, the total weight of post-resupinate flowers (1.92 ± 0.23) was 31.51 % larger than that of pre-resupinate (1.46 ± 0.24) ($t = 4.91$, $p < 0.001$; Fig. 3F). However, post-resupinate ovaries (0.08 ± 0.01 g) were not different from those pre-resupinate (0.08 ± 0.01 g) ($U = 80$, $p = 0.32$; Fig. 3G).

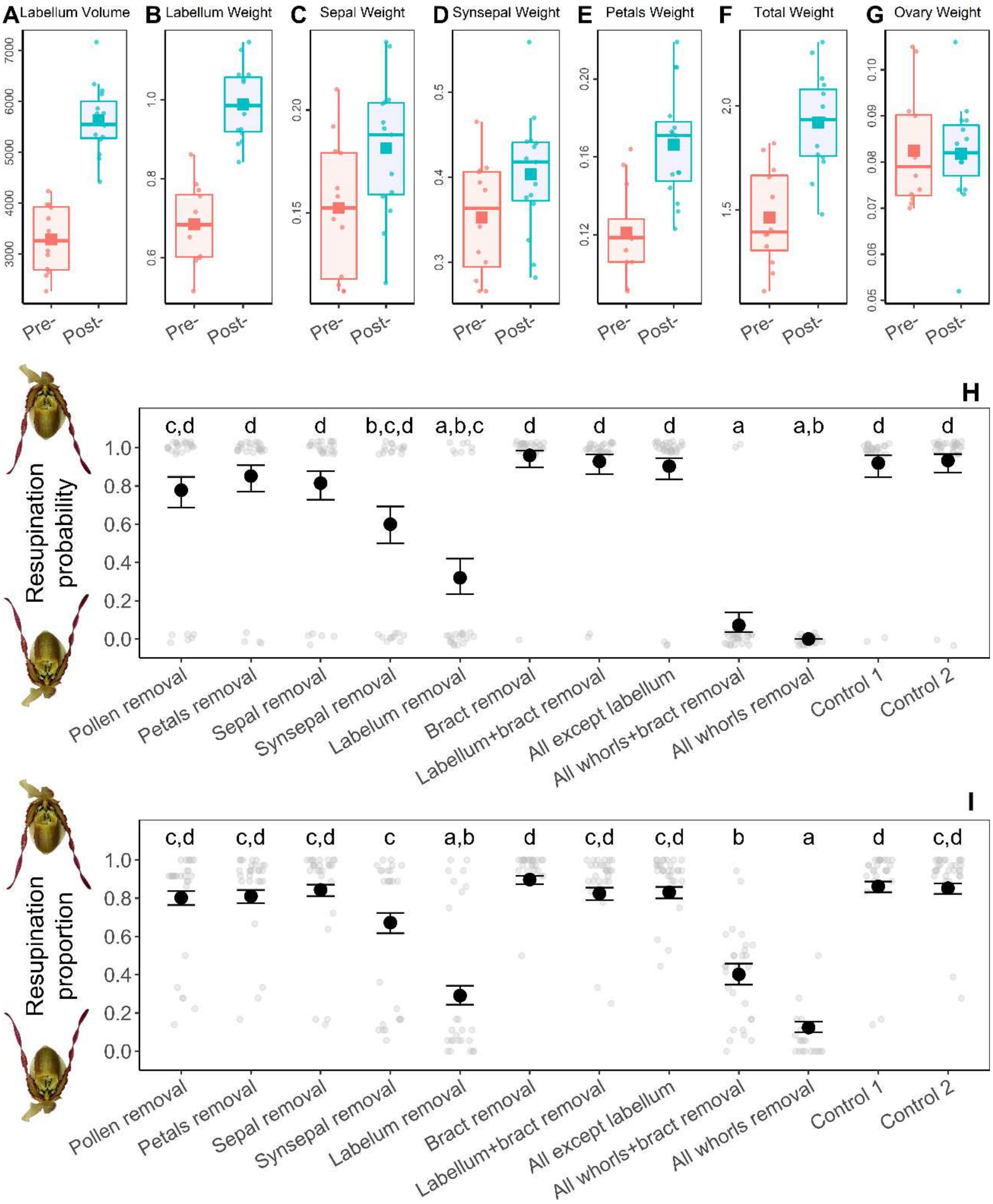


Figure 3. Results from the comparison of pre- and post-resupination of buds regarding (A) labellum volume, and (B) labellum, (C) sepal, (D) synsepal, (E) petals, (F) total and (G) ovary weights. Box plots show median quartiles and outliers. Squares show means and dots the original data points. Results from the experiment alternating the removal of the distinct flower parts of *Phragmipedium vittatum* (H, I). Probability of resupination (1.0) according to the treatment levels (H). y axis show the resupination absence (0.0) or presence (1.0). Proportion of resupination in arc degrees twisted (I). y axis ranges from 0 (0º - flowers facing up) until 1 (180º - flowers facing down). Black dots and line segments (in H and I) show back-transformed marginal (model adjusted) means and standard errors, respectively. Grey dots show observed data points.

We found highly positive correlations regarding all pairwise combinations among whorls and also regarding those with flower total weight and labellum volume (all above 0.815) (Fig. S1J). We found significant differences in the weight of the floral whorl types ($\chi^2 = 1345.6$, df = 3, $p < 0.001$, Fig. S1 K). The labellum was the heavier structure (mean ± SD: 1.20 ± 0.31 g), followed by synsepal (0.46 ± 0.12 g), petals (0.26 ± 0.08 g) and them by sepal (0.23 ± 0.06 g).

Taking resupination as reference level, we did not find any effects of either bract size or position on the inflorescence on both non- and half-resupination (Table 1). However, labellum volume was positively related to the probability of both non- and half-resupination ($\chi^2 = 132.24$; $p < 0.001$; $R^2_{\text{McFadden's}} = 0.17$; Table 1; Fig. 2B). we may infer that weight is also related to resupination since labellum volume was positively correlated to all variables of weight including sepal (0.84), synsepal (0.84), petals (0.88), labellum (0.90) and specially flower total weight (0.92) (Fig. 2J).

Table 1. Standard Error, t and p values results of multinomial logistic regression for non- and half-resupination.

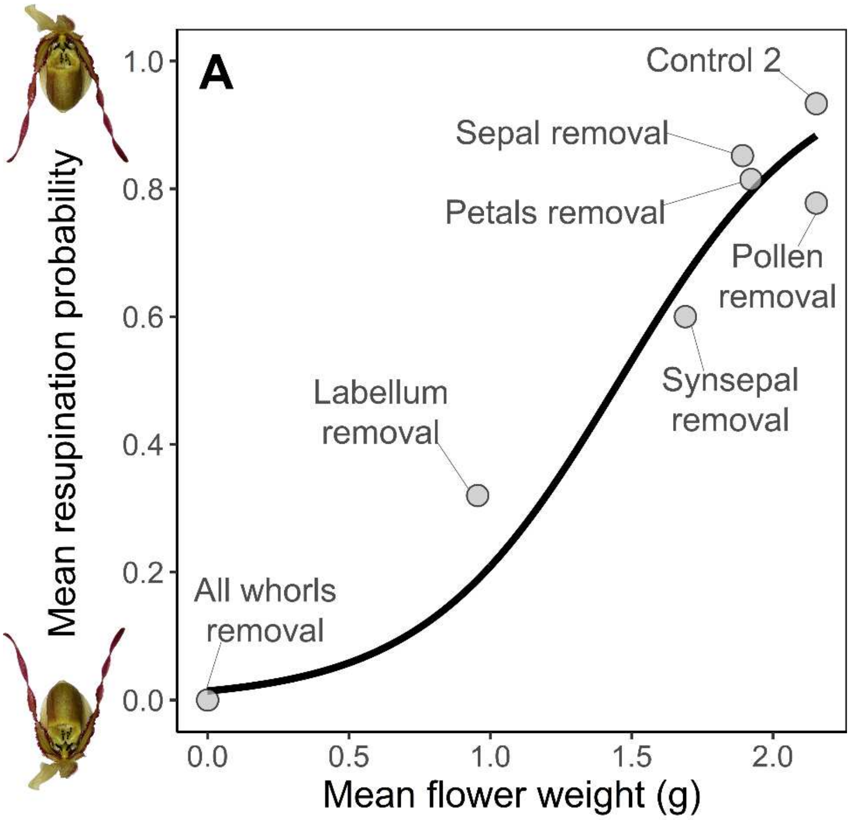
	Non-resupiation			Half-resupination		
	Error	*t*	*p*	Error	*t*	*p*
Bract	0.17	0.62	0.54	0.19	-0.41	0.68
Position	0.12	0.98	0.32	0.13	0.95	0.34
Volume	0.1	-7.01	< 0.001*	0.11	-7.32	< 0.001*

We found a significant effect on the probability of resupination according to the different treatment groups in the whorl removal experiment ($\chi^2 = 165.13$, df = 11, $p < 0.001$, Fig. 3H). The group with all whorls removal had the smallest probability of resupination (0 %) together with the group of all whorls + bract removal (7.14 % of flowers resupinate). Not significantly different from these, is the group of the labellum removal (32.00 %). The next group is that with the removal of synsepal (60.00 %). Them, the next groups all have high rates of resupination including pollen removal (77.78 %), sepal removal (81.48 %), petals removal (85.19 %), removal of bract and all whorls except the labellum (90.32 %), control 1 (92.00 %), labellum + bract removal (92.86 %), control 2 (93.33 %) and bract removal (96.00 %).

We also found significant effects considering the analysis of this data quantitatively (χ^2 = 195.70, df = 11, $p < 0.001$, Fig. 3I). We found that the proportion of degrees twisted along the semicircle was lower in the group with the removal of all whorls (μ = 14.75º), which was not significant from the group with labellum removal (μ = 59.8º). The groups with the removal of all whorls + bract (μ = 71.79º) and synsepal removal (μ = 112.6º) had intermediate proportions of angles twisted. Them, the higher values of twist proportion were those of pollen removal (μ = 142.96º), sepal removal (μ = 153.70º), petals removal (μ = 154.81º), labellum+bract removal (μ = 160.18º), control 1 (μ = 161), removal of bract and all whorls except the labellum (μ = 162.58), control 2 (μ = 164.5) and bract removal (172.8).

We found that the mean flower weight across the different groups positively determined both the mean probability of resupination (χ^2 = 21.32, df = 1, $p < 0.001$, $R^2_{\text{Efron's}} = 0.94$, Fig. 4A) and the mean proportion of resupination arc degrees (χ^2 = 20.13, df = 1, $p < 0.001$, $R^2_{\text{Efron's}} = 0.96$, Fig. 4B). Thus, these results highlight that groups with heavier flowers tend to resupinate more frequently and twist more degrees, respectively.

Taken together, after the observation of the system and the results of our experiments, we found that although the whole ovarium bends to get the flower in resupinate position, the bending (or elongating) zone is main located at the tip of the ovarium (Fig. 5A,B). In this region it is possible to discern the differential growth of upper and lower flanks typical to a gravistimulated organ, which leads to the characteristic curvature (Fig. 5B). This can be seen evidenced by the moderate twist of some groups such as in all whorls + bract removal and all whorls removal (Fig. 5G,H). However, we noticed that even in flowers non- (Fig. 5C,D) and half-resupinate (Fig. 5E,F), the tip of the ovarium still moves, highlighting that there is a natural movement during the development in the last moments before anthesis. Thus, it is possible to notice that ovary pre-determined gravitropism naturally occurs at some level in all treatment groups and even when flowers are trapped in the bracts. However, the flower weight plays a hole incrementing the amount of twist towards the ground (Fig. 5A, B, H).


A
Mean resupination probability
1.0
0.8
0.6
0.4
0.2
0.0
Control 2
Sepal removal
Petals removal
Pollen removal
Synsepal removal
Labellum removal
All whorls removal
0.0
0.5
1.0
1.5
2.0
Mean flower weight (g)

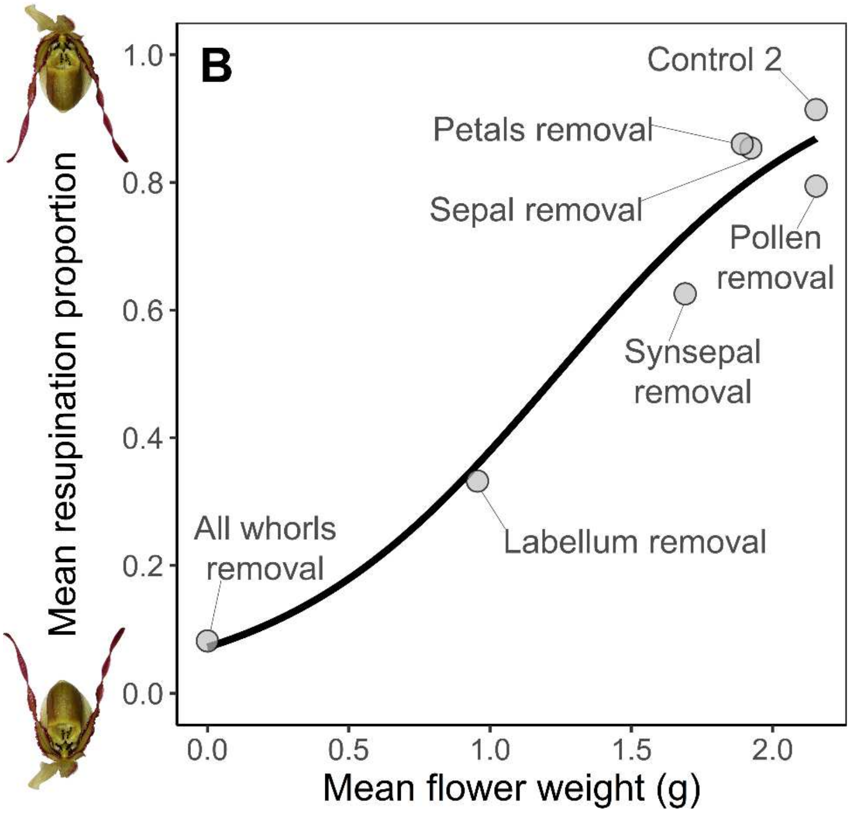

B
Mean resupination proportion
1.0
0.8
0.6
0.4
0.2
0.0
Control 2
Petals removal
Sepal removal
Pollen removal
Synsepal removal
Labellum removal
All whorls removal
0.0
0.5
1.0
1.5
2.0
Mean flower weight (g)

Figure 4. Positive relationship between the mean resupination proportion of the different treatment groups and the respective mean flower weight. Gray dots show observed data points and the black line is adjusted to the beta distribution.

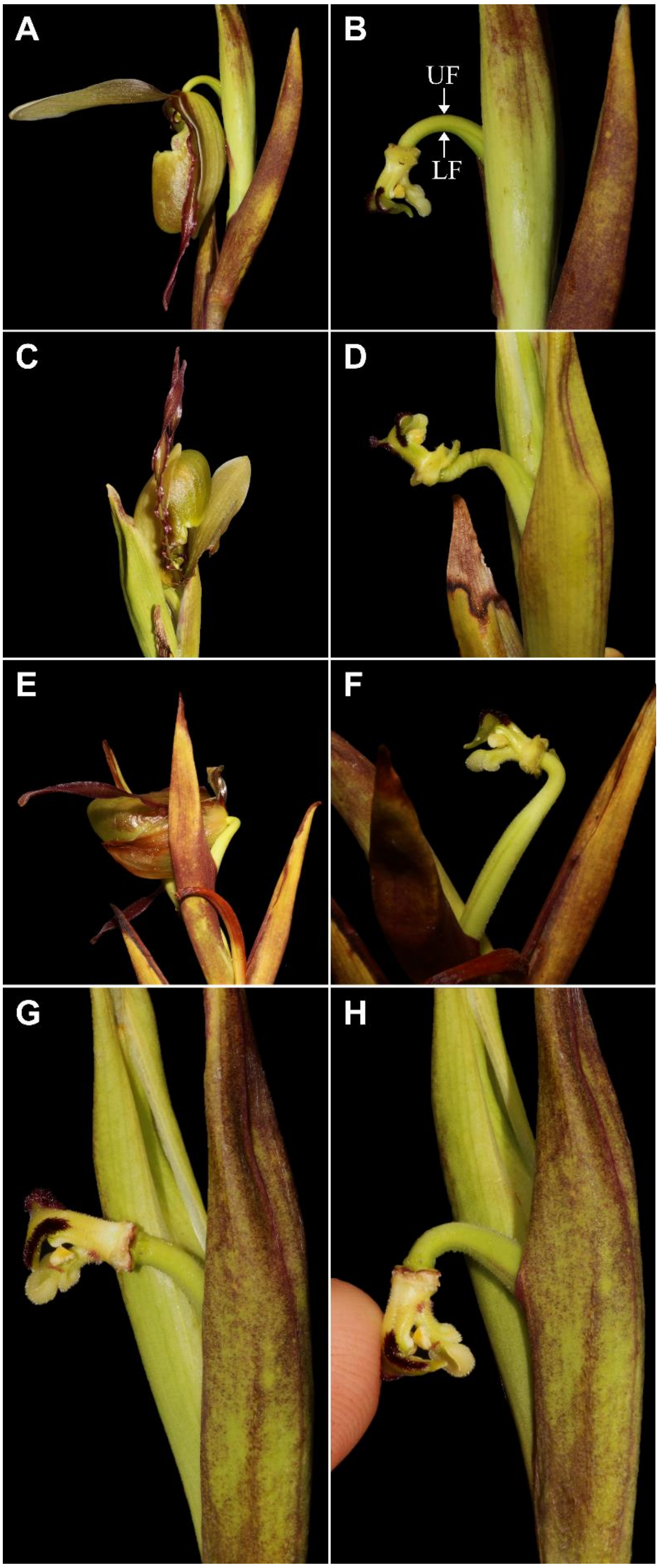


Figure 5. Flowers, column, and ovaria of *Phragmipedium vittatum*. (A) Resupinate flower and (B) detail of the respective column and ovary after the removal of non-reproductive whorls. UF and LF indicate respectively the upper and lower flanks of the bending zone. (C) Non-resupinate flower and (D) detail after removal of whorls. (E) Half-resupinate flower and (F) detail after removal of whorls. (G) Flower in which all whorls were removed from the bud (bract left; treatment 10 in Table S1) and left to twist. (H) The same flower showing the supposed position of column and ovarium twist if resupination had taken place completely. The column has been positioned with the tip of the first author's finger to illustrate the process.

Discussion

The direction and intensity of gravity have been constant throughout the evolutionary history of life on Earth, acting on all organisms. Thus, life forms are not only shaped by this important physical force but adapted to use it for their own benefit (Morey-Holton, 2003; Bhaskaran et al 2009). Plants respond to gravity in different levels that range since intracellular mechanisms until growth and development, in a way that arranges their spatial orientation and survival (Bhaskaran et al 2009). In this study, we show that gravity influences flower resupination in two different but complementary ways: gravitropism and gravitation. First, the tip of the ovarium naturally bends due to gravitropism, which causes the remaining flower parts to move accordingly. However, only this movement is not enough to cause the complete twist. As flowers increment their weights progressively before anthesis, such mass drives the rest of the twist as suggested by our manipulative experiment controlling flower weights and the increase in the probability of successful resupination in larger/heavier flowers. These findings corroborate our predictions and demonstrate how flower weight is an important trait determining flower orientation though resupination. Below, we discuss our results in detail and their implications.

We found that asymmetric organ growth, the typical response plants show to gravity (Nakamura et al 2019), occurs in *P. vittatum* flowers. The typical bending zone where differential growth occurs on the upper flank can be noticed especially at the tip of the ovarium, which redirects the column and therefore the connected whorls accordingly. In the lady's slippers, it is probably that the ovarium twist is also driven by auxins, which then may lead to the expansion of raphide-bearing cells and the ovarium twist. Importantly, we observed that this occurs even if the flower whorls have their orientation fixed, as in those naturally non- and half-resupinate flowers which remained trapped in the bracts, which causes the tip of the ovary to curl (Fig 5D,F). Thus, ovarium bending is part of the flower development schedule before anthesis.

However, the movement of the ovarium tip is not enough to promote the complete resupination. Here we show that gravitation pull is important completing the twist and leaving the flowers in a resupinate position. We experimentally demonstrate the influence of weight by removing different flower parts. This approach allowed us to obtain different flower weight averages and contrast them to the resupination patterns. We found that both resupination probability and proportion are higher as flowers get heavier. This finding is in agreement with the results assessing which mechanisms explain the natural flower twist fail. The probability of finding resupinate flowers increases with their volume. Since volume in highly positively correlated to flower total weight (correlation = 0.92) and specifically with labellum weight (correlation = 0.90), we may infer the role of weight in the process. Thus, lighter flowers have higher chances of either become non- or half-resupinate.

However, our results do not exclude the influence of physiological and anatomical processes. In fact, gravitropism followed by gravitational pull are complementary mechanisms that lead to resupination. Resupination is known to occur in zygomorphic flowers (Ames 1938; Van der Pijl & Dodson 1966; Mondragón-Palomino & Theißen, 2009), which arranges them into a horizontal position with the plane of symmetry perpendicular to the ground (Endress, 2012). For instance, in the Apostasioideae, the most basal subfamily of the Orchidaceae, resupination occur in the zygomorphic genus Neuwiedia but it is absent in the actinomorphic genus Apostasia (Kocyan and Endress, 2001). As weight is equally distributed in all planes of actinomorphic flowers, their center of mass is located the middle of the flower, close to the point where whorls are inserted. On the other hand, zygomorphy promotes a differential weight distribution in the flower. In this sense, the heavier part of the flower (*i.e.*, the labellum) shifts the center of gravity away from the insertion point of the whorls. The larger the distance between the flower's center of mass and the main axis of the inflorescence, the greater the gravitational pull. This weight arrangement allowed by zygomorphy leads to the typical resupination movement. However, if this heavier part is positioned upwards, which is on the same plane as the main axis of the inflorescence (*i.e.*, 0º), the gravitational pull is canceled by the support offered by the inflorescence below, and flowers do not twist. This highlights the importance of the ovarium initial twist that moves the center of mass of flowers away from the inflorescence axis in a way that the gravitational pull may act on the weight.

Under weightless conditions, the twist conferred naturally only by the ovarium goes to a certain point (*i.e.*, 71.79º in all whorls + bract removal). After that, flower keeps twisting because the ovarium keeps bending, but this time influenced by the gravitational pull. Thus, if the ovarium initial gravitropic twist latter allows the gravitational pull, the weight-mediated movement then twists even more the ovarium. Such negative bending moment causing convexity on the upper flank of the ovarium tip is only possible because of the malleability of the young vegetal tissue, which has not hardened yet.

We demonstrate that the weight gain that triggers ovarium bending and the consequent resupination of the flower occurs quickly. Most of flowers resupinate early in the morning. The comparison of pre- and recently post-resupinate buds show that there is a weight increment of 31.51 % in flower total weight. This was observed in all whorls and especially in the labellum, which had the highest increment in weight (45.59 %). Such quickly weight gain of whorls occurs because the flower acts like a sink, especially during anthesis (Galen et al. 1999). Such water uptake then boosts several movements including petal opening and resupination as we show here. Importantly, we did not find an increment in the weight of ovarium, which indicates that it is already fully formed at this point and serves only as a passage for water. As weight and volume are highly correlated, we also found that labellum volume increased more than 70 %. Linking this result with that of smaller/lighter flowers having higher changes of getting stuck on bracts, we may infer that the increment in volume may be important to flowers to move away from the bracts during the beginning of the twist.

The labellum is the heavier floral part, accounting for 55.81 % of flower total weight. Artificially removing the labellum, decreased resupination probability to 32.00 % and resupination proportion to only 59.80º of the possible 180º. These results confirm that the labellum is the most important whorl contributing to both resupination probability and proportion. Accordingly, the labellum is one of the most important structures promoting the zygomorphic arrangement in orchids (Mondragón-Palomino & Theißen, 2009). Besides these functions, here we show that labellum is important promoting the resupination of flowers. Thus, from a biological viewpoint the lowermost labellum is a consequence of resupination, but from a physical viewpoint the labellum (as the whole flower weight) is also the cause of resupination.

The labellum is associated to the pollination process of orchids, being adapted to different pollinators (Mondragón-Palomino & Theißen, 2009). Although it can play several functions including pollinator attraction and rewarding, one of the most important is to act as a landing platform in resupinate flowers (Ames 1938; Darwin 1877; Van der Pijl & Dodson 1966; Mondragón-Palomino & Theißen, 2009). Such platform constricts the direction of approach of the pollinator to a single plane and restricts its landing behavior to a specific site (Cardoso et. al, in prep). Although the placement of the labellum lowermost is one of the main aspects in a resupinate flower, the reversal rearranges the whole zygomorphic flowers including placing the column uppermost (Mondragón-Palomino & Theißen, 2009), which may be further related to precise pollen placement on pollinator body (Cardoso et al, in prep). Recently, using the same study system investigate here, Cardoso et al. (in prep) provided the first evidence that resupination is important in reproductive success. They conducted an experiment showing that pollen smears removal and deposition in *P. vittatum* only occurs in resupinate flowers (≈ 180º oriented) when compared to half- (≈ 90º) and non-resupinate flowers (≈ 0º). Although these later two groups may still attract the syrphid fly pollinators, the trapping mechanism that involves the falling of insects into the pouch-like labellum is meditated by gravity and only works on resupinate flowers. In addition, they twisted resupinate flowers which had pollinators inside and found that they can get out in few seconds by the same entrance hole, without contacting the reproductive parts. They surveyed 4984 flowers and report that together non-and half-resupinate flowers account for ca. of 10 % of the total number of flowers. Since the species is xenogamous and non-spontaneous selfer, those flowers that fail to resupinate are faded to not reproduce (Cardoso et al, in prep). This highlights how failures during the flower ontogenetic developmental may further prevent reproduction. The effects of resupination fail cannot be overlooked, specially in orchids in which a single fruit can bear tens or hundreds of thousands of seeds (Cardoso et al., in prep). Finally, since resupination fail is related to lighter and less voluminous flowers, we may suggest that there might be a selection favoring for larger/bigger flowers, which will successfully complete the twist.

Although we did not find that the size of the bract influences the probability of finding non- and half-resupinate flowers in the population, its presence seems to be the determinant of the unusual resupination fail found uniquely on this species so far. In our manipulative experiment, we found that the group with “all whorls removal” twisted 14.75º (8.19 % of the semicircle), on average. However, when we remove all whorls and also the bract, the twist level increases to 71.79º (39.88 % of the semicircle). The effect of the bract impairing the twist can also be noticed when comparing the “labellum removal” group (59.80º; 33.22 % of the semicircle) *vs.* the “labellum + bract removal” (160.18º; 88.99 %). Thus, we confirm that the unusual long bract is the responsible for impairing resupination. The bract allows that when at bud stage, flowers keep soaked in mucilage. This is probably an anti-florivore strategy since these enemies are common in the open grassy regions the pant occurs (Cardoso et al, in prep).

Although here we found resupination to be related to the twist of the ovary in *P. vittatum*, it may be caused by the movement of several pant organs, including the inflorescence rachis (Ames 1938). In fact, flowers of some other lady’s slippers with one-flowered inflorescences resupinate by bending the top of the inflorescence while in other species with several flowers this may occur only in the top flowers while the lower flowers twist in the traditional way (Kurzweil & Kocyan, 2002). These observations corroborate that the weight-mediated resupination patterns found here may be expanded to other systems as well. However, most of research on resupination in orchids has been carried out with members of the Epidendroidae, the most derived and diverse subfamily of Orchidaceae (Pérez-Escobar, 2021). *Phragmipedium vittatum* belongs to the Cypripedioideae subfamily, which is positioned in the middle of the Orchidaceae phylogeny. Our study also brings phylogenetic insights. For instance, one of the differences we found is that the removal of the pollen smears does not hinder resupination.

Thus, the early gravitropism does not depend on hormonal production by the male function. In the Cypripedioideae, the pollen smears are loosely attached to the anther, without forming a true pollinarium (Dressler 1981). This is a much simpler structure when compared to the sophisticated pollinarium of the Epidendroidae with its several accessory structures (Mosquera-Mosquera et al. 2019) and responsible to produce large hormonal quantities (Nair & Arditti 1991; Arditti, 2003; Yam, et al. 2009). Still in the phylogeny, we may further ask if weight mediated resupination is present in both more derived and basal conditions, which could help us to understand the evolution of the phenomenon.

Mayr (1961) postulated that the biological phenomena may have a proximate and an ultimate causation. While the first is related to the immediate mechanisms such as the genetic, anatomical and physiological traits, the second is related to the evolutionary explanations involving natural selection. Applying the nature of causation to floral resupination, we may infer that the ultimate cause is that the upside-down floral orientation allows pollinator landing and accurate pollen deposition on their bodies, which ultimately leads to seed set and plant reproduction. However, both proximate and ultimate set of causes must be clarified for a complete understanding of biological phenomena (Mayr, 1961). So, the proximal causes of resupination include the dominant genetic control (Kim et al. 2010), auxin-driven gravitropism (Nair & Arditti 1991; Nair & Arditti 1992) and raphide-bearing cells expansion (Dines et al., 1994). We now add a not mutually exclusive physical explanation related to weight weight, in which the Earth's gravitational pull aids in the ovary turning movement and rearranges the flowers into a resupinate position. Our study opens new avenues into the field of flower orientation. Since our flowers weight ca. 2.15 g and orchids show a remarkably variation in size, weight may be also important on those much heavier flowers that weight dozens of grams.

On the other hand, is the hormonal control enough to promote resupination in much lighter flowers such as the micro-orchids?

Conclusions

Floral resupination is present in thousands of species of monocots and eudicots, determining how flowers interact with the physical and biotic environment, being an important trait in flower evolution. In this study, we provide an example of how gravity may influence flower resupination through both gravitropism and gravitational pull, with makes flower weight an essential trait arranging flowers on a proper position. Our findings provide new insights into the working machinery of flower orientation and resupination, highlighting the several complex mechanisms flowers undergo to get in a proper position that further allows the efficient attraction and morphological fit of pollinators, guarantying their reproduction.

Acknowledgements

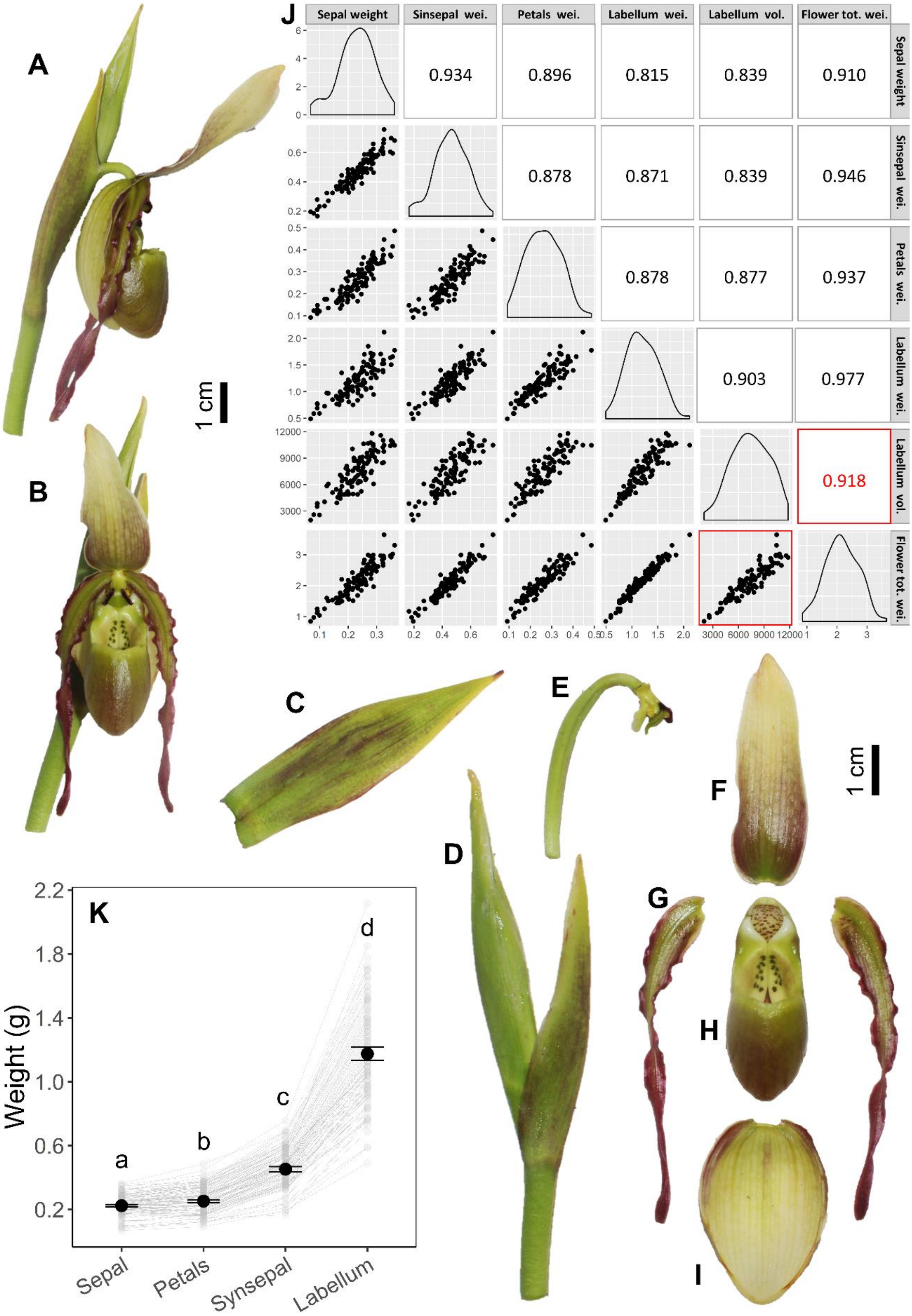
We are grateful to D. C. Oliveira for lending us the microbalance. JCFC is grateful to CAPES (*Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior*) – Finance Code 001 – for his PhD grant. SDJ is grateful to the South African Research Chairs Programme (NRF grant 46372). PEO is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for his research grant and financial support.

Supplementary Material

Table S1. Treatments of the flower whorls removal experiment including a detailed description of the procedure adopted, the sample size of each group, and the results including mean resupination probability and mean resupination proportion.

		Methods		Results	
Group	**Treatment**	**Description**	**Sample size**	**Resupination probability (mean)**	**Resupination degrees (mean)**
1	Pollinia removal	Both left and right pollinia were removed.	27	77.78 %	142.96º
2	Petals removal	Both left and right petals were removed.	27	85.19 %	154.81º
3	Sepal removal	Only the sepal was removed.	27	81.48 %	153.70º
4	Synsepal removal	Only the synsepal was removed.	25	60.00 %	112.60º
5	Labellum removal	Only the labellum was removed.	25	32.00 %	59.80º
6	Bract removal	Only the adjacent bract was removed.	25	100 %	172.80º
7	Labellum + bract removal	Labellum and adjacent bract were removed.	28	92.86 %	160.18º
8	All except labellum	Adjacent bract and whorls (petals, sepal, synsepal) were removed, except the labellum.	31	90.32 %	162.58º
9	All whorls + bract removal	Removal of all whorls (petals, sepal, synsepal and labellum) in addition to the bract	28	14.29 %	71.79º
10	All whorls removal	Removal of all whorls including petals, sepal, synsepal and labellum. Bract left intact.	20	0.00 %	14.75º
11	Control 1	Intact/non-manipulated flowers.	30	92.00 %	161.00º
12	Control 2	Flowers were opened with forceps at the junction between the synsepal and the sepal and without any further treatment.	25	93.33 %	164.50º

Figure S1. Flower structures and weight-size relationships of *Phragmipedium vittatum*. Lateral (A) and frontal (B) views of a flower and the adjacent bract in the inflorescence. Detail of the bract (spread) (C), the inflorescence (D), the ovarium with the column and pollinia (E), the sepal (F), petals (G), labellum (H) and synsepal (I). Scale bar on the left refers to A and B while that in the right refers to the remaining pictures. Correlation matrix among the weighs of whorls, the flower total weight, and the labellum volume (further used to refer to flower weight) (J). Differences in the weight of the distinct whorls (K). Black dots and line segments show back-transformed marginal (model adjusted) means and standard errors, respectively. Grey dots show observed data points and lines connect the same flower. Distinct letters show significant differences at 0.05 level.


A
B
1 cm
J
Sepal weight
Sinsepal wei.
Petals wei.
Labellum wei.
Labellum vol.
Flower tot. wei.
0.934
0.896
0.815
0.839
0.910
0.878
0.871
0.839
0.946
0.878
0.877
0.937
0.903
0.977
0.918
C
D
E
F
1 cm
G
H
I
K
Weight (g)
2.2
1.8
1.4
1.0
0.6
0.2
a
b
c
d
Sepal
Petals
Synsepal
Labellum

References

Ames, O. (1938). Resupination as a diagnostic character in the Orchidaceae with special reference to *Malaxis monophyllos*. Botanical Museum Leaflets, Harvard University, 6(8), 145-183. https://doi.org/10.5962/p.295120

Araújo, G. M., Barbosa, A. A. A., Arantes, A. A., & Amaral, A. F. (2002). Composição florística de veredas no Município de Uberlândia, MG. Revista Brasileira de Botânica, 25, 475–493. https://doi.org/10.1590/S0100-84042002012000012

Arditti, J. (2003). Resupination. Lankesteriana 7: 95-96.

Bhaskaran, S., Jagtap, S. S., & Vidyasagar, P. B. (2009). Life and gravity. Biophysical Reviews and Letters, 4(04), 299-318. https://doi.org/10.1142/S179304800900106X

Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Maechler M, Bolker BM (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. The R Journal, 9(2), 378-400. https://doi.org/10.32614/RJ-2017-066

Cohen, I. B., and Whitman A. 1999. Isaac Newton. The Principia. Mathematical Principles of Natural Philosophy. A New Translation. Berkeley/Los Angeles/London: University of California Press.

Crawley MJ (2013) The R book. Chichester: John Wiley & Sons, Chichester

Croissant Y (2020). “Estimation of Random Utility Models in R: The mlogit Package.” _Journal of Statistical Software_, *95*(11), 1-41. https://doi.org/10.18637/jss.v095.i11

Darwin, C. (1877). On the various contrivances by which British and foreign orchids are fertilised by insects. John Murray. https://doi.org/10.5962/bhl.title.60877

Dias, M.C.; Smidt, E.C. 2020. *Phragmipedium* in Flora do Brasil 2020. Jardim Botânico do Rio de Janeiro. Available at: http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB32001. Accessed on May 27, 2021

Dines, T. D., & Bell, A. D. (1994). Differential cell enlargement and its possible implication for resupination in *Lemboglossum bictoniense* (Orchidaceae). Botanical journal of the Linnean Society, 114(1), 67-79. https://doi.org/10.1111/j.1095-8339.1994.tb01924.x

Dressler RL (1981) The orchids: natural history and classification. Harvard University Press, Cambridge, Mass, USA.

Dworaczek, E., & Claßen-Bockhoff, R. (2016). 'False resupination' in the flower-pairs of *Thalia* (Marantaceae). Flora, 221, 65-74. https://doi.org/10.1016/j.flora.2015.08.007

Efron, B. (1978). Regression and ANOVA with zero-one data: Measures of residual variation. Journal of the American Statistical Association, 73, 113-121. https://doi.org/10.1080/01621459.1978.10480013

Endress, P. K. (2012). The immense diversity of floral monosymmetry and asymmetry across angiosperms. The Botanical Review, 78(4), 345-397. https://doi.org/10.1007/s12229-012-9106-3

Galen, C., Sherry, R. A., & Carroll, A. B. (1999). Are flowers physiological sinks or faucets? Costs and correlates of water use by flowers of *Polemonium viscosum*. Oecologia, 118(4), 461-470. https://doi.org/10.1007/s004420050749

Gerlach, G. (2011). The genus *Coryanthes*: a paradygm in ecology. Lankesteriana. https://doi.org/10.15517/lank.v11i3.18280

Goebel, K., 1924. Die Entfaltungsbewegungen der Pflanzen und deren teleologische Deutung. Gustav Fischer Verlag. Jena, Germany.

Hartig F (2020). DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.3.3.0. https://CRAN.R-project.org/package=DHARMa

Hervé, M. 2020. RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R package version 0.9-78. https://CRAN.R-project.org/package=RVAideMemoire

Hill, A. W. (1939). Resupination studies of flowers and leaves. Annals of Botany, 3(4), 871-887. https://doi.org/10.1093/oxfordjournals.aob.a085096

Kim, S. Y., Endo, M., Yun, P. Y., & Kanno, A. (2010). Production of intraspecific hybrids between wild-type and petaloid-sepal cultivars in *Habenaria radiata*. Scientia horticulturae, 124(3), 415-418. https://doi.org/10.1016/j.scienta.2010.01.022

Kiss J. Z. 2000. Mechanisms of the early phases of plant gravitropism. Critical Reviews in Plant Sciences 19, 551–573. https://doi.org/10.1080/07352680091139295

Kocyan, A., & Endress, P. K. (2001). Floral structure and development of *Apostasia* and *Neuwiedia* (Apostasioideae) and their relationships to other Orchidaceae. International Journal of Plant Sciences, 162(4), 847-867. https://doi.org/10.1086/320781

Kurzweil H., Kocyan A. (2002) Ontogeny of orchid flowers. In: Orchid Biology: Reviews and Perspectives, Viii. p. 83–138. https://doi.org/10.1007/978-94-017-2500-2_2

Lenth RV (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.5.3. https://CRAN.R-project.org/package=emmeans

Linné, C., 1753. Species Plantarum. Impensis Laurentii Salvii. Stockholm.

Lüdecke D (2018). “ggeffects: Tidy Data Frames of Marginal Effects from Regression Models.”. Journal of Open Source Software, 3(26), 772. https://doi.org/10.21105/joss.00772

Lüdecke D, Makowski D, Ben-Shachar MS, Patil I, Waggoner P, Wiernik BM, Arel-Bundock V, & Jullum M (2020). performance: Assessment of Regression Models Performance. R package version 0.4.8. https://CRAN.R-project.org/package=performance

Mayr, E. (1961). Cause and effect in biology. Science, 134(3489), 1501-1506. https://doi.org/10.1126/science.134.3489.1501

Mondragón-Palomino, M., & Theißen, G. (2009). Why are orchid flowers so diverse? Reduction of evolutionary constraints by paralogues of class B floral homeotic genes. Annals of Botany, 104(3), 583-594. https://doi.org/10.1093/aob/mcn258

Morey-Holton, E. R. (2003). The impact of gravity on life. In *Evolution on Planet Earth* (pp. 143-159). Academic Press. https://doi.org/10.1016/B978-012598655-7/50036-7

Mosquera-Mosquera HR, Valencia-Barrera RM, Acedo C (2019) Variation and evolutionary transformation of some characters of the pollinarium and pistil in Epidendroideae (Orchidaceae). Plant Syst Evol 305:353–374. https://doi.org/10.1007/s00606-019-01575-5

Nair H, Arditti J (1992) Effects of auxin transport and H+ secretion inhibitors, a calcium chelator and an anti-auxin on buds of *Aranda* Kooi choo. Lindleyana. 7: 185–193.

Nair H, Arditti J. 1991. Resupination in orchids. III. Effects of indoleacetic, naphthaleneacetic and gibberellic acids and benzyladenine on buds and flowers of *Aranda* Kooi Choo. Lindleyana 6:154–161.

Nakamura, M., Nishimura, T., & Morita, M. T. (2019). Gravity sensing and signal conversion in plant gravitropism. Journal of experimental botany, 70(14), 3495-3506. https://doi.org/10.1093/jxb/erz158

Newton, I. (1687). Philosophiae naturalis principia mathematica. William Dawson & Sons Ltd, London. https://doi.org/10.5479/sil.52126.39088015628399

Nyman, L. P., Soediono, N., & Arditti, J. (1984). Opening and resupination in buds and flowers of *Dendrobium* (Orchidaceae) hybrids. Botanical Gazette, 145(2), 215-221. https://doi.org/10.1086/337449

Nyman, L. P., Soediono, N., & Arditti, J. (1985). Resupination in flowers of two *Dendrobium* (Orchidaceae) hybrids: effects of nodal position and removal of floral segments. Botanical Gazette, 146(2), 181-187. https://doi.org/10.1086/337513

Pérez-Escobar, O. A., Dodsworth, S., Bogarín, D., Bellot, S., Balbuena, J. A., Schley, R. J., ... & Baker, W. J. (2021). Hundreds of nuclear and plastid loci yield novel insights into orchid relationships. American Journal of Botany, 108(7), 1-15. https://doi.org/10.1002/ajb2.1702

Pfeifer, H. W. (1966). Revision of the North and Central American hexandrous species of *Aristolochia* (Aristolochiaceae). Annals of the Missouri Botanical Garden, 115-196. https://doi.org/10.2307/2394940

R Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Romero, G.A., Carnevali Fernández-Concha, G., Pridgeon, A.M., 2009. *Catasetum*. In: Pridgeon, A.M., Cribb, P.J., Chase, M.W., Rasmussen, F.N. (Eds.), Genera Orchidacearum. Volume 5. Epidendroideae (Part Two). Oxford University Press, Oxford, pp. 22–25.

Schloerke B, Di Cook, Joseph Larmarange, Francois Briatte, Moritz Marbach, Edwin Thoen, Amos Elberg and Jason Crowley (2021). GGally: Extension to 'ggplot2'. R package version 2.1.0. https://CRAN.R-project.org/package=GGally

Smyth, D. R. (2016). Helical growth in plant organs: mechanisms and significance. Development, 143(18), 3272-3282. https://doi.org/10.1242/dev.134064

Van der Pijl, L, and C. H. Dodson. 1966. Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables, Fla.

Wickham H., Seidel D. (2020). scales: Scale Functions for Visualization. R package version 1.1.1. https://CRAN.R-project.org/package=scales

Yam, T. W., Arditti, J., & Cameron, K. M. (2009). "The orchids have been a splendid sport"—an alternative look at Charles Darwin's contribution to orchid biology. American Journal of Botany, 96(12), 2128-2154. https://doi.org/10.3732/ajb.0900122

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. & Smith, G.M. (2009) Mixed effects models and extensions in ecology with R. *Springer Science & Business Media*, New York, USA. https://doi.org/10.1007/978-0-387-87458-6

Conclusões

As orquídeas são mundialmente conhecidas por apresentarem estratégias de sobrevivência fascinantes e histórias misteriosas. Nessa tese, mostramos como poucas espécies representantes de um único grupo de organismos que ocorre nos ambientes de Vereda nos traz histórias de vida únicas, descoberta de processos, e funcionam como modelo para teste de hipóteses.

Usando *C. hatschbachii* como modelo, demonstramos como dois fatores que não estão primariamente relacionados a polinização como a herbivoria e a chuva, podem juntos promover a formação de frutos por autogamia em uma espécie com limitação de polinização, funcionando como segurança reprodutiva. Até onde sabemos, essa é primeira vez onde é demonstrado que dois fatores que são detrimentais em separado, podem juntos facilitar a autogamia em uma planta. Usando *P. vittatum* como modelo, nós discutimos as fases do processo de polinização de flores semi-armadilha. Nós demonstramos as várias estratégias que essas flores adotam e que as deixam com superfícies escorregadias ou aderentes, dependendo do local onde o polinizador se encontra. Nós mostramos como *P. vittatum* aplica um sistema de mimetismo de afídeos que engana fêmeas grávidas de sirfídeos durante a entrada e saída da flor, uma vez que estas estão a procura de sítios para oviposição e suas larvas são predadoras de afídeos. Ainda estudando *P. vittatum*, encontramos que esta espécie se trata de um modelo único que apresenta variação intrapopulacional no processo de ressupinação, o tradicional giro de aproximadamente 180º que algumas flores fazem. Aproximadamente 10% das flores da população não completam o giro, permanecendo com o labelo voltado para cima ou para o lado. Nós demonstramos experimentalmente como apenas flores que estão inteiramente ressupinadas apresentam sucesso reprodutivo masculino e feminino. Esses resultados são importantes pois corroboram que a evolução da ressupinação provavelmente está relacionada a polinização, uma vez que o labelo funciona como uma plataforma de pouso ou uma armadilha bem elaborada capaz de capturar os polinizadores e a coluna se encontra voltada para baixo, promovendo a deposição precisa de pólen. Por fim, demonstramos que a probabilidade das flores não ressupinarem na população está negativamente relacionada ao peso das mesmas. Até onde sabemos, essa é a primeira vez que o peso é posto como um fator determinante do processo de ressupinação floral.

O fato de as espécies estudadas serem ameaçadas demonstra a diversidade de diferentes histórias que esses organismos à beira do desaparecimento podem nos contar. Isso sugere que a extinção desses organismos não se trata apenas de números a menos, mas acarreta na perda de processos importantes, muitos deles afetando organismos em cascata. Assim, a conservação dessas espécies ameaçadas e dos seus respectivos ambientes é de extrema relevância para a manutenção de tais processos e interações, que certamente estão associados a um ambiente equilibrado e provedor de numerosos serviços ecossistêmicos. Por fim, a perda ou deterioração dos diversos sistemas especializados encontrados nas Veredas podem ser indicadores de mudanças ambientais em curso, podendo ser usados como norteadores de estratégias de conservação.