UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL

ANA CAROLINA SABINO DE OLIVEIRA

O PAPEL DE ATRIBUTOS FLORAIS NA REPRODUÇÃO DE FLORES DE ÓLEO E NA PROTEÇÃO CONTRA A PERDA DE PÓLEN

Recife- PE
2025

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Biologia Vegetal. Área de Concentração: Ecologia e Conservação

Orientador (a): Dra. Isabel Cristina Sobreira Machado

Coorientador (a): Dr. Vinícius Lourenço Garcia de Brito

Recife- PE

2025

Catalogação de Publicação na Fonte. UFPE - Biblioteca Central

Sabino-Oliveira, Ana Carolina.
O papel dos atributos florais na reprodução de flores de óleo e na proteção contra a perda de pólen / Ana Carolina Sabino de Oliveira. - Recife, 2025.
150f.: il.

Tese (Doutorado) - Universidade Federal de Pernambuco, Centro de Biociências, Programa de Pós-Graduação em Biologia Vegetal, 2025.
Orientação: Isabel Cristina Sobreira Machado.
Coorientação: Vinícius Lourenço Garcia de Brito.
Inclui referências.

1. Atributos florais; 2. Abelhas coletoras de óleo; 3. Cor floral; 4. Longevidade floral; 5. Proteção do pólen; 6. Sinalização visual. I. Machado, Isabel Cristina Sobreira. II. Brito, Vinícius Lourenço Garcia de. III. Título.

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Aprovada em:

BANCA EXAMINADORA

Profa. Dra. Isabel Cristina Sobreira Machado (Orientadora)
Universidade Federal de Pernambuco (UFPE)

Prof. Dr. Anselmo Nogueira (1ª avaliador)
Universidade Federal do ABC Paulista (UFABC)

Prof. Dr. Pedro Joaquim Bérgamo (2º avaliador)
Universidade Estadual de São Paulo (UNESP)

Dra. Pamela Santana (3º avaliador)
Universidade de Lund- Suécia

Prof. Dr. Paulo Eugênio Alves Macedo de Oliveira (4º avaliador)
Universidade Federal de Uberlândia (UFU)

Aos meus pais, Elmo e Elena, e a minha irmã
Débora, que estão e estarão sempre comigo!

AGRADECIMENTOS

Àquele que me deu o dom da vida, obrigada por cuidar de mim e dos meus!

Aos meus pais, Elmo e Elena, e à minha irmã Débora, que mesmo em momentos delicados e não conhecendo o caminho da pós-graduação por não terem exemplos próximos pra se espelharem, acreditaram no que eu amava fazer e que a trajetória que escolhi seguir era o melhor que eu podia fazer por mim e por nós. Como diz Emicida, ‘tudo que nóis tem é nóis’!

À minha querida orientadora, Professora Isabel Machado, que abriu as portas pra mim sem mesmo me conhecer, e me deu a oportunidade de trabalhar com essa grande mulher da polinização no Brasil. Muito obrigada por enxergar meu potencial e por me permitir aprender com a senhora durante esses anos de Doutorado, mas além de tudo, obrigada pelo seu ímpeto de ver seus orientandos crescerem, nunca esquecerei disso.

Ao meu coorientador Vinícius, por também abrir as portas do seu grupo de pesquisa pra mim, que logo comecei a me sentir parte dele e amada, e também por um dos maiores aprendizados que eu poderia ter de alguém que também escolheu a ciência: o percurso do cientista pode ser leve, prazeroso e ainda assim de altíssimo nível. Obrigada pelo companheirismo e pela caminhada, Vini.

Ao Professor Bruce Anderson, que me recebeu na Universidade de Stellenbosh na África do Sul durante o meu Doutorado sanduíche, e que com seu espírito de naturalista curioso e apaixonado por entender a reprodução das plantas, me permitiu viver experiências acadêmicas que jamais esquecerei, e acreditou no meu potencial e no trabalho que realizamos juntos, thanks Bruce.

Á Banca examinadora, que gentilmente aceitou participar da minha defesa de Doutorado e por todas as contribuições dadas ao nosso trabalho. Obrigada Anselmo, Pedro, Pâmela e Paulo Eugênio, que privilégio tê-los conosco.

Aos meus amigos que o Lab Polinizar me deu, que tornaram a jornada mais prazerosa e acolhedora. Nando e Sandro, Recife me deu o sentimento de casa durante esses anos, e boa parte disso foi graças ao acolhimento de vocês no início, que bom que nos encontramos, obrigada por serem família, por serem apoio, por serem vocês!!! Isadora e Ailza, minhas pollination girls rsrs compartilhar as dores e as delícias da vida acadêmica com vocês sempre foi e é um prazer imenso, agradeço muito por nosso encontro!

Aos meus amigos Mikael e Sabrina, fomos apoio uns dos outros desde muito antes da pós graduação, quando ainda não tínhamos perspectivas de oportunidades. A realidade a qual viemos nos uniu, e isso fez com que entender um ao outro fosse mais simples, pois conseguíamos nos colocar nesse lugar. Que prazer compartilhar a vida com vocês, pois sei que ainda acompanharemos muito as dores e conquistas uns dos outros, sempre!

Aos meus amigos Lucas e Felipe, que de vizinhos geólogos desconhecidos se tornaram irmãos, família. Obrigada por compartilharem a vida comigo, não só a vida acadêmica, mas principalmente a vida de quem precisa estar longe dos seus, e encontra nos amigos uma família, por serem o respiro no fim do dia, sou muito feliz pelo nosso encontro.

Aos meus amigos do NERP, que desde o nosso primeiro encontro me fizeram sentir amada, obrigada por todas as trocas acadêmicas e de vida, pelos campos compartilhados em Diamantina com direito a muito afeto e bordões eternos haha, a ciência feita coletivamente é muito mais legal e prazerosa!

À Liga Mineira de Polinização e a FAPEMIG por todo o apoio logístico durante os campos que fizemos em Diamantina, em especial ao Laboratório CAFESIN.

Ao PELD Catimbau (Programa Ecológico de Longa Duração/Catimbau, 403770/2012-2), por todo o apoio logístico durante esses anos. Agradeço também por todas as experiências compartilhadas com amigos que, assim como eu, passavam semanas no Catimbau

durante suas coletas, e isso nos permitia criar momentos e memórias que tornavam todo o processo mais leve.

À Universidade Federal de Pernambuco- UFPE e ao Programa de Pós-graduação em Biologia Vegetal- PPGBV, em nome dos professores e funcionários, por toda a contribuição na minha formação e aquisição do título de Doutora.

À Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior- CAPES, pelo financiamento durante os seis meses do meu Doutorado sanduíche na África do Sul.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico- CNPq, pela bolsa concedida.

E por fim, e mais importante, agradeço a mim!!! Principalmente por nunca ter tido dúvidas de que a educação era o único caminho possível, desde a infância foi onde sempre me agarrei e depositei toda a minha esperança em mudar de realidade. Também por nunca ter pensado em desistir, pois isso jamais poderia ter sido uma opção. Dona Elena e Seu Elmo agora tem uma filha Doutora, porque a educação me salvou!!!

Vejo a vida passar num instante
Será tempo o bastante que tenho pra viver?
Não sei, não posso saber
[...]

Então, será tudo em vão? Banal? Sem razão?
Seria, sim, seria se não fosse o amor
O amor cuida com carinho, respira o outro, cria o elo
No vínculo de todas as cores, dizem que o amor é amarelo
[...]

Mas eu o miro, de frente, na minha fragilidade
Eu não tenho a bolha da proteção
Queria eu guardar tudo que amo
No castelo da minha imaginação
Mas eu vejo a vida passar num instante
Será tempo o bastante que tenho para viver?
Eu não sei, eu não posso saber
Mas enquanto houver amor
Eu mudarei o curso da vida
[...]

Porque eu descobri o segredo que me faz humano
Já não está mais perdido o elo
O amor é o segredo de tudo
E eu pinto tudo em amarelo

Principia, Emicida

RESUMO

Os atributos florais, como estrutura, cor e longevidade, desempenham papéis cruciais nas interações com polinizadores, influenciando tanto na atração quanto na eficiência reprodutiva das flores. Além disso, esses atributos também podem moldar a estrutura das comunidades de plantas polinizadas por abelhas. Nesta Tese, investigamos como diferentes atributos, especialmente a cor, longevidade floral e compostos químicos do óleo, influenciam a interação planta-polinizador em flores de óleo, além de um caso interessante de uma estrutura floral evitando a perda abiótica de pólen. No primeiro capítulo, analisamos como a cor floral determina o papel das espécies em duas comunidades da família Malpighiaceae. No segundo capítulo, exploramos o papel da longevidade floral na sinalização, produção de óleo e sucesso reprodutivo na espécie *Stigmaphyllon paralias* (Malpighiaceae), que mantém a cor ao longo de sua vida floral. No terceiro capítulo, investigamos se os compostos presentes no óleo floral de uma espécie de Melastomataceae, família caracterizada por flores que ofertam apenas pólen, atuam como um filtro na exclusão ativa de polinizadores. Por fim, no quarto capítulo exploramos a função do lábio superior de *Salvia chamelaeagnea* (Lamiaceae) na proteção contra a perda de pólen pelo vento, antes da chegada dos polinizadores. Esses capítulos, em conjunto, evidenciam como diferentes atributos florais influenciam a atração de polinizadores e a reprodução de flores produtoras de óleo, além de destacar a importância de estruturas florais que maximizam o sucesso reprodutivo masculino.

Palavras-chave: Atributos florais; abelhas coletoras de óleo; cor floral; estratégias reprodutivas; longevidade floral; proteção do pólen; redes mutualísticas; sinalização visual

ABSTRACT

Floral attributes, such as structure, color, and longevity, play crucial roles in pollinators' interactions, influencing the attraction and reproductive efficiency of flowers. Furthermore, these attributes can also shape the structure of bee-pollinated plant communities. In this thesis, we investigate how different attributes, especially color, floral longevity, and chemical compounds in the oil, influence the plant-pollinator interaction in oil flowers, in addition to an interesting case of a floral structure preventing abiotic pollen loss. In the first chapter, we analyze how floral color determines the role of species in two communities of the family Malpighiaceae. In the second chapter, we explore the role of floral longevity in signaling, oil production, and reproductive success in the species *Stigmaphyllon paralias* (Malpighiaceae), which maintains color throughout its floral life. In the third chapter, we investigate whether the compounds present in the floral oil of a species of Melastomataceae, a family characterized by flowers that offer only pollen, act as a filter in the active exclusion of pollinators. Finally, in the fourth chapter, we explore the function of the upper lip of *Salvia chamelaeagnea* (Lamiaceae) in protecting against wind-borne pollen loss before pollinators arrive. Together, these chapters highlight how different floral attributes influence pollinator attraction and reproduction of oil-producing flowers, and highlight the importance of floral structures that maximize male reproductive success.

Keywords: Floral attributes; oil-collecting bees; floral color; reproductive strategies; floral longevity; pollen protection; mutualistic networks; visual signaling

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1 INTRODUÇÃO GERAL

Os atributos florais desempenham um papel crucial na mediação das interações planta-polinizador, influenciando a atração dos polinizadores e o sucesso reprodutivo das plantas (ASHMAN,; SCHOEN, 1994; MUCHHALA; JOHNSEN; SMITH, 2014; WEISS, 1995). Cada característica floral pode atuar de forma independente ou em conjunto para maximizar a eficiência de polinização e minimizar o desperdício de recursos (BERGAMO *et al.*, 2018; SCHAEFER; SCHAEFER; LEVEY, 2004). Diante disso, esta tese investiga como diferentes atributos, especialmente a cor, longevidade floral e os compostos químicos do óleo, influenciam a interação planta-polinizador em flores de óleo, além de mostrar um caso interessante em que uma estrutura floral evita a perda abiótica de pólen.

Dentre os atributos florais, a cor é um dos mais importantes na comunicação entre plantas e polinizadores, auxiliando na eficácia da obtenção de recursos (LUNAU; WESTER, 2017; WESTER; LUNAU, 2017). O arranjo das cores em comunidades pode atuar fortemente na preferência de polinizadores (BRADSHAW-JR; SCHEMSKE, 2003; HORTH; CAMPBELL; BRAY, 2014; KANTSA *et al.*, 2017; KEMP; BERGH; SOARES; ELLIS, 2019), como as abelhas, que utilizam as pistas cromáticas como principal característica para detecção de alimento (BUKOVAC *et al.*, 2017; DYER; STREINZER; GARCIA, 2016). Além disso, a variação nas cores florais pode promover especialização de interações, levando a diferentes padrões de centralidade e conectância em redes mutualísticas (SCHIESTL; JOHNSON, 2013). No primeiro capítulo, investigamos como a cor da flor determina a estrutura das interações e ~~como a cor da corola~~ explica o papel que as espécies exercem em redes mutualísticas de Malpighiaceae, família que apresenta muitas espécies com flores de óleo.

Outra estratégia reprodutiva relevante é a longevidade floral, que influencia a disponibilidade de recursos ao longo do tempo. Embora a produção e manutenção de flores exijam um custo energético elevado (ASHMAN; SCHOEN, 1996; SONG *et al.*, 2022), a duração em que uma flor permanece aberta e atrativa pode ser fundamental para o sucesso da polinização, especialmente em espécies que não apresentam mudança de cor ao longo da vida da flor (PRIMACK, 1985; WEISS, 1995). No segundo capítulo, abordamos este atributo floral e sua função na reprodução de uma espécie produtora de óleo floral. Mais especificamente, investigamos como a longevidade floral influencia na sinalização visual para polinizadores, a produção de óleo e nos componentes reprodutivos, usando como modelo *Stigmaphyllon paralias,* uma espécie de Malpighiaceae que não apresenta mudança de cor floral.

Além de atributos de sinalização visual, como a cor floral, e atributos da história de vida dos organismos, como a longevidade floral, as características químicas são cruciais na comunicação planta-polinizadores. Compostos químicos em flores podem ter diversas funções, dentre elas atuar como filtros florais excluindo visitantes indesejados (RHOADES, 1981; JOHNSON; HARGREAVES; BROWN, 2006; TIEDEKEN *et al.*, 2016). No entanto, o conhecimento sobre filtros químicos ainda está majoritariamente restrito ao aroma floral e a recursos amplamente difundidos em angiospermas, como o néctar. Diante disso, no terceiro capítulo investigamos se o óleo floral da espécie *Pleroma candolleanum* (Melastomataceae), pertencente a uma família característica de flores que ofertam apenas pólen como recurso, atua como um filtro floral na exclusão ativa de visitantes.

Por fim, a estrutura física das flores também desempenha um papel crucial nas interações planta-polinizador. Mecanismos como a alavanca estaminal pode restringir o acesso aos recursos, garantindo que apenas polinizadores com características morfológicas específicas consigam realizar a polinização de forma eficiente (THIMM, 2008). Além disso, antes que a fertilização do óvulo possa ocorrer, o pólen pode ser perdido por diversas vias como o vento (REYNOLDS *et al.*, 2009) ou chuva (MAO; HUANG, 2009), e estruturas florais podem atuar na prevenção dessa perda. No quarto capítulo, portanto, investigamos como o lábio superior, estrutura que compõe a alavanca estaminal em espécies de *Salvia* (Lamiaceae), protege contra a perda abiótica de grãos de pólen pelo vento.

Em síntese, esta tese aborda como diferentes atributos florais e estratégias reprodutivas, como a cor floral, a longevidade e a estrutura das flores afetam a sinalização visual para polinizadores, estrutura das comunidades e sucesso reprodutivo, com foco especial nas flores de óleo. Dessa forma, ao investigar como diferentes atributos florais modulam as interações planta-polinizador, esta tese contribui para a compreensão de como a variação nos traços florais afeta a estabilidade das redes mutualísticas e a resiliência das comunidades ecológicas.

2 REFERENCIAL TEÓRICO

Neste referencial teórico são abordados aspectos relacionados aos diferentes atributos florais como cor, longevidade, morfologia e filtros e suas influências nas interações planta-polinizador, especialmente em flores de óleo. Foram utilizados artigos científicos publicados entre 1793 e 2024, consultados nas bases de dados: Google Scholar, Web of Science, SciELO e sites de periódicos acadêmicos.

2.1 Cor floral

2.1.1 Panorama geral

A partir da comunicação entre plantas e polinizadores, processos coevolutivos podem ser gerados por pressões seletivas, nos quais sinais florais e polinizadores se adaptam mutuamente (AGUIAR *et al.*, 2020). Dentre estes sinais, a cor floral é um dos mais importantes nesta comunicação e auxilia na eficácia da obtenção de recursos (WESTER, P.; LUNAU *et al.*, 2017). A cor floral é um atributo chave na atração de polinizadores, com implicações diretas na reprodução das plantas (BRADSHAW-JR; SCHEMSKE, 2003; FENSTER *et al.*, 2004; OBERRATH; BÖHNING-GAESE, 1999). Trata-se de uma das principais características que definem as interações entre polinizadores e flores, além de refletir a influência da história evolutiva na sinalização visual (LARUE-KONTIĆ; JUNKER, 2016; YAN *et al.*, 2016). A cor das flores exibe uma enorme diversidade na natureza, sendo um dos traços mais marcantes da radiação adaptativa das angiospermas, e essa diversidade se traduz em padrões de cores que abrangem não apenas o espectro visível aos humanos, mas também aos polinizadores (KOOI, VAN DER; SPAETHE; LEONHARDT, 2022; SCHIESTL; JOHNSON, 2012). Além disso, a cor floral pode variar consideravelmente em escalas geográficas e temporais (TRUNSCHKE *et al.*, 2021), desempenhando um papel fundamental na estruturação das comunidades vegetais.

A vasta gama de cores florais é formada por uma diversidade de pigmentos que, ao absorver determinados comprimentos de onda da luz e refletir outros, criam cores que ocupam diferentes partes do espaço visual dos polinizadores (KOOI, VAN DER; SPAETHE; LEONHARDT, 2022; NARBONA *et al.*, 2021). Esses pigmentos, compostos químicos presentes nas flores, incluem clorofilas, carotenoides, flavonoides e betalaínas, cada um com características específicas relacionadas à absorção de luz e à cor resultante (GROTEWOLD, 2006; NARBONA *et al.*, 2021). As clorofilas absorvem luz nas regiões azul e vermelha, gerando cores verdes para a visão humana, enquanto os carotenoides absorvem principalmente na região azul, resultando em cores amarelo-alaranjadas, enquanto as betalaínas, por sua vez, absorvem luz nas regiões azul ou verde, originando tons amarelos ou rosa (GROTEWOLD, 2006; NARBONA *et al.*, 2021). Já os flavonoides, que são a classe de pigmentos mais difundida entre as angiospermas (IWASHINA, 2015), abrangem subgrupos como as antocianinas, que absorvem luz na faixa do verde e geram cores que variam entre azul, rosa, laranja e vermelho (GROTEWOLD, 2006). Outros subgrupos incluem as auronas e chalconas, que absorvem na região azul e produzem flores amarelas, bem como flavonóis, flavonas e flavanonas, que absorvem no ultravioleta, gerando flores com reflexos UV, brancas ou amarelo-claras (NARBONA *et al.*, 2021; TANAKA; SASAKI; OHMIYA, 2008).

Diante da sua diversidade química e estrutural, a cor floral desempenha um papel crítico na mediação das interações planta-polinizador, uma vez que a eficácia da sinalização depende da capacidade dos polinizadores em detectar e discriminar cores específicas (SCHIESTL; JOHNSON, 2013). Diante desse contexto, a conexão entre a produção de cores, a percepção animal e as pressões seletivas destaca a importância das cores florais como um dos principais pilares na coevolução de plantas e polinizadores (FENSTER *et al.*, 2004;

NARBONA *et al.*, 2021), e compreender como as cores das flores são produzidas e percebidas pelos animais é essencial para entender as interações planta-polinizador.

2.1.2 Visão em cores de abelhas

A cor floral parece ter evoluído sob uma forte pressão seletiva exercida pelos sistemas de visão de cores dos insetos (SCHIESTL; JOHNSON, 2012). A capacidade de enxergar cores está amplamente disseminada entre os animais e está diretamente relacionada ao uso da composição espectral da luz para orientar a tomada de decisões (KOOI, VAN DER *et al.*, 2021). A percepção das cores florais varia de acordo com os diferentes sistemas visuais dos grupos de polinizadores, incluindo abelhas, aves e morcegos, cada um com sensibilidades específicas a determinados comprimentos de onda (BRISCOE; CHITTKA, 2001). Nos insetos, a forma mais comum e ancestral de visão em cores é tricomática, baseada em três fotorreceptores sensíveis aos comprimentos de onda ultravioleta, azul e verde (CHITTKA, 1996). Assim, a maneira como os polinizadores percebem e respondem às cores das flores em populações vegetais influencia diretamente a força da seleção mediada pelos polinizadores, moldando a evolução dos atributos florais.

As abelhas estão entre os insetos mais bem estudados em relação à percepção visual e aos comportamentos derivados de seu sistema neural e desempenho visual (AVARGUÈS-WEBER; MOTA; GIURFA, 2012; AVARGUÈS; MARTIN, 2014; GIURFA; LEHRER, 2001; MOTA, *et al.*, 2013). Estes polinizadores possuem neurônios oponentes de cor em áreas visuais de ordem superior no cérebro, responsáveis pela geração das sensações de cor (KIEN; MENZEL, 1977). Esses neurônios recebem informações de três tipos de fotorreceptores e apresentam campos receptivos e características de resposta temporal amplamente variáveis (HEMPEL DE IBARRA; VOROBYEV; MENZEL, 2014). Com uma visão tricromática, os fotorreceptores das abelhas atingem picos de sensibilidade em 344 nanômetros (nm) na região de onda curta (ultravioleta), 436 nm na região de onda média (azul) e 544 nm na região de onda longa (verde) (PEITSCH *et al.*, 1992). Portanto, a extensa variação de cores das flores na natureza versus a variação nos sistemas visuais, sobretudo de abelhas, sugere que as cores florais estão ajustadas à visão dos polinizadores (KOOI, VAN DER *et al.*, 2021).

Além da visão cromática, as abelhas utilizam o contraste acromático, baseado na intensidade luminosa, para localizar flores (GIURFA *et al.*, 1997; SPAETHE, J.; TAUTZ; CHITTKA, 2001). O contraste acromático é essencial para a detecção de flores à distância, enquanto a percepção cromática torna-se predominante quando as abelhas estão próximas das flores ou diante de alvos florais de grande porte (HEMPEL DE IBARRA; LANGRIDGE; VOROBYEV, 2015). Cerca de 20% das angiospermas exibem mudanças de cor ao longo da antese (WEISS, 1995), uma estratégia comum para ajustar a sinalização floral aos polinizadores (SUZUKI; OHASHI, 2014; WEISS; LAMONT, 1997). No entanto, a maioria das espécies mantém a coloração ao longo da vida útil da flor. À distância, essas mudanças de cor aumentam o tamanho aparente do display floral, atraindo mais visitas dos polinizadores (GORI, 1983; OBERRATH; BÖHNING-GAESE, 1999). Em distâncias curtas, essa estratégia melhora a eficiência de forrageamento ao reduzir visitas desnecessárias a flores sem recursos (WEISS, 1995).

Para representar a visão cromática das abelhas, Chittka (1992) desenvolveu o hexágono de cores, um modelo de cromaticidade que mapeia as excitações dos três fotorreceptores de sistemas de visão tricromática. Esse modelo organiza as respostas dos fotorreceptores ultravioleta, azul e verde de himenópteros em um espaço cromático em forma de hexágono (TRUNSCHKE *et al.*, 2021). A distância euclidiana entre dois pontos no hexágono reflete a diferença percebida entre duas cores, fornecendo uma métrica objetiva para avaliar como abelhas distinguem estímulos visuais Chittka (1992). Amplamente adotado, este modelo tem sido uma ferramenta essencial para explorar como variações na cor floral moldam a percepção e a preferência das abelhas, permitindo avanços na compreensão dos mecanismos de seleção mediada por polinizadores e na evolução da diversidade cromática das flores (DYER *et al.*, 2012; KOOI, VAN DER *et al.*, 2021).

2.1.3 Propriedades de cor

A cor floral é tradicionalmente descrita com base em três propriedades principais que influenciam a percepção visual humana: matiz, brilho e saturação (KELBER; OSORIO, 2010). No entanto, avanços em estudos comportamentais e de modelagem visual têm permitido investigar quais características espectrais das flores e quais receptores visuais são mais relevantes para direcionar as decisões dos polinizadores (KOOI, VAN DER *et al.*, 2019). Nesse contexto, um dos grandes desafios da ecologia cognitiva da polinização é identificar os atributos visuais biologicamente relevantes para a evolução das flores, a percepção dos polinizadores e como esses fatores interagem com outros sinais visuais (KOOI, VAN DER *et al.*, 2019).

O modelo hexagonal de cores, desenvolvido para modelar a visão de abelhas, oferece uma ferramenta valiosa para estimar diferentes propriedades de cor na percepção desses insetos (CHITTKA, 1992). Nesse modelo, o matiz é representado pelo ângulo formado entre o eixo x do hexágono e a linha que conecta o centro do hexágono aos loci da cor (CHITTKA, 1992). A pureza espectral, por sua vez, é medida pela distância perceptual entre o locus de cada mancha floral e o centro do hexágono, normalizada pela distância até o locus espectral de pureza máxima correspondente (LUNAU, K.; WACHT; CHITTKA, 1996). O brilho é calculado pela soma das respostas dos três tipos de fotorreceptores presentes na visão das abelhas (SPAETHE, J.; TAUTZ; CHITTKA, 2001). Já o contraste com o plano de fundo, definido como a distância euclidiana entre o locus floral e o ponto central do hexágono que representa o fundo verde padrão, desempenha um papel crucial na localização de flores a longas distâncias . Em muitos casos, o contraste de uma flor e seu fundo é semelhante à sua pureza espectral, mesmo que os mecanismos subjacentes da visão de abelhas possuam diferenças (LUNAU, K.; DYER, 2024). Dessa forma, integrar estudos sobre as propriedades ópticas das flores com o entendimento dos sistemas visuais e comportamentos dos polinizadores é essencial para compreender as interações planta-polinizador e os mecanismos subjacentes à seleção de flores pelos polinizadores (KOOI, VAN DER *et al.*, 2019).

2.1.4 Cor floral em comunidades

A montagem das cores florais é conhecida na literatura como um atributo-chave que influencia a estrutura de algumas comunidades vegetais (MUCHHALA; JOHNSEN; SMITH, S. D., 2014b). Estudos sugerem que o arranjo das cores dentro de comunidades vegetais pode desempenhar um papel determinante nas preferências dos polinizadores . Esses arranjos podem variar entre configurações de cores superdispersas (MUCHHALA; JOHNSEN; SMITH, 2014) e agrupadas (KEMP; BERGH; SOARES; ELLIS, Allan G., 2019), refletindo a plasticidade evolutiva desse atributo floral sob seleção mediada por polinizadores (MUCHHALA; JOHNSEN; SMITH, 2014).

A seleção imposta por polinizadores dominantes pode resultar em convergência evolutiva de características florais entre as espécies de uma comunidade (JAGER; ELLIS; ANDERSON, 2022). Em particular, a sobreposição de cores florais afeta significativamente o comportamento de forrageamento dos polinizadores. Flores com cores semelhantes podem intensificar os movimentos entre espécies cofloridas, promovendo tanto a polinização cruzada como a produção de sementes (COETZEE; SEYMOUR; SPOTTISWOODE, 2021; JAGER; ELLIS, 2011; JOHNSON *et al.*, 2003). Esses agrupamentos de cores podem surgir devido à facilitação, que aumenta a aptidão das plantas, à filtragem ecológica ou à convergência evolutiva impulsionada pelas preferências dos polinizadores (JAGER; ELLIS; ANDERSON, 2022; KEMP *et al.*, 2019). No entanto, os benefícios proporcionados pelo aumento de visitação de polinizadores podem ser neutralizados em certas circunstâncias. O aumento do movimento entre espécies cofloridas pode elevar a deposição de pólen heteroespecífico, o que reduz a produção de sementes (ASHMAN; ARCEO-GÓMEZ, 2013; MUCHHALA, 2019). Isso pode resultar em competição interespecífica causada pela perda de pólen efetivo (JAGER; ELLIS; ANDERSON, 2022). Portanto, para compreender plenamente os efeitos da sobreposição de cores florais entre espécies em comunidades, é fundamental considerar os contextos espaciais e temporais, bem como os aspectos específicos da biologia floral de cada espécie.

Além disso, o arranjo de cores florais em comunidades pode influenciar significativamente sua estrutura (BERGAMO *et al.*, 2018; KEMP *et al.*, 2019; MUCHHALA; JOHNSEN; SMITH, 2014) e, potencialmente, o papel que as espécies desempenham nas redes de interações mutualísticas. Embora o conhecimento sobre como características morfológicas das flores afetam a estrutura das comunidades tenha avançado (KANTSA *et al.*, 2017; KEMP; BERGH; SOARES; ELLIS, Allan G., 2019), os atributos relacionados à sinalização visual para polinizadores ainda são pouco explorados em relação às suas contribuições para a funcionalidade das espécies nas redes ecológicas (SHRESTHA *et al.*, 2019).

2.2 Longevidade floral

2.2.1 Panorama geral acerca da longevidade floral

A longevidade floral refere-se ao período em que a flor permanece aberta, funcional e acessível aos polinizadores, variando amplamente entre táxons vegetais (ASHMAN;

SCHOEN, 1994; PRIMACK, 1985). Esse intervalo pode ser de apenas algumas horas até vários meses (ASHMAN; SCHOEN, 1994; PRIMACK, 1985; SONG *et al.*, 2022). Grande parte dessa variação é influenciada pelo contexto do serviço de polinização ao qual a espécie está sujeita, refletindo pressões seletivas distintas e a plasticidade de características florais que modulam a atração de polinizadores (SPIGLER, 2017). Por exemplo, espécies submetidas a alta limitação de pólen frequentemente prolongam a vida útil das flores, aumentando o período de exibição floral para atrair mais polinizadores (ASHMAN; SCHOEN, 1996; RATHCKE, 2003; SPIGLER, 2017). Em contrapartida, espécies em ambientes com alta eficiência de polinização tendem a reduzir a duração floral, minimizando os riscos de autofecundação (SPIGLER, 2017).

Além disso, a longevidade floral é um atributo dinâmico que influencia diretamente a atratividade das flores e as chances de receber visitas de polinizadores (PRIMACK, 1985). Flores abertas desempenham um papel ativo na atração de polinizadores, e a duração do período floral determina o número de flores simultaneamente disponíveis em um indivíduo, influenciando assim a eficácia da exibição floral (PRIMACK, 1985). Dessa forma, a longevidade floral é uma característica crucial na atratividade das plantas, pois afeta diretamente as oportunidades de forrageamento dos polinizadores dentro de um mesmo indivíduo (HARDER; BARRETT, 1995; ISHII; SAKAI, 2000).

2.2.2 Influência da longevidade floral na reprodução vegetal

O período em que a flor permanece aberta e funcional é uma característica fundamental com importantes implicações para o sucesso reprodutivo das plantas (ASHMAN; SCHOEN, 1994, 1996; PRIMACK, 1985). A duração da vida útil das flores contribui para aumentar as chances de recepção adequada de pólen nos estigmas, além de promover maior exportação de pólen e diversidade de doadores (ASHMAN; SCHOEN, 1994; MARSHALL *et* *al.*, 2010; RATHCKE, 2003). Contudo, a longevidade floral não apenas influencia os componentes reprodutivos, mas também está associada a um equilíbrio de custos e benefícios relacionados à manutenção das flores em estado funcional e atrativo (GORI, 1983).

A longevidade floral pode impactar de maneira distinta os diferentes componentes reprodutivos das plantas. Em relação ao sucesso reprodutivo feminino, geralmente, a longevidade floral não apresenta uma relação direta com esse componente (SONG *et al.*, 2022). Um possível motivo para isso é a elevada proporção pólen-óvulo característica da maioria das plantas polinizadas por animais (CRUDEN, 1977). Por outro lado, enquanto algumas espécies induzem a senescência floral precoce após a deposição de pólen no estigma (ARROYO *et al.*, 2013; CASTRO, S.; SILVEIRA; NAVARRO, 2008), outras mantêm as flores abertas para maximizar a exportação de pólen (DOORN, VAN, 1997), destacando um benefício diferencial para o sucesso masculino. A longevidade floral está mais diretamente associada a este componente reprodutivo (ISHII; SAKAI, 2000; SONG *et al.*, 2022), pois flores com maior duração podem aumentar a exportação de pólen ao receberem múltiplas visitas (BELL, 1985; QUELLER, 1997; SONG *et al.*, 2022). Assim, enquanto o impacto da longevidade floral no sucesso masculino pode ser tanto positivo quanto negativo, seu efeito sobre a função feminina tende a ser mínimo ou neutro, dependendo da espécie.

2.3 Filtros florais e a exclusão ativa de polinizadores

A transferência de pólen em angiospermas é um processo altamente ineficiente, com apenas cerca de 1% dos grãos de pólen atingindo os estigmas coespecíficos (HARDER; BARRETT; COLE, 2000; HARGREAVES; HARDER; JOHNSON, 2009; HOLSINGER; THOMSON, 1994; JOHNSON; NEAL; HARDER, 2005). Essa baixa eficiência pode ser atribuída a diversos fatores, incluindo a visita de vetores ineficazes no transporte de pólen, o que reduz a aptidão reprodutiva da planta (LAU; GALLOWAY, 2004). Para mitigar essas perdas e aumentar a eficiência da polinização, muitas plantas desenvolveram diversas estratégias, dentre elas filtros florais, estruturas ou mecanismos que restringem o acesso às recompensas florais exclusivamente aos polinizadores legítimos, otimizando o processo de transferência de pólen (JOHNSON; HARGREAVES; BROWN, 2006; JOHNSON; STEINER, 2000).

A evolução de filtros florais pode resultar na exclusão ativa de polinizadores. Esse processo é uma consequência de um trade-off de oportunidades, um tipo de trade-off mediado por visitantes, no qual diferentes grupos de visitantes, que variam em sua eficácia para a reprodução das plantas, competem por oportunidades limitadas de contribuir para o sucesso reprodutivo da planta (OHASHI; JÜRGENS; THOMSON, 2021). Nesse contexto, polinizadores ineficientes atuam como parasitas funcionais, ou antagonistas, ao competir com visitantes mais eficientes. Os filtros florais, portanto, desempenham um papel crucial ao excluir esses visitantes menos eficazes, maximizando o sucesso reprodutivo da planta (OHASHI; JÜRGENS; THOMSON, 2021).

Dentre os filtros florais já descritos na literatura, é possível encontrar tanto filtros estruturais quanto de sinalização, olfativas ou visuais. Os filtros visuais já foram relatados em cores florais, desencorajando polinizadores indesejados a visitar determinadas espécies, ou favorecer outras (CAMARGO *et al.*, 2019; JOHNSON, HARGREAVES; BROWN, 2006; LUNAU *et al.*, 2011). Filtros morfológicos atuam como barreiras que impedem o encaixe morfológico dos visitantes de forma adequada, como o comprimento dos tubos florais (WESTER *et al.*, 2020). E os filtros químicos atuam de diversas formas, dentre elas repelindo visitantes indesejados (RHOADES, 1981; JOHNSON; HARGREAVES; BROWN, 2006; TIEDEKEN *et al.*, 2016). Porém, ainda é escasso o conhecimento de barreiras químicas associadas à compostos além do aroma floral, e de recursos altamente difundidos em angiospermas, como o néctar.

2.4 Influência da morfologia floral na proteção do pólen

Dentre as primeiras etapas da reprodução masculina em angiospermas está a apresentação do pólen, no qual as plantas polinizadas por animais enfrentam o desafio de transferir o pólen de maneira eficiente para maximizar o sucesso da polinização (MINNAAR *et al.*, 2019). Entretanto, antes mesmo de o pólen ser transportado, as estruturas reprodutivas associadas à sua apresentação podem estar sob pressão seletiva para protegê-lo contra perdas abióticas causadas por fatores como chuva e vento (MAO; HUANG, 2009; MINNAAR *et al.*, 2019; REYNOLDS *et al.*, 2009). Nesse contexto, estruturas florais que desempenham funções como a atração de polinizadores ou o ajuste mecânico na deposição do pólen podem também atuar na proteção do pólen, garantindo sua integridade até o momento da visitação por polinizadores efetivos (MAO; HUANG, 2009).

Os primeiros estudos sobre perda abiótica de pólen focaram no impacto da chuva nesse processo. Sprengel (1793) foi pioneiro ao identificar que a chuva não apenas lavava os grãos de pólen, comprometendo sua qualidade, como também diluía o néctar. Desde então, diversas investigações têm explorado o papel de estruturas florais na proteção do pólen contra a chuva. Exemplos incluem flores conhecidas como "guarda-chuva", que evitam a exposição do pólen à umidade (HAGERUP, 1950), flores que fecham a corola para proteger o pólen (BYNUM; SMITH, 2001; HASE, VON; COWLING; ELLIS, 2006) e flores com estruturas em forma de capuz que cobrem as anteras, característica observada em algumas flores tubulares (MARILAUN, VON; OLIVER, 1895).

Embora a função de estruturas florais na proteção contra a perda de pólen por chuva ou neblina tenha sido explorada em alguns contextos, ainda carecemos de estudos empíricos sobre estruturas que atuem especificamente na proteção do pólen contra a perda pelo vento. Já é conhecido que o vento pode causar perdas significativas de pólen, como mostrado por Reynolds e colaboradores (2009), que relataram uma redução de 50% no pólen presente nas anteras devido à ação do vento. No entanto, ainda não foram investigadas as adaptações estruturais das flores que possam minimizar essas perdas até o momento da apresentação do pólen aos polinizadores. Assim, estudos focados na função de estruturas florais para proteger o pólen contra perdas ambientais são indispensáveis, considerando que a etapa de apresentação do pólen aos polinizadores é uma das mais cruciais para o sucesso reprodutivo das angiospermas.

2.5 Flores de óleo

2.5.1 Panorama geral

As angiospermas que dependem de animais para a transferência de pólen utilizam recompensas florais como estratégia para atrair polinizadores (MACHADO, 2004). Por muito tempo, acreditava-se que néctar e pólen eram as únicas recompensas nutritivas disponíveis. No entanto, essa visão mudou com o trabalho pioneiro de Vogel (1964), que, ao estudar a espécie *Angelonia angustifolia* (Plantaginaceae), identificou que uma substância anteriormente descrita como néctar não se misturava com água. Esse achado levou a investigações mais detalhadas e, em 1969, Vogel descreveu um novo sistema de polinização em que flores possuíam glândulas especializadas na secreção de óleo. Posteriormente, ele realizou uma série de estudos (VOGEL, 1969, 1974, 1990) para compreender os aspectos morfológicos e ecológicos desse recurso floral até então pouco conhecido.

Espécies produtoras de óleo floral surgiram diversas vezes ao longo da evolução das angiospermas, sendo, portanto, uma característica polifilética (RENNER; SCHAEFER, 2010). O óleo floral é secretado por estruturas especializadas chamadas elaióforos (Vogel, 1974), presentes em aproximadamente 1800 espécies distribuídas em 11 famílias botânicas (MACHADO, 2004; POSSOBOM; MACHADO, 2017; RENNER; SCHAEFER, 2010). Dependendo da espécie, os elaióforos podem estar localizados no androceu, na corola ou no cálice, variando em sua estrutura secretora (CARNEIRO; MACHADO, 2023). Os elaióforos apresentam dois tipos morfológicos. Os elaióforos epiteliais, os mais comuns, são pequenas regiões de células epidérmicas especializadas onde os lipídios secretados se acumulam em grande quantidade sob uma fina camada de cutícula protetora, e podem ser encontrados em famílias como Krameriaceae, Malpighiaceae, Melastomataceae e Orchidaceae (BUCHMANN, 1987). Já os elaióforos tricomais consistem em extensos agrupamentos de tricomas glandulares, que podem reunir centenas a milhares de estruturas que secretam lipídios, e ocorrem em famílias como Iridaceae, Cucurbitaceae, Primulaceae, Plantaginaceae e Solanaceae (BUCHMANN, 1987). Algumas espécies da família Orchidaceae apresentam ambos os tipos de elaióforos, exibindo morfologias altamente complexas (CASTRO; PERDOMO; SINGER, 2022; GOMIZ; TORRETTA; ALISCIONI, 2017).

Dentre as famílias com flores de óleo, Malpighiaceae se destaca como uma das principais representantes. As flores de Malpighiaceae tendem a possuir uma similaridade estrutural quanto a arquitetura floral, atração e recompensa aos polinizadores, como por exemplo os elaióforos epiteliais que, quando presentes, estão pareados e aderidos na face abaxial das sépalas, o que é chamado de conservatismo floral (ANDERSON, 1979; BEZERRA; MACHADO, 2008). O sucesso das espécies do grupo nos neotrópicos teve como principal causa a coevolução dessas flores de óleo e as abelhas coletoras de óleos florais (VOGEL, 1990b). No entanto, apesar do amplo conhecimento existente sobre aspectos da história natural dessa relação intrínseca entre espécie de Malpighiaceae e abelhas coletoras de óleo, ainda são necessários estudos que abordem estas interações de forma mais ampla para entender os padrões e determinantes das interações de óleo e os papeis funcionais das espécies.

2.5.2 Características dos óleos florais

Os óleos florais são compostos de lipídeos como ácidos graxos livres, mono e diglicerídeos, que constituem o segundo maior grupo de constituintes florais (BUCHMANN, S. L., 1987; VOGEL, 1974, 1990a). Além disso, também são encontradas pequenas quantidades de compostos fenólicos, aminoácidos (prolina, alanina e glicina) e traços de glicose (HALEEM, 2012). Os óleos florais variam entre grupos, e tais variações na quantidade e composição química se tornam um aspecto relevante para compreender o papel do óleo na história evolutiva das abelhas coletoras de óleo, como espécies da tribo Centridini (ALVES-DOS-SANTOS; MACHADO; GAGLIANONE, 2007). Em Malpighiaceae, os gêneros utilizam ácidos graxos distintos, que poderiam levar a uma diferença química entre as espécies simpátricas (HALEEM, 2012). Por exemplo, espécies do gênero *Banisteriopsis* possuem ácidos graxos do tipo A, enquanto espécies de *Byrsonima* possuem ácidos graxos do tipo B (HALEEM, 2012), e tais diferenças podem ocasionar polinização preferencial em algumas espécies.

O conhecimento atual sobre a composição dos óleos florais está concentrado principalmente em algumas famílias de plantas, com destaque para Malpighiaceae. Entretanto, há registros de flores produtoras de óleo também na família Melastomataceae, conhecida principalmente por possuírem flores de pólen. Até recentemente, os registros de óleo floral em Melastomataceae estavam restritos à subfamília Memecyloideae (BUCHMANN; BUCHMANN, 1981). No entanto, Oliveira e colaboradores (2022) descreveram a composição e a função do óleo em um clado distinto, na espécie *Macairea radula* (Marcetieae, Melastomatoideae). Esses avanços destacam a necessidade de estudos que investiguem os aspectos ecológicos da produção de óleo floral em uma família tipicamente de flores de pólen e sua influência nas interações com polinizadores.

2.5.3 Relação das flores de óleo com as abelhas coletaras de óleo

As descobertas de Vogel (1969, 1974) sobre a existência de flores que secretam óleo como recurso abriram um amplo campo de pesquisa sobre as relações entre as espécies produtoras de óleo e os gêneros de abelhas que o coletam (MACHADO, 2004; RENNER & SCHAEFER 2010). Cerca de 330 espécies de abelhas são especialistas na coleta de óleo em flores (ALVES-DOS-SANTOS; MACHADO; GAGLIANONE, 2007). As abelhas utilizam o óleo produzido pelos elaióforos como alimento para suas larvas e/ou como material para revestimento celular (BUCHMANN, 1987; SIMPSON; NEFF, 1981; VOGEL, 1974, 1990a). Nos neotrópicos, essas abelhas estão distribuídas entre as tribos Centridini, Ctenoplectrini, Tapinotaspidini e Tetrapediini (Apidae), sendo os gêneros *Centris* e *Epicharis* destacados como os principais polinizadores das flores produtoras de óleo (ALVES-DOS-SANTOS; MACHADO; GAGLIANONE, 2007; CARNEIRO; MACHADO, 2023).

O comportamento das abelhas durante a coleta de óleo floral é semelhante entre os diferentes gêneros especializados, mas varia em função das estruturas florais das espécies visitadas (BEZERRA 2008). As abelhas, normalmente fêmeas, possuem pêlos em suas pernas, que funcionam como pentes simples ou ramificadas, conhecidos como escopas (ALVES-DOS-SANTOS *et al.*, 2006). Utilizando essas estruturas, as abelhas raspam os elaióforos, armazenam o óleo coletado em suas escopas das pernas posteriores, e misturam esse óleo com pólen para alimentar suas larvas (ALVES-DOS-SANTOS; MACHADO; GAGLIANONE, 2007; BUCHMANN, 1987; MACHADO, 2004; NEFF; SIMPSON, 1981; VOGEL, 1974). Um exemplo é o comportamento das abelhas do gênero *Centris*, um dos principais grupos coletores de óleo. Essas abelhas raspam os elaióforos ao pousar na flor, garantindo a exploração completa das glândulas. Ainda na flor, realizam a limpeza das pernas anteriores e medianas, transferindo o óleo coletado para as pernas posteriores (ALBUQUERQUE et al., 2023; TEIXEIRA; MACHADO, 2000; VILHENA; AUGUSTO, 2007).

Nesse contexto, observa-se que as flores produtoras de óleo e as abelhas coletoras desse recurso estão profundamente interligadas, formando uma relação altamente especializada (POSSOBOM; MACHADO, 2017). Espécies de Malpighiaceae evoluíram em paralelo ao surgimento de um proto-Centridini, indicando que o sucesso dessa interação está ligado à coevolução entre as espécies dessa família e as abelhas da tribo Centridini (NEFF; SIMPSON, 1981). Assim, é de extrema importância o desenvolvimento de pesquisas que explorem os aspectos evolutivos e ecológicos dessa relação altamente especializada, para contribuir na compreensão dos mecanismos que sustentam essas interações mutualísticas.

2.6 Referências

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Capítulo 1

The corolla color explains the functional role of species in Malpighiaceae mutualistic networks

Manuscrito a ser submetido ao periódico: Functional Ecology

Fator de impacto: 4.6

The corolla color explains the functional role of species in Malpighiaceae mutualistic networks

Sabino-Oliveira, A. C.[1*]; Brito, V. L. G.[2] Camargo, M. C. G[3].; Morelatto, P.[3] & Machado, I. C.[1]

1: Biosciences Center, Department of Botany, Postgraduate Program in Plant Biology, Federal University of Pernambuco, Recife, Pernambuco 50670-420, Brazil

2: Institute of Biology, Federal University of Uberlândia, Uberlândia, Minas Gerais 38400-902, Brazil

3: Centro de Pesquisa em Biodiversidade e Mudanças Climáticas e Departamento de Biodiversidade, Laboratório de Fenologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), São Paulo, Brazil

Abstract

Among the floral attributes that can influence the structure of mutualistic networks, floral color stands out as one of the most relevant. In this study, we investigated whether floral color determines interactions and how color explains the functional role of species in mutualistic networks of Malpighiaceae. We used floral color and data on interactions between species of this family and their bees in two communities. We found that floral color determines interactions with bees. In addition, we found that flowers with higher hue (yellow) and lower spectral purity, as well as flowers with lower hue (white) and higher spectral purity, are those with greater strength and centrality in these mutualistic networks. These results suggest that visual similarity between species, mediated by color compensation mechanisms, may be an effective strategy to optimize pollinator attraction and reduce competition for resources.

Keywords: floral color; mutualistic networks; visual signaling

Introduction

All species interact within ecological communities, forming complex networks, and understanding the factors that drive these interactions is crucial for comprehending community functioning (de Jager et al., 2011; Tylianakis et al., 2010). Species characteristics, such as functional, behavioral, and morphological traits, can influence the structure of interaction networks (Dehling et al., 2016; Hagen et al., 2012; Sebastián-González, 2017). In plant-pollinator networks, floral traits can affect the pollination niche and interactions with other species (Kemp et al., 2019), resulting in more dispersed or clustered trait distributions (Armbruster et al., 1994; Muchhala & Potts, 2007). Although morphological traits have already been highlighted as determinants of species function within ecological communities (Dehling et al., 2016; Ricklefs, 2012), the contribution of individual traits to specific species roles is still not fully understood (Mello et al., 2015; Ricklefs, 2012; Sebastián-González, 2017).

Among the factors that can influence the structure of plant-pollinator interactions, one of them is the temporal availability of floral resources, in which species that flower at the same time may be more likely to share pollinators, shaping interaction networks (Albor et al., 2022). Furthermore, several floral traits have been suggested as determinants of ecological function, influencing how species interact within communities. For example, central species that connect multiple members within an ecological network play a crucial role in facilitating indirect interactions (Cirtwill et al., 2018; Martín González et al., 2010). Furthermore, bilateral symmetry (Ornai & Keasar, 2020) and floral tube size (Ornai & Keasar, 2020; Watts et al., 2016) tend to attract more specialized pollinators, providing more efficient access to floral resources. However, despite recent advances in understanding how floral traits structure communities and influence species roles (Kantsa et al., 2017; Kemp et al., 2019), the relative contribution of visual floral signaling to pollinators and its role in shaping mutualistic networks remain poorly understood.

Floral color stands out as one of the most evolutionarily labile characteristics, due to natural selection mediated by pollinators (Muchhala et al., 2014). The arrangement of colors is already known as a structuring trait in some plant communities, presenting as either overdispersed (Muchhala et al., 2014) or clustered, and varying across landscapes (Kemp et al., 2019) or along altitudinal gradients (Bergamo et al., 2018). Moreover, these colors can act as filters in community composition and influence pollinator visitation rates (Dyer, Boyd-Gerny, et al., 2016; Jager et al., 2022). Among pollinating insects, bees are the most studied regarding the visual perception of flowers (Avarguès-Weber et al., 2012; Avarguès & Martin, 2014; Giurfa & Lehrer, 2001; Mota et al., 2013). In visual signaling in bee-pollinated systems, chromatic cues are recognized as the primary characteristics for flower detection (Bukovac et al., 2017; Dyer, Streinzer, et al., 2016) and discrimination (Dyer & Spaethe, 2008; Spaethe et al., 2014). That said, despite being one of the main characteristics modulating plant-pollinator interactions, the functional role of floral color in defining the ecological roles of species and the structure of mutualistic networks remains poorly understood (Shrestha et al., 2019). Exploring this relationship may reveal flower choice criteria in this context, information that is crucial to understanding the dynamics of plant-pollinator interactions.

Species of the Malpighiaceae family exhibit a wide range of colors in communities and have been described as a “small world”, especially due to the conservatism of floral characters (Bezerra et al., 2009). These Malpighiaceae species are intrinsically linked and form specialized interactions with oil-collecting bees of the tribe Centridini, in which floral conservatism allows interactions of these bees with both basal and derived groups of Malpighiaceae (Anderson, 1979; Buchmann, 1987; Carneiro & Machado, 2023; Mello et al., 2012; Possobom & Machado, 2017; Vogel, 1990). However, although bees are specialized in collecting floral oil, Malpighiaceae flowers also attract different bee species, with some of them focusing on specific flowers (Bezerra, 2008), which suggests that even with the floral conservatism of Malpighiaceae, there are other floral characteristics that determine bee attraction and that remain unexplored.

Given this context, we investigated how floral color properties explain the structure and role of species within plant-pollinator interaction networks, using Malpighiaceae species communities as a model. We aimed to address the following questions: 1) How do floral color and species phenology influence the structure of interactions within the Malpighiaceae mutualistic network? We used phenology to control the effect of this variable and understand more precisely the contribution of floral color to community structuring; and 2) How are floral color properties related to the functional roles of species within the Malpighiaceae mutualistic network? We hypothesize that: 1) Species with greater similarity in floral color and phenology will exhibit greater similarity in interactions with pollinators. Thus, the structure of the plant-pollinator interaction matrix will be partially explained by the matrix of color distances between species and the phenological matrix of the community. Furthermore, we hypothesize that 2) Floral color properties will be related to the roles that species occupy within the Malpighiaceae mutualistic network. Specifically, we presume that the greater the color hue, spectral purity, and contrast with the background, the stronger the species' strength, specialization, resource use, and centrality.

Methods

Study Areas

We collected data in two distinct areas to characterize mutualistic networks of Malpighiaceae. The first area was the Catimbau National Park (PARNA Catimbau; 8º32'14" to 8º35'12" S and 37º14'42" to 37º15'02" W), located in Northeastern Brazil. This semi-arid region lies at the transition zone between the "agreste" and "sertão" of the state of Pernambuco, with vegetation consisting of a mosaic of Caatinga, Cerrado, and rupestrian fields (Rodal et al., 1998). The park covers 62,000 hectares of dry Caatinga forest (Vieira et al., 2022), harboring both xeric and non-xeric species (Rito et al., 2017).

The second network comprised species in seven areas from the Serra do Cipó National Park and its buffer zone, "Morro da Pedreira", located in the central-eastern region of Minas Gerais, in the southern end of the Serra do Espinhaço (PARNA Serra do Cipó; 19º12'-19º35'S, 43º30'-43º60'W), Minas Gerais, Southeastern Brazil. The park's dominant vegetation includes Cerrado and native forests up to 900 m altitude, transitioning to rocky outcrops and rupestrian grasslands in sandy and stony soils, with predominant rupestrian fields above 1100m (Silveira et al., 2019).

Data Collection

Mutualistic Networks of Malpighiaceae

To characterize the mutualistic network of Malpighiaceae in the PARNA Catimbau, we used data published by Bezerra et al. (2009) on this community, which includes nine species of the family. Bezerra et al. (2009) recorded the interaction during three distinct periods, totaling 29 months: from January to December 2003, from January to December 2005, and from

August to December 2006. Focal observations were made on 138 individuals in natural clumps during the peak of the flowering period, with the number of visits by bees to the flowers recorded over four consecutive days, from 5 am to 5 pm, totaling 1392 hours of focal observations.

Similarly, to characterize the second mutualistic network of Malpighiaceae in Serra do Cipó, we collected data on interactions of eight species. We sampled interactions from early October to mid-December 2012 during the peak of the flowering season through focal observations. In each area, ten 1 m² plots were sampled along a 200 m curved transect. We observed pollinators on flowering plants for 15 minutes over six days, from 9 am to 2 pm, with 252 hours per area per individual.

Reflectance Curves, Color Hexagon, and Color Properties

To characterize the reflectance curves of the 17 Malpighiaceae species, i.e., of the species from both areas, and determine their loci in the bee vision model, we measured the spectral reflectance of three flowers from 10 individuals (30 flowers per species) in PARNA Catimbau, and two flowers from five individuals (10 flowers per species) in Serra do Cipó. The measurements were taken in situ using a spectrophotometer (JAZEL200, Ocean Optics, USA), with standard light and the green leaves of the plants as background (D65, Wyszecki & Stiles, 1982). The probe was positioned at a 45° angle in the center of a petal, excluding the standard petal, to capture corolla reflectance (Chittka & Kevan, 2005). The equipment was calibrated with an integration time of 30, boxcar of 5, and averaging of 30. We used a white matte diffuse reflectance standard as the white reference and removed the probe to measure the black reference. All measurements were taken at the center of the petal.

We then generated the reflectance curves and bee color vision models using the pavo package in R (Maia *et al.* 2019). We calculated the mean color loci of each Malpighiaceae species in the color hexagon, considering the spectral sensitivity functions of bee photoreceptors (Chittka, 1992; Chittka & Kevan, 2005), which are phylogenetically conserved in Hymenoptera (Briscoe & Chittka, 2001; Peitsch et al., 1992).

Next, we extracted the color properties of each species from the bee color vision model. We selected characteristics described in the literature as relevant for bee visual perception: hue (Raine & Chittka, 2007; Van Der Kooi et al., 2019), spectral purity (Koethe et al., 2018; Rohde et al., 2013), and chromatic contrast with the background (Bukovac et al., 2017; Dyer, Streinzer, et al., 2016). Hue was obtained through the h.theta metric from the pavo package, which generally corresponds to what humans classify as "color" (Wyszecki & Stiles, 2000). Spectral purity was calculated based on the X and Y coordinates of the vision model using the pdist package in R (Wong, 2013). This property is derived from the perceptual distance between the measured locus and the background, divided by the distance to the locus' maximum purity point (Lunau et al., 1996). Lastly, we obtained chromatic contrast with the background using the r.vec metric from the pavo package, which represents the Euclidean distance between the color locus and the center of the hexagon (Chittka, 1992).

After obtaining the color properties of each species, we tested whether they were correlated. We identified a strong positive correlation between spectral purity and chromatic contrast with the background ($t = 317.35$, $df = 1$, $p = 0.002$). This pattern has been observed previously (van der Kooi and Spaethe 2022), suggesting that these properties are not completely independent and contain redundant information. Given this, we chose to follow subsequent analyses with spectral purity, due to its fundamental role in flower perception by bees, especially in color detection and discrimination (Lunau et al., 1996; Lunau & Dyer, 2024; Rohde et al., 2013).

Interaction Matrices, Color Distances, and Phenology

To investigate how floral color and phenology influence pollinator interactions in the mutualistic network of Malpighiaceae, we constructed three matrices for each variable, both for the PARNA Catimbau and the Serra do Cipó networks. Color matrices were based on Euclidean distances between species in the color hexagon, calculated using the coldist function from the pavo package (Maia et al., 2019). Phenological matrices recorded the presence or absence of flowers for each species in each month, with data obtained from the phenology of the PARNA Catimbau (Bezerra, 2008). For the Serra do Cipó community, data were extracted from exsiccata deposited in herbaria, recording the presence or absence of flowers monthly in the flowering periods from 2010 to 2013. Lastly, interaction matrices comprised Malpighiaceae species and their respective bee pollinator species, with the number of interactions represented in the cells.

Species' Roles in the Interaction Network

Among the available metrics at the species level, we selected four widely used in the literature for their empirical relevance in understanding the structure of plant-pollinator networks. The first metric was (a) species strength (species.strength), which refers to the sum of dependencies between plants and animals, measured by the number of interactions per species, in which the higher the value, the more generalized a plant species is (Bascompte et al., 2006; Jordano et al., 2003; Watts et al., 2016). The second metric was (b) Blüthgen's d' index (d'), which measures the specialization of interactions by estimating their complementarity. This index indicates the extent to which the specialization of a focal species differs from the expectations of a null model, in which interactions occur proportionally to the availability of partners (Blüthgen et al., 2006). The third metric was (c) resource range, which measures the breadth or variation of resources (or partners) of a species within the network, corresponding to the number of links established between a species and the immediately lower trophic level (Novotny et al., 2002). Finally, the fourth metric was (d) weighted closeness centrality, which measures the proximity of nodes within the network, taking into account the weight of interactions, in which low values indicate more specialized species. In contrast, high values reflect a more central position in the network (Opsahl et al., 2010). Therefore, these metrics were chosen for their relevance in understanding the role played by species in mutualistic interaction networks.

Data analyses

Floral Color and Phenology of Species X Structure of Interactions

To examine the relationship among the color matrices, phenology, and interactions based on each plant species within each mutualistic network of Malpighiaceae, we initially transformed each matrix into a dissimilarity matrix. For the interaction and color distance matrices, we employed the dist function with the Euclidean distance method suitable for continuous data. For the phenological matrix, we utilized the vegdist function from the vegan package (Oksanen et al., 2013) with the Jaccard method, which is appropriate for binary data. Subsequently, we applied Multiple Regression on Distance Matrices (MRM), constructing two models using the *MRM* function from the ecodist package (Goslee & Urban, 2020), one for each network. The color and phenological distance matrices were used as predictor variables, while the interaction matrix served as the response variable. The significance of the relationships was assessed through 999 random permutations, generating a null distribution for obtaining adjusted p-values.

To visualize the similarity among species within each network, we conducted Principal Coordinates Analysis (PCoA) based on the interaction distance matrix. PCoA was performed using the cmdscale function, and the principal axes were utilized to represent the variation in species interactions. Graphical visualizations were generated using the ggplot2 package (Wickham & Chang, 2016).

Color Properties X Network Metrics

To investigate the correlation between color properties and the four species-level metrics describing the role of plant species within the mutualistic network, we fitted four generalized linear mixed models (GLMM) using the glmmTMB package in R (Brooks et al., 2023). As predictor variables, we included the interaction between hue and spectral purity. The response variables used were: (a) species strength, (b) d' index, (c) resource range index, and (d) weighted closeness centrality. The mutualistic networks (PARNA Catimbau and PARNA Serra do Cipó) were included as a random factor, and we employed a Gaussian distribution across all models, given that the response variable data were continuous. Additionally, we calculated the marginal and conditional R^2 for each model using the performance package (Ludecke, 2020) to evaluate the proportion of variance explained by the fixed effects and the combination of fixed and random effects, respectively.

We assessed the model adequacy by checking the dispersion of residuals using the *simulationResiduals* function from the DHARMa package (Hartig & Lohse, 2022) and tested for overdispersion using the *testDispersion* function. For graphical visualization, we utilized the ggplot2 package (Wickham & Chang, 2016). Furthermore, we used the effects package (Fox & Al., 2016) to visualize the effect of the interaction between the predictor variables. All analyses were conducted in R version 4.3.1 (R Development Core Team, 2023).

Results

Mutualistic networks

The mutualistic network of Malpighiaceae at PARNA Catimbau comprised nine plant species and 13 bee species, totaling 21.764 interactions during the observations (Figure 1). At PARNA Serra do Cipó, we observed 106 interactions in the eight species of Malpighiaceae and 38 floral oil-collecting bees (Figure 2).

Color Hexagon

The color hexagon of Hymenoptera showed that plant species from the two mutualistic networks of Malpighiaceae predominantly stimulated the green photoreceptor of bees. Species from PARNA Catimbau occupied three different color sections: *Banisteriopsis muricata*, *Banisteriopsis stellaris*, and *Byrsonima gardneriana* are in the cyan section; *Carolus chasei*, *Diplopterys pubipetala*, *Heteropterys eglandulosa*, *Stigmaphyllon auriculatum*, and *Stigmaphyllon paralias* are in the green section, while only *Janusia. anisandra* occupies the UV green section. Conversely, the species from PARNA Serra do Cipó exhibited a similar pattern, with most species occupying the green section of the hexagon, except for *Byrsonima sp2*, which occupied the UV green section, and *Byrsonima vacciniifolia*, which occupied the cyan section (Figure 3).

Floral Color and Species Phenology X Structure of Interactions

In the mutualistic network of Malpighiaceae in Catimbau National Park, the constructed model indicates that 43.7% of the variation in species interactions can be explained by the color and phenology of the species. However, we observed a strong positive relationship only between color distances and the dissimilarity between interactions, with an estimated coefficient = 3.585 (p = 0.004). In contrast, phenology has no effect on the interactions (p = 0.342), indicating that phenology does not contribute significantly to the structure of plant-pollinator interactions in this network (Figure 4-a).

In contrast, in the Malpighiaceae mutualistic network in Serra do Cipó National Park, floral color did not show a significant effect on the structure of interactions (coef = 25.220, p = 0.239), nor did species phenology (coef = 0.849, p = 0.935).

Color Properties X Network Metrics

Species with higher hue exhibited an increase in interaction strength (β = 0.304, z = 4.627, p < 0.001). Similarly, an increase in spectral purity was accompanied by a substantial growth in species strength (β = 137.56, z = 5.060, p < 0.001), suggesting that flowers with purer colors are associated with stronger interactions, i.e., with a greater number of interactions per species. However, the interaction between hue and purity resulted in a variation of 68.5% in species strength, indicating that as hue increases, the effect of purity on strength decreases, and vice-versa (β = -1.154, z = -4.998, p < 0.001). The constructed model explained 64.8% of the observed pattern (Figure 5-a).

When analyzing the d' index, neither hue (z = 0.654, p = 0.513) nor spectral purity (z = 0.464, p = 0.643) showed significant effects, indicating that these chromatic variables do not directly influence the specialization index (Figure 5-b).

In contrast to species strength, the resource range index exhibited an opposite behavior. As the color hue increases, the resource range index decreases (β = -0.008, z = -2.272,

$p < 0.05$). Likewise, an increase in spectral purity resulted in a decrease in resource range ($\beta = -5.075$, $z = -3.192$, $p < 0.001$). However, the interaction between color hue and spectral purity resulted in a 4.81% increase in resource range, indicating that as color properties vary jointly, resource utilization intensifies ($\beta = 0.0478$, $z = 3.544$, $p < 0.001$). This model explained 67.5% of the variation in this index (Figure 5-c).

Regarding the weighted centrality of species, as color hue increases, the centrality of species also increases ($\beta = 0.0028$, $z = 2.694$, $p < 0.001$). Furthermore, greater spectral purity also results in increased centrality ($\beta = 1.616$, $z = 3.718$, $p < 0.001$), indicating that flowers with purer colors occupy more central positions in the network. However, the interaction between color properties is significant ($\beta = -0.0129$, $z = -3.480$, $p < 0.001$), suggesting that the impact of hue on centrality decreases by 1.20% as purity increases. The model explained 63% of the variation in species centrality (Figure 5-d).

Discussion

Our findings highlight that corolla color properties are important floral traits that determine the roles of species within the mutualistic interaction network between Malpighiaceae and oil-collecting bees. In the Malpighiaceae community of PARNA Catimbau, we found that the greatest similarity in floral color explained about half of the organization of these interactions in the network, confirming our expectations. In contrast, in the PARNA Serra do Cipó network, neither color nor phenology explained the structure of the network, indicating that other factors influence these interactions. However, regarding the role of species in the network, we corroborate our hypotheses that the properties of the corolla color can largely explain them. Yellow flowers (high hue) with lower spectral purity and white flowers (low hue) with higher spectral purity were the most influential in the structure of the network, as they had greater interaction strength, indicated by the total number of interactions, and also a greater number of partners. Furthermore, predominantly yellow flowers with high spectral purity and greater contrast with the background interacted with a wider range of partners. These patterns reinforce the role of floral color in modulating the selectivity of oil-collecting bees and in structuring interactions in the network, in addition to showing that different combinations of strategies can be equally effective in maximizing the spectral response of bees, favoring attraction and interaction with pollinators.

The color of the corolla of Malpighiaceae flowers in the Catimbau National Park played an important role in structuring interactions, influencing the way species connect in the mutualistic network. Although color dissimilarity occurs due to processes such as ecological filtering or evolutionary divergence due to the reduced discrimination capacity of pollinators (de Jager et al., 2011), the similarity of floral colors can increase the efficiency of pollinator visits and reduce pollen limitation (Jones & Reithel, 2001; Moeller, 2004). Flowers with similar colors tend to attract a greater number of pollinators, reducing the cognitive cost associated with the search for floral rewards, facilitating the transport of pollen between species (Ghazoul, 2006). This phenomenon of similarity of floral colors between species acts as one of the main structuring factors of mutualistic networks, promoting more frequent interactions between plants with similar colors (de Jager et al., 2011; 2022), which can result in an increase in reproductive fitness by decreasing the probability of receiving heterospecific pollen and increasing the deposition of conspecific pollen (Ghazoul, 2006; Jager et al., 2022). Although Malpighiaceae species show clear differences in corolla color from the bee's perspective, similarity between species subgroups appears to be one of the main factors guiding the preferences of oil-collecting bees, resulting in a network structure driven by these visual patterns.

However, although the distance between species' floral color was not a determining factor in interactions in the plant-pollinator network at PARNA Serra do Cipó, spectral attributes such as purity, hue, and contrast with the background continued to be related to the role of species in the community. Floral color can influence other important ecological factors, such as floral specialization or sensory capabilities, contributing to shaping pollinator behavior (Trunschke et al., 2021; Van der Kooi et al., 2021). Variation in pollinator color perception can result in different visitation patterns and, consequently, alter the centrality and strength of species in interaction networks (Schiestl & Johnson, 2013). Thus, the relationship between spectral attributes and the roles of species in the network may be associated with other ecological processes, such as the sensory capacity of pollinators, which may explain the influence of variables such as spectral purity and contrast with the background, even when the similarity between floral colors is not the main factor determining interactions.

The strongest and most central species were those with the highest hue (yellow flowers) and lowest spectral purity or, conversely, those with the lowest hue (white flowers in the studied community) and highest spectral purity. In yellow flowers, such as the species *Carolus chasei*, *Janusia anisandra* and *Byrsonima vacciniifolia*, which have high hue, spectral purity and contrast with the background were lower in the strongest and most central species. In contrast, white flowers, which have low hue but higher intensities of spectral purity and contrast, also occupied this position in the network. It is known that pollinator preferences can be influenced by several factors that affect decision-making (Van Der Kooi et al., 2019). While bees exhibit an innate preference for short-wavelength colors (Dyer, Boyd, et al., 2016; Menzel, 1967), a common aspect among yellow flowers pollinated by bees is the presence of UV patterns, possibly an adaptation to the visual system of these pollinators, enabling an increase in visitation (Papiorek et al., 2016). Here, we observed small peaks of reflectance in the UV range in yellow flowers, suggesting that this characteristic can increase the attractiveness of these flowers even with low spectral purity, ensuring their relevance in the community. Furthermore, although bee-pollinated white flowers have lower chromatic detectability due to low spectral contrast, their high spectral purity may contribute to attractiveness, especially in combination with other signals such as UV patterns (Kevan et al., 1996). Since bees prefer flowers with high spectral purity (Lunau, 1990; Rohde et al., 2013), even without the color intensity of colorful flowers, the high spectral purity of white flowers still makes them attractive targets for bees. Therefore, different combinations between color properties can be equally successful in efficiently exploiting the spectral sensitivity of bees, even with contrasting chromatic characteristics.

Moreover, although bees show a preference for flowers with high spectral purity (Lunau, 1990; Rohde et al., 2013) and for high contrast with the background, as these color parameters are strongly correlated (Lunau & Dyer, 2024; van der Kooi et al., 2022), some species may invest more in abundant flower production and less in pigments. Species of the Malpighiaceae family are characterized by the abundant production of flowers in each flowering cycle (Bezerra, 2008; Buchmann, 1987; Vogel, 1974, 1990). While flower abundance alone may not be sufficient to predict pollinator visitation (Justino et al., 2012), it increases the likelihood of encounters between pollinators and flowers, contributing to the structuring of plant-pollinator networks (Vázquez et al., 2009; Vizentin-Bugoni et al., 2018). Therefore, although floral color is recognized as an essential attribute for the detection of flowers in the environment, the metrics that comprise this floral trait may not fully explain the perceptual capabilities of pollinators in natural environments (Lunau & Dyer 2024), and in this case, in the yellow flowers of this community.

In terms of species specialization, although we did not find a relationship between color properties and the d' index, we did observe a correlation with the range of resources used. Flowers with higher spectral purity and contrast tend to interact with more partners. This broader use of resources may be linked to better color detection by bees, as seen in flowers with purer corollas (Lunau, 1990; Rohde et al., 2013). Pollinators more easily recognize flowers with higher spectral purity due to their increased visibility (Chittka & Raine, 2006). Additionally, pure colors play a critical role in flowers that compete for visually oriented pollinators, such as bees (Schaefer & Ruxton, 2011), and thus may be more generalist in their partner interactions within the network. Therefore, flowers with greater spectral purity may be more generalist within communities, as their high detectability by bees facilitates interactions with a wider range of partners.

In summary, our findings show that, although floral color explained the structure of the mutualistic network only in the Malpighiaceae community of PARNA Catimbau, its role in defining the role of species within the network is evident. Our results indicate that different mutations in flower color properties influence the choice of oil-collecting bees, directing pollinator selectivity, and can be equally efficient. Furthermore, chromatic characteristics also explain the generalist behavior of some species, reinforcing the functional importance of this floral attribute in community organization. The similarity of nuclei between species may represent an advantageous strategy to improve pollinator attraction and reduce competition for resources. Thus, we show that, although floral color structured the mutualistic network in one of the scientific communities, its role goes beyond network organization, as it also modulates the behavior of oil-collecting bees and influences the role of species, being a relevant floral trait in the dynamics of these interactions.

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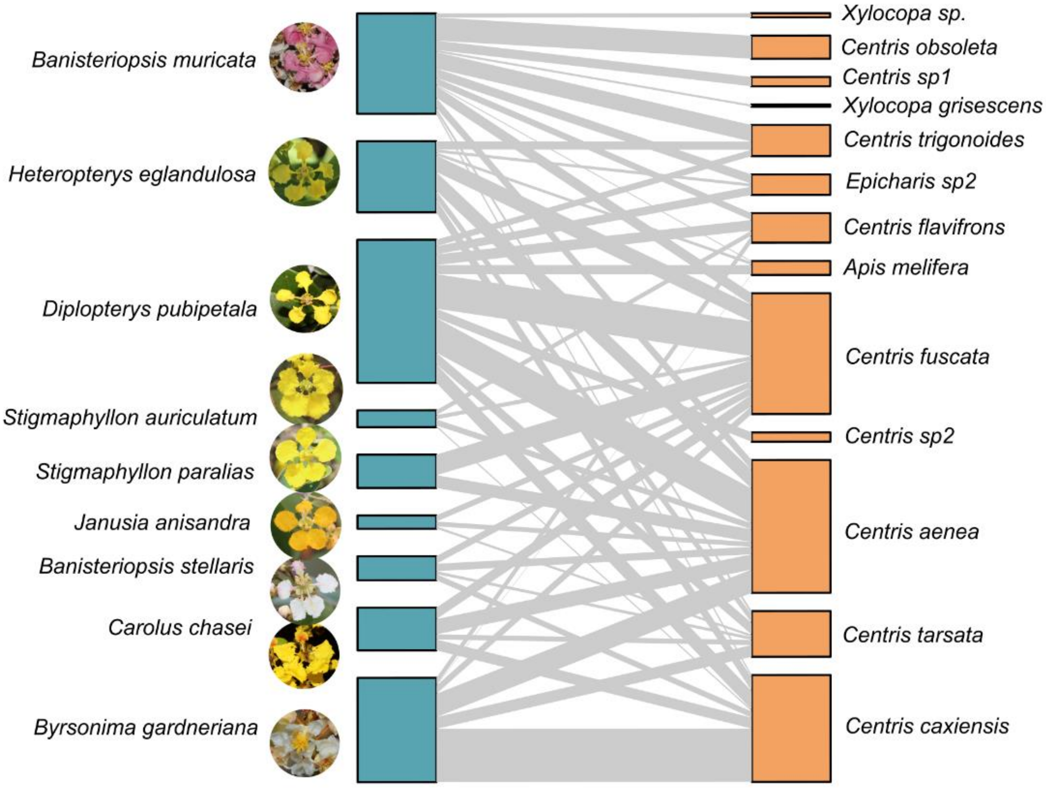

Figure 1: Mutualistic network of Malpighiaceae species and bees in Catimbau National Park-Pernambuco- Brazil. Data from Bezerra et al. (2009).

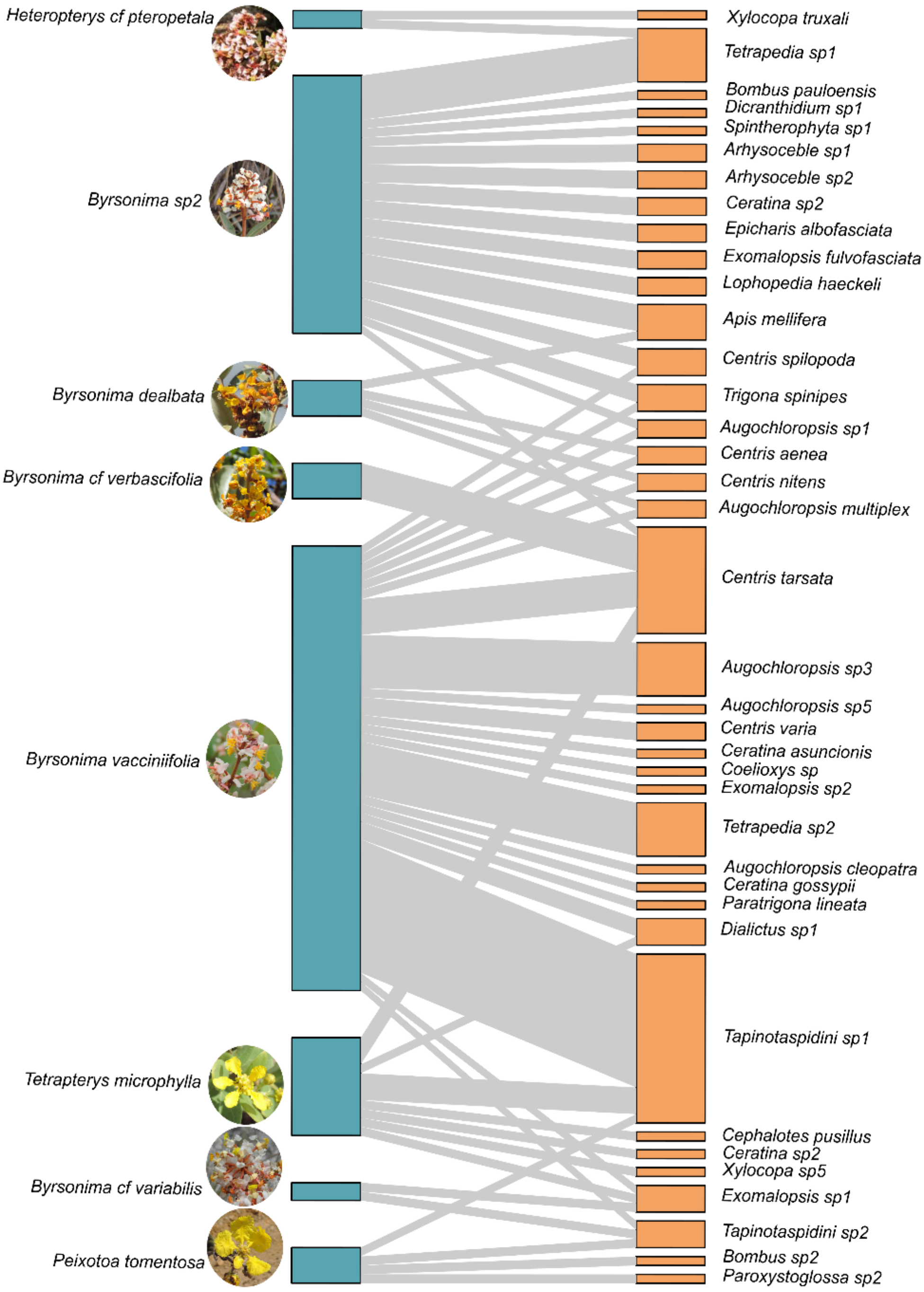

Figure 2: Mutualistic network of Malpighiaceae species and bees in Serra do Cipó National Park- Minas Gerais- Brazil.

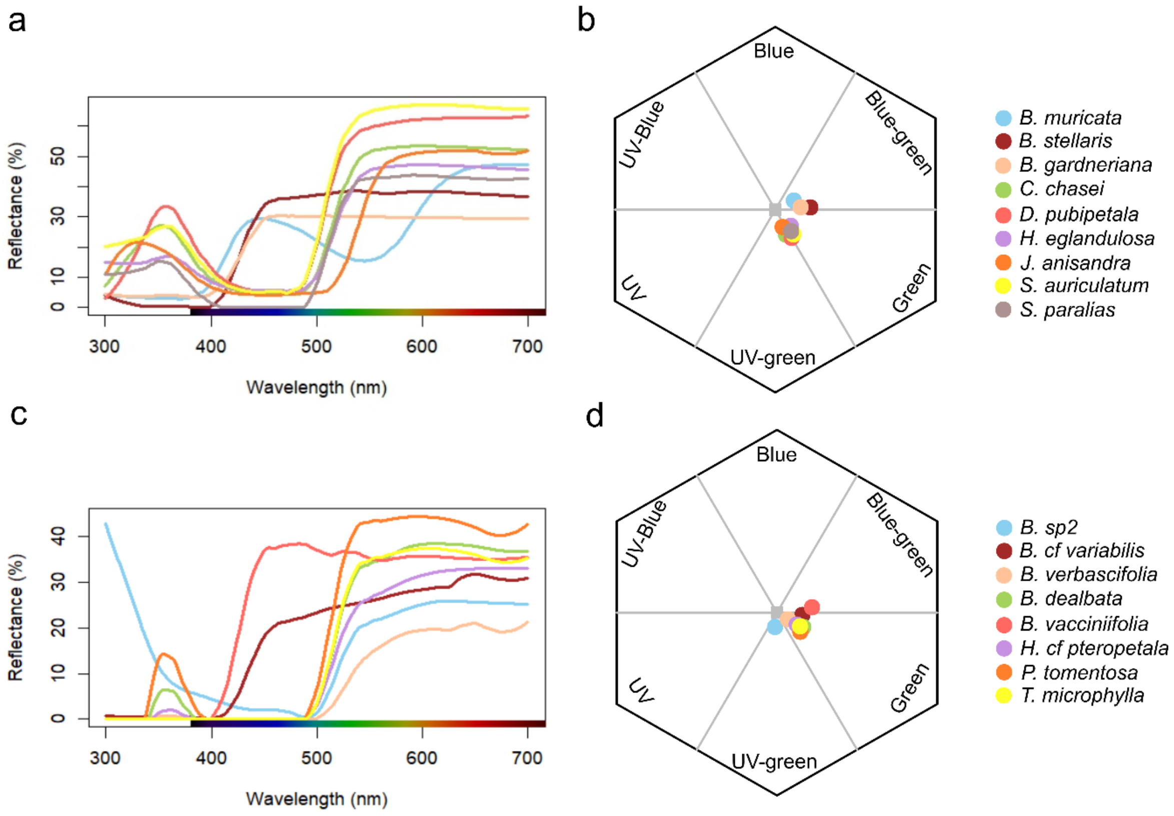

Figure 3: Reflectance curves and vision model of bees. a) Corolla curves and b) Color hexagon in the vision model of species from Catimbau National Park (Pernambuco- Brazil); c) Corolla curves and d) Color hexagon in the vision model of species from Serra do Cipó National Park (Minas Gerais- Brazil).

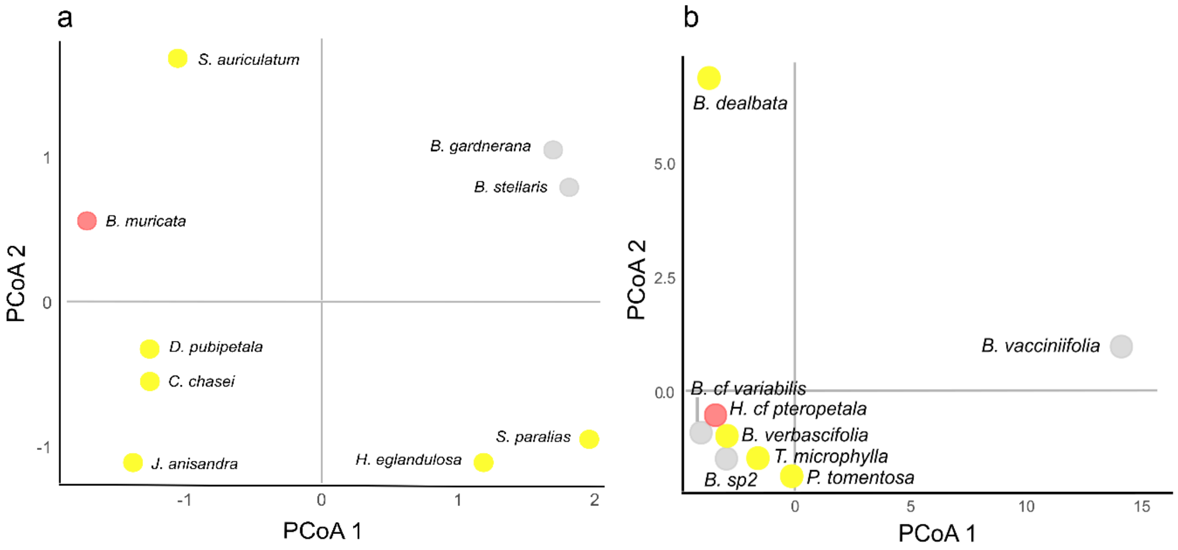

Figure 4: PCoA result for the relationship between the interactions of Malpighiaceae species and their floral color. a) Species from Catimbau National Park (Pernambuco- Brazil), and b) Species from Serra do Cipó National Park (Minas Gerais- Brazil).

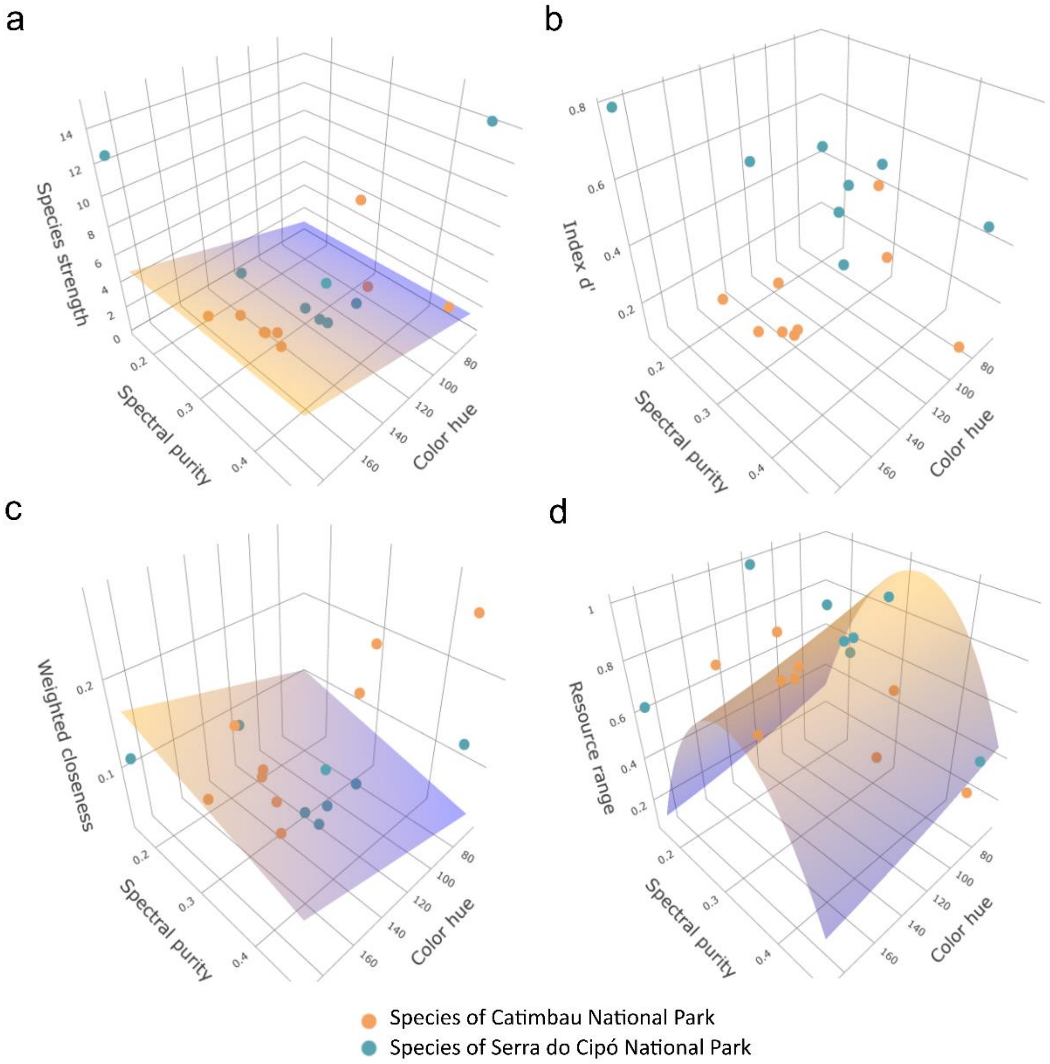

Figure 5: Relationships between color metrics of hue and spectral purity and the role of species in the Malpighiaceae mutualistic networks. a) Species strength; b) d' index; c) Weighted centrality; and d) Resource range index.

Capítulo 2

Flower age increases male but not female performance through resource consumption in a floral oil-producing species

Manuscrito submetido ao periódico: Plant Biology

Fator de impacto: 3.9

Flower age increases male but not female performance through resource consumption in a floral oil-producing species

Sabino-Oliveira, A. C.[1*]; Brito, V. L. G.[2] & Machado, I. C.[1*]

1: Biosciences Center, Department of Botany, Postgraduate Program in Plant Biology, Federal University of Pernambuco, Recife, Pernambuco 50670-420, Brazil

2: Institute of Biology, Federal University of Uberlândia, Uberlândia, Minas Gerais 38400-902, Brazil

Running title: Floral color longevity increases male performance

For correspondence: carolsabino.bio@gmail.com, icsmachado@yahoo.com

Keywords: floral longevity; flower age; bee vision; floral oil consumption; male performance; non-changing floral color; reproductive performance

key message of the paper: The flower age of *Stigmaphyllon paralias* directly impacts male performance through oil consumption and increased pollen release, which may be associated with the maintenance of floral color in the eyes of bees throughout the floral lifespan.

Abstract:

- The high costs of floral maintenance modulate floral longevity. In some species with massive flowering and long floral lifespans, floral longevity increases overall display by changing petal color which ultimately outbalances the costs of floral maintenance. However, the color of petals remains unchanged throughout the floral lifespan in some species with long floral longevity, and the reproductive consequences of such combined traits remain elusive. In this study, we investigated how the flower age affects resource availability and reproduction in the species *Stigmaphyllon paralias* A.Juss. (Malpighiaceae), a floral oil-producing species in which petal color does not change throughout its 2-day longevity.
- We followed the petal color properties (spectral purity, brightness, chromatic contrast with the background and saturation), resource availability to pollinators, and female and male reproductive performance along the lifespan of 180 flowers exposed or isolated from pollinators.
- Despite petal saturation decreasing throughout the floral lifespan, the color hexagon model predicts no perceptual color change in flowers isolated or exposed to bee pollinators. However, pollen release and oil consumption increase along the floral lifespan, while the amount of pollen on stigmas does not change.
- These results suggest that the maintenance of floral color in the vision of bees may be related to increased male performance.

Keywords: floral longevity; flower age; bee vision; floral oil consumption; male performance; non-changing floral color; reproductive performance.

Introduction

Producing and maintaining flowers for pollinators require significant energy investment (Primack, 1985; Ashman and Schoen, 1994; Song *et al.*, 2022). Despite being costly, the entire period in which a flower remains open and functionally attractive to pollinators (i.e. floral longevity) can be crucial for pollination success (Primack, 1985; Ashman and Schoen, 1996; Blair and Wolfe, 2007). Several studies have shown that floral longevity can range from a few hours to several months across angiosperms (Primack, 1985; Ashman and Morgan, 2004; Song *et al.*, 2022). Floral longevity is a dynamical event in which advertisement traits, floral arrangement, and resource availability vary, regulating pollinator visitation as long as flowers age (Ashman and Schoen, 1996; Spigler, 2017) and potentially affecting reproductive success. For example, floral color can change with flower age in some species in two ways: as flowers wither or as parts of the flower change color prior to senescence (Weiss 1995; Van Doorn 1997; Weiss & Lamont 1997; Marshall *et al.* 2010). While floral color change occurs in about 20% of angiosperms (Weiss 1995), most species do not undergo such changes, which may be an alternative strategy to maintain pollinator attraction and maximize abundance, although this involves costs and uncertainties, such as resource allocation to keep flowers viable for longer (Ashman & Schoen 1994). However, the functional role of floral longevity remains largely unexplored.

It is expected that the longer the flower remains attractive to pollinators, the greater the chance of exporting and receiving more pollen. However, the relationship between flower longevity and female and male performance depends on the magnitude of pollen–ovule ratio and flower visit quality (i.e., pollinator efficiency and behavior) (Castro *et al.* 2008). For example, plants with a high pollen–ovule ratio require more pollinator visits to achieve satisfactory levels of pollen release compared to ovule fertilization (Cruden 1977). This is because just a few early visits during a flower's lifespan would be sufficient to saturate the female function. However, the differential outcomes in male and female performance during flowers' lifespan likely depends on the quality of flower visits, which is influenced by the efficiency of pollinators in exporting and depositing high-quality pollen, as is often achieved by specialized pollinators. Flower viability time may be an important factor for plant reproductive success and influenced by the balance between investment in reproduction (pollen and ovule production) and pollination quality. Thus, floral longevity may affect male function, while the impact on female function is expected to be minimal or neutral. Male fitness has received little attention in pollination biology studies (Pearson *et al.*, 2023), but the impact of floral longevity on this reproductive component may be significant, especially if flowers continuously produce floral resources over their lifespan. Therefore, it is important to consider the success of reproductive functions separately, since recent studies have shown that the male and female components can be affected differently depending on the traits related to pollination success.

Pollination success at the flower level is influenced not only by the duration flowers are available to pollinators but also by their traits and resource availability, which both keep them attractive. For example, the maintenance of floral color properties and continuous resource production are key to optimizing flower viability over time. Although some plants alter floral color properties and 'manipulate' pollinator behavior by changing resource availability (e.g., variations in nectar volume) to maximize pollination success (Weiss 1995; Brito *et al.* 2015; de Melo *et al.* 2018; Santana *et al.* 2022), most species may invest in maintaining a fresh appearance of flowers. Additionally, resource availability plays a crucial role; in some environments, floral longevity and resource levels fluctuate throughout the lifespan, impacting pollinator visitation patterns (Pyke 2016; Endres *et al.* 2021).

Such changes can also be shaped by interactions with pollinators: some flowers cease their longevity once their reproductive role is fulfilled, allowing for resource conservation

(Ishii & Sakai 2000; Stpiczyńska 2003; Abdala-Roberts *et al.* 2007; Castro *et al.* 2008). This dynamic adjustment reflects an adaptive strategy that balances floral maintenance costs with the fertilization opportunities offered by the environment (Ashman & Schoen 1994). When pollinators are scarce, flowers may prolong their lifespan with low maintenance costs (Ashman & Schoen 1994). Therefore, the effects of floral longevity on attraction traits, floral resource variation, and reproductive performance need to be assessed not only as intrinsic characteristics of floral biology but also as a result of interactions with pollinators. Considering that, it is of great value to understand the functional role of flower age and the associated variation in floral traits to ensure plant reproductive success.

Here, we investigated how the flower age alters resource availability and reproductive performance in the floral oil-producing *Stigmaphyllon paralias* A.Juss. (Malpighiaceae), which also does not present a noticeable color change in human vision throughout its lifespan. Initially, we tested whether floral color maintenance also occurred in properties based on the bee vision model. Subsequently, to see the effect of the flower age on reproductive attributes, we tested the following hypotheses: 1) Oil consumption, pollen receipt, and release increase throughout the floral lifespan; and 2) The longer the flower maintains oil consumption, only the male performance of the flower will be greater.

Methods

Study area and studied species

We carried out the data collection in the Catimbau National Park (PARNA Catimbau), Pernambuco, Northeast Brazil (8º32’14’’ to 8º35’12’’ S and 37º14’ 42’’ to 37º15’02’’ W), during December 2022 (Appendix 1- Supplementary material). This conservation unit comprises 62,000 ha of dry Caatinga forest (Vieira *et al.*, 2022), where the annual temperature averages 25 °C, and average annual precipitation ranges from 240 to 1500 mm (Sampaio 1995)Plant communities are diverse in the study site, including species native to different biomes (Caatinga, Cerrado, and Campos rupestres) (Rodal *et al.*, 1998).

Stigmaphyllon paralias A. Juss. (Malpighiaceae) is a subshrub species with annual flowering that lasts from one to five months (Bezerra 2008). Flowers are yellow, slightly zygomorphic and last 2-3 days available to pollinators (Costa *et al.* 2006). The heteromorphic androecium is composed by three sterile staminodes and seven fertile stamens with well-developed connectives and anthers of different sizes, with longitudinal dehiscence (Bezerra, 2004). The gynoecium has three green styles, two lower lateral and one central, with modified leaf-shaped apices, where the stigmas are found (Anderson 1997; Carvalho *et al.* 2005). The species has only three ovules per flower (Bezerra 2004)(Figure 1, A-B). This floral oil-producing species is self-incompatible and thus pollination by oil-collecting bees (Centridini, Apidae) is required as for most neotropical representatives of Malpighiaceae (Figure 1, C) (Vogel, 1990; Bezerra *et al.*, 2009; Albuquerque *et al.,* 2023; Carneiro & Machado, 2023).

Data collection

To evaluate the maintenance of floral color in the bee color vision, resource availability, and female and male performance over flower longevity, we quantified color properties, measured oil volume, and estimated reproductive success at different times. Sampling began immediately after flower opening to capture any changes in color and reproductive properties. Sampling times were separated by five hours on the first day (1, 6, and 11 h after the beginning of anthesis) and on the second day (24, 29, and 34 h after the beginning of anthesis). A total of 180 flowers were sampled from approximately 60 individuals of *S.*

paralias within the same population. Half of these flowers (n = 90) were isolated with mesh bags before opening (pre-anthesis bud stage) to control for any changes in color and reproductive properties due to pollination. We sampled 30 flowers at six different time points over their lifespan (i.e., 15 bagged and 15 non-bagged flowers per time interval).

Floral reflectance and color properties

To test wether *S. paralias* flowers remain similar in the vision of bees over their lifespan, we measured (*in situ*) the spectral reflectance in all 180 flowers (open and bagged) using a spectrophotometer (JAZEL200, Ocean Optics, USA). We used a diffuse reflectance white matte surface available with the spectrophotometer as a white pattern, and light absence as a black pattern. We adjusted the parameters during calibration by setting values of 30 for integration times, 5 for boxcar and 30 scans for averages. We performed measurements in the center of a petal, at an angle of 45º to the surface of the petal (Chittka and Kevan, 2005).

We obtained the reflectance curves and the bee color vision models using the *pavo* R package (Maia *et al.*, 2019). We calculated the mean of the color loci in the color hexagon for each group of flowers collected at different times within floral longevity, considering the spectral sensitivity functions of honeybee photoreceptors (Chittka ,1992; Chittka and Kevan, 2005), which are phylogenetically conserved in Hymenoptera (Peitsch *et al.*, 1992; Briscoe and Chittka, 2001). We used the default green from the *pavo* R package as the green leaves background.

We then collected the saturation from the reflectance, and the color properties from the bee vision model: spectral purity, brightness, and contrast with the background. Details of how each color property is calculated are provided in the supplementary material (Appendix 2).

Among the variables collected from the vision and bee model, we obtained a high correlation between brightness and contrast with the background (Pearson correlation coefficient: $r = 0.46$; $df = 170$; $p < 0.001$), as well as between contrast and spectral purity ($r = 0.76$; $df = 170$; $p < 0.001$) (Appendix 5 - Supplementary material). Therefore, we chose to consider only contrast, since this variable plays an essential role in flower detection by bees (Bukovac *et al.* 2017; van Der Kooi *et al.*, 2019).

Floral oil

To evaluate the relationship between different flower ages and variation in oil volume, and female and male performance in *S. paralias*, we quantified floral oil availability using a single elaiophore per flower ($n = 180$). We gently pressed a cigarette rolling paper onto the oil-secreting surface of one elaiophore per flower to absorb all the available oil. After drying for 24 h, we took scaled photographs of all paper samples and measured the area of oil spots using the Image J software to the nearest 1 mm^2. We estimated the oil volume (µL) through a calibration curve, obtained from *Ricinus communis* L. oil, which shares similarities in chemical and physical properties with floral oils (Vogel 1974, Ferreiro *et al.*, 2015, 2017). The following formula was used to estimate oil volume: $\text{Volume} = (10^{\wedge -0.37})*(10^{\wedge 1.39})^{\wedge \text{area}}$ (Ferreiro *et al.*, 2015, 2017).

Female and male performance

We used each flower's central stigma and quantified the number of pollen grains deposited to estimate female success. To measure male failure, we counted the remaining pollen grains present in two anthers (one large and other small) from each flower. We collected both anthers using tweezers and immersed them into a microcentrifuge tube containing 70% alcohol. The pollen was carefully removed from anthers and counted under the microscope. To estimate the total number of pollen grains remaining on the flower, we multiplied the number of pollen grains obtained from each anther size by the total number of anthers of each size.

Effect of oil consumption on reproductive performance

We obtained female and male performance by subtracting the reproductive successes and failures of each component (Opedal *et al.*, 2023). Regarding female performance, we considered success as the total number of pollen grains deposited on the stigma, while failure was the maximum number of grains deposited on the stigma of all flowers subtracted by the number of pollen grains deposited on the stigma. The resulting subtraction corresponds to the number of potential pollen grains that did not reach the stigma during the lifespan, and therefore can be considered failures. For the male component, success was the maximum number of pollen grains obtained from the anthers of a flower subtracted by the number of pollen grains in each sample, while failure was the number of pollen grains in each sample, which correspond to the grains that were not released during lifespan, and therefore, are considered failures. Finally, to obtain oil consumption through collection by bees, we used the maximum volume of oil produced by a gland subtracted by the volume observed in each gland sampled.

Data analysis

To initially assess whether flowers remain similar throughout floral lifespan in the bee vision, and subsequently the effect of the flower age on resource availability and reproductive variables, also considering pollination as a variable of interest, we constructed seven generalized linear models (GLM), one for each of the following response variables: (a) contrast, (b) saturation, (c) oil volume, (d) number of pollen grains on the stigma; (e) number of pollen grains in anthers. We performed transformation logarithmic scale (log) in the saturation, and square root (sqrt) for the oil volume, as well as excluding one sample from each variable that were outliers, in order to normalize the residuals of the models (Zuur *et al.*, 2010). For a, b, and c, we built linear models using the Gaussian family, since these response variables are continuous, and models reached good fit. As variables d and e are counting variables, we built linear models with the negative binomial family after bad model fit using Poisson distribution. To verify the existence of an effect of the predictor variables, which we considered the time period and the exposure to pollinators, we used likelihood-ratio tests with the *Anova* function, type III. Subsequently, we perform a *post-hoc* test, to verify the differences between time periods and between pollinator exposure treatments. In this sense, we chose the Tukey Test for comparisons of means.

To test the influence of floral oil consumption on the performance of female and male sexual functions, we constructed a generalized linear mixed model (GLMM), using the binomial family. For this, we used oil consumption as a predictor variable, the performance of each sexual function as a response variable, and the identity of the flower as a random variable. In this model, we use the *cbind* function to combine the reproductive performance successes and failures values of each sexual component. To verify the influence of oil consumption on female and male sexual performance, we used likelihood tests (likelihood-ratio test) with the Anova function.

All statistical analyses were performed in R version 4.3.1 (R Development Core Team 2023) using standard packages and the following: glmmTMB (Brooks *et al.* 2023), car (Fox 2019) and emmeans (Lenth & Al. 2022). In addition, we used DHARMa (Hartig and Lohse, 2022) to check the residual dispersion around the fitted models and ggplot2 (Wickham and Chang, 2016) for graphical constructions.

Results

Floral color properties

The flowers of *Stigmaphyllon paralias* showed lower reflectance percentage peaks in the ultraviolet range (300 to 400 nm) at the beginning of floral lifespan, with a variation between 15% and 25%, and higher peaks in the green range (500 to 600 nm), varying between 35% and 60% (Figure 1, D-E) (Appendix 3- Supplementary material). At the end of the lifespan of *S. paralias,* the characteristics of the reflectance curves were maintained as those of flower opening. In the hexagon color space, flowers occupied the UV green section throughout their entire lifespan (Figure 1, F-G) (Appendix 4- Supplementary material).

We found no relationship between color metrics considering bee perception and flower aging (Table 1). There is no evidence that the chromatic contrast with the background changed among sampling times and between exposed and isolated flowers (Table 1) (Appendix 6- Supplementary material). However, their saturation decreased significantly along floral lifespan. In general, flowers from the first sampling period showed higher saturation than flowers from subsequent sampling periods, with a 50.72% decrease in saturation over the entire lifespan. On the other hand, there is no evidence that floral saturation is different between pollinator exposure treatments (Table 1) (Figure 2, A).

Floral oil volume and reproductive performance over floral lifespan

Oil volume decreases more quickly in exposed flowers, with 93.3% collected by pollinators over floral lifespan, while the decrease was mild over time in bagged flowers, totalizing 21.1% (Table 1) (Figure 2, B). Differences in oil volume were found between flowers sampled at each floral age and the age sampled later, showing a gradual decrease over floral ages (Table 2 - Supplementary material).

The number of pollen grains on stigmas did not vary over time. However, flowers exposed to pollinators had 5% more pollen grains on the stigmas (Table 1) (Figure 2, C). The amount of pollen grains in anthers gradually decreased during the floral lifespan, but this decrease was dependent on the pollination treatment (Table 2- Supplementary material). In the exposed treatment, the decrease was more prominent (17,4%) than in the isolated treatment (9.9%) (Table 1) (Figure 3, D).

Relationship between oil consumption and female and male performance

Oil consumption by bees over floral lifespan in *S. paralias* flowers positively affected reproductive performance, but this effect is dependent on the sexual function. Oil consumption did not alter female performance. However, male performance increased as long as oil consumption become more intense (Table 2) (Figure 3).

Discussion

Flower age directly impacted male performance through oil consumption and increased pollen release in *Stigmaphyllon paralias*. This may be associated with the maintenance of floral color in the vision of bees throughout the floral lifespan. We partially corroborate the first hypothesis that resource consumption and pollen release increase as flower ages, whilst pollen receipt did not vary. In addition, we corroborate our second hypothesis, in which the greater the oil consumption, the greater male reproductive performance. Although flowers acquire an aged appearance due to decreased saturation, this change was unlikely to affect the perception of pollinators, which continued to visit them regardless of flower aging, as evidenced by the constant oil consumption. Over flower lifespan, the maintenance of floral color in the vision of bees occurred simultaneously with increased male reproductive performance. Therefore, our findings show that flower aging in *S. paralias* contributes to male reproductive success by prolonging pollen release potentially through efficient signaling to pollinators, maximizing the use of floral resources over time.

We did not observe changes in female reproductive performance in terms of magnitude of pollen receipt throughout the flower lifespan of *S. paralias*. From the beginning of floral life, both isolated and exposed flowers presented similar amounts of pollen grains on the stigmas, indicating that the obstruction may be caused by self-deposition of pollen, since isolated flowers, without visits from pollinators, also accumulate equivalent pollen. Stigma clogging may occur to limit access of subsequent pollen arrival (Ashman *et al.*, 1993; Minnaar *et al.*, 2019). The studied population of *S. paralias* is self-incompatible (Bezerra, 2004), and receiving self-pollen can reduce seed production due to stigma clogging, abortion of self-pollinated ovules and pollen waste (de Jong *et al.*, 1992; Charpentier, 2002). Therefore, interaction with pollinators in the exposed flowers increases the possibility of improving the efficiency of pollen deposition and modifying the proportion of self-incompatible and compatible pollen in the stigma and increase the chances of efficient fertilization (Rodet *et al.*, 1998). Here, we did not test the change in the proportion of pollen on the stigmas, but we showed that the amount of pollen is similar from the opening to the end of the floral lifespan, which shows that the female function is fulfilled regardless of the age of the flower.

Pollen removal and oil collection by bees occurred throughout the floral lifespan in flowers exposed to pollinators, evidenced by the decrease in pollen quantity on anthers and oil in flowers. Although floral longevity requires a high cost to maintain its functioning and attractiveness (Ashman and Schoen, 1997), long-lasting flowers can increase pollinator attraction by increasing total display (Harder and Johnson, 2005; Suzuki and Ohashi, 2014), elevating the chances of receiving pollinator visits. It has been reported that plants that change floral color, flower retention contributes mainly to male reproductive success, as it reduces the number of consecutive visits to the flowers (Ida and Kudo, 2003). However, our results suggest that color maintenance can also enhance male performance, hence increasing pollen release over time due to long attractiveness to pollinators.

Moreover, maintaining visually identical flowers but varying in oil content can contribute to increase outcrossing rates. Oil-producing plants require precise and efficient resource allocation due to the relatively high energetic investment required for oil production compared to nectar (Buchmann 1987). In nectar-producing species, the strategy of presenting flowers in the same individual with varying amounts of resource and exhibiting color maintenance, was seen as a stimulus for pollinators to leave the plant when finding flowers with less resource and then reducing geitonogamy (i.e. pollen transfer between flowers of the same individual) (Biernaskie *et al.*, 2002; Suzuki and Ohashi, 2014). Although not directly tested in this study, this strategy is likely to occur in *S. paralias* since oil volume was different between flowers within the same individual at different ages. Therefore, oil-collecting bees may also be discouraged from continuing to visit flowers of the same individual which may increase outcrossing rates.

Our results further suggest that pollinators can be less efficient in pollen export when compared to pollen receipt. Although this difference has been rarely tested in pollination ecology, less efficiency in pollen release should indeed impact plants positively. For example, pollen release in small amounts over time may benefit male reproductive success by reducing early depletion of pollen grains and increasing the rates of pollen export to conspecific stigmas (Lebuhn & Holsinger 1998). This is particularly interesting since no apparent morphological mechanism has been described to trigger gradual pollen release in *S. paralias* (Bezerra 2004), although its anthers are partially covered by the leaf-shaped stigmas during the entire flower lifespan. Whereas the importance of this morphology to male function is yet to be tested, gradual pollen export as long as flowers age may be an indirect strategy to maximize male success by ensuring greater fertilization coverage in recipient flowers (Ashman & Schoen 1994). Thus, floral longevity may function as an adaptive mechanism to extend the opportunity for pollen export and increase reproductive success.

The maintenance of floral color over time also seems to play an important role in the reproductive success of *S. paralias*. Although its floral color does not change, the notable decrease in saturation gives their flowers an aged appearance, which occurred in both exposed and isolated flowers, indicating that this process does not depend on pollination. In the absence of significant floral color changes, there is evidence that insects do not discriminate fresh and aged flowers (Suzuki and Ohashi, 2014), which may occur in the studied system given bees continuously forage throughout floral lifespan. Although it receives less attention, continuous variation in floral color caused by small differences in pigment concentrations that confer an aged appearance is more common than a shift to a distinct color hue (Tourjee *et al.*, 1993; Tastard *et al.*, 2008). This continuous variation of pigments can affect the color properties seen by bees (Papiorek *et al.*, 2013), as there is a necessary amount of pigment for the signal to be visible (van Der Kooi *et al.*, 2019). However, this phenomenon does not seem to happen in *S. paralias*, as the variation in pigment concentrations did not change the color in the vision of bees, and only changed the appearance of the flower throughout the floral lifespan.

In summary, we showed that *S. paralias* flower colors remain similar to bees' vision throughout floral lifespan and this floral color longevity may be related to increased male performance. The visible changes of floral aging do not reflect what is seen by pollinators, as was evidenced by the variation in corolla saturation between *S. paralias* flowers and, on the other hand, by the maintenance of floral color for bees throughout the floral lifespan color. Therefore, in this study we show that the cost of maintaining flowers with similar appearance for bees throughout the floral lifespan is balanced with the increase in the performance of the male function, contributing to the understanding of the reproductive strategies of plants to balance an effective attraction of pollinators and an increase in the possibilities of pollen dispersal.

Acknowledgments

We would like to thank the logistical support for data collection from the Long-Term Ecological Program (PELD/Catimbau, 403770/2012-2). We thank Dr. Pedro Bergamo and Dra. Maria Gabriela Camargo for review and improvement of previous versions of the manuscript. We also would like to thank the National Council for Scientific and Technological

Development - CNPq, for the PhD scholarship granted to Sabino-Oliveira AC and for a Research Grant to Machado IC (310508/2019-3).

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Table 1: Results of generalized linear models (GLM) that evaluated how floral lifespan and exposure to pollinators alter signaling variables and reproductive variables in *Stigmaphyllon paralias* (Malpighiaceae). Significant results between variables are in bold.

	Floral lifespan (FL)			***Exposure to pollinators (EP)***			***Interaction (FL + EP)***		
	χ^2	**df**	**p**	**χ^2**	**df**	**p**	**χ^2**	**df**	**p**
Contrast- background	11.71	5	0.0822	0.006	1	0.2164	7.28	5	0.1775
Corolla saturation	**43.28**	**5**	**< 0.001**	0.17	1	0.2894	7.79	5	0.1516
Oil volume	**69.45**	**5**	**< 0.001**	**25.06**	**5**	**< 0.001**	10.80	5	0.0553
Pollen grains- stigma	0.80	5	0.9766	**5.25**	**1**	**< 0.01**	8.91	5	0.1124
Pollen grains- anthers	**138.527**	**5**	**< 0.001**	**5.569**	**1**	**< 0.001**	**21.264**	**5**	**< 0.001**

Table 2: Results from generalized linear mixed models (GLMM) that evaluated how the performance of reproductive components varies according to oil consumption in *Stigmaphyllon paralias* (Malpighiaceae)

	Oil consumption (OC)			***Sexual function (SF)***			***Interaction (OC + SF)***		
	χ^2	**df**	**p**	**χ^2**	**df**	**p**	**χ^2**	**df**	**p**
Relative sexual performance	0.0698	1	0.7916	1217.2862	1	< 0.001	1627.1388	5	< 0.001

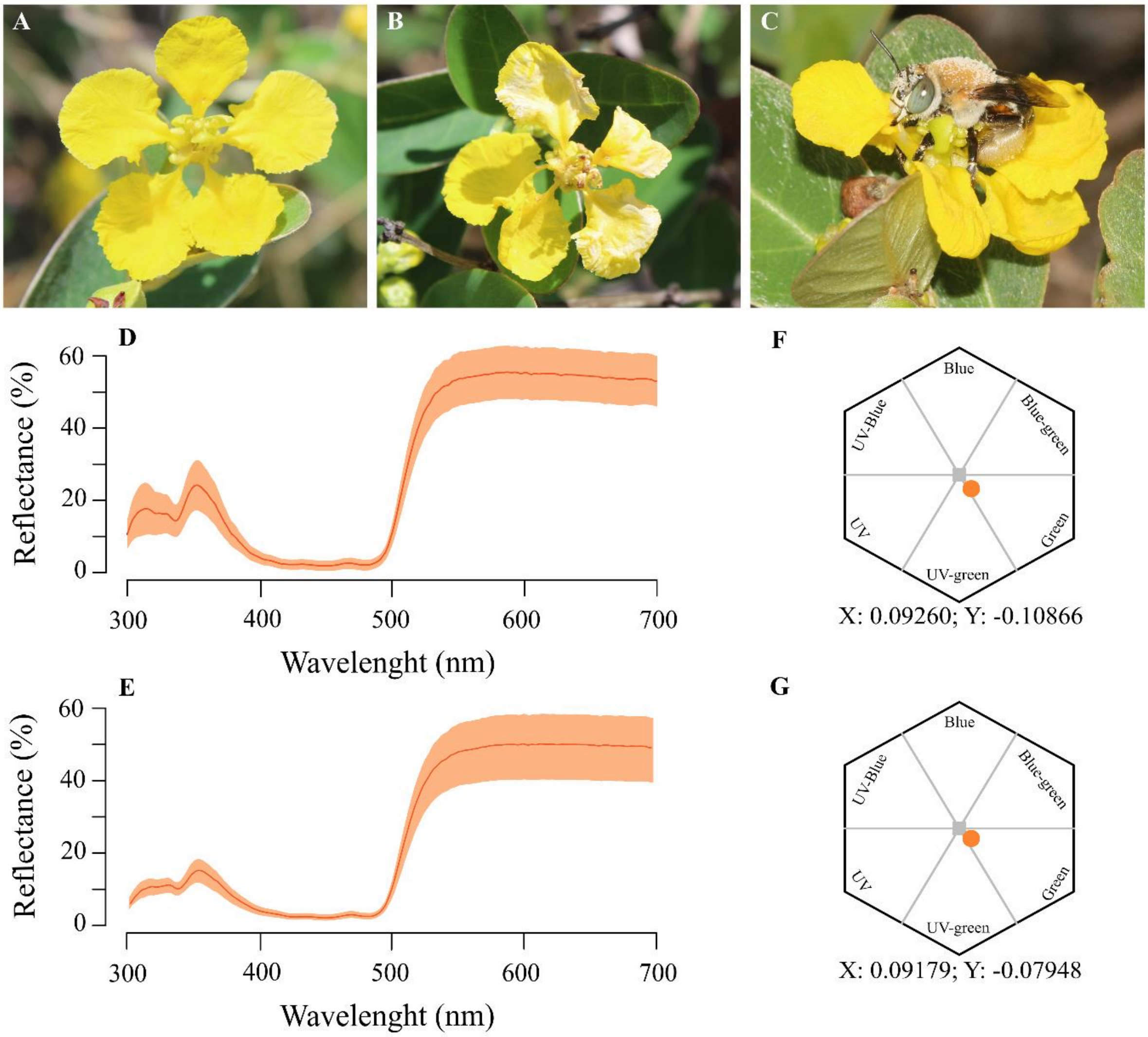

Figure 1: *Stigmaphyllon paralias* A.Juss. A) Flower on the first day of the floral lifespan; B) Flower on the second day of the floral lifespan; C) Visit of a bee of the genus *Centris* on a flower at the beginning of the floral lifespan; D) Reflectance curve of flowers exposed to pollinators at the beginning of the floral lifespan; and E) At the end of the floral lifespan. F) Color hexagon of flowers exposed to pollinators at the beginning of the floral lifespan; and G) At the end of the floral lifespan. Reflectance curves and color hexagons for all collected periods and treatments are in the supplementary material.

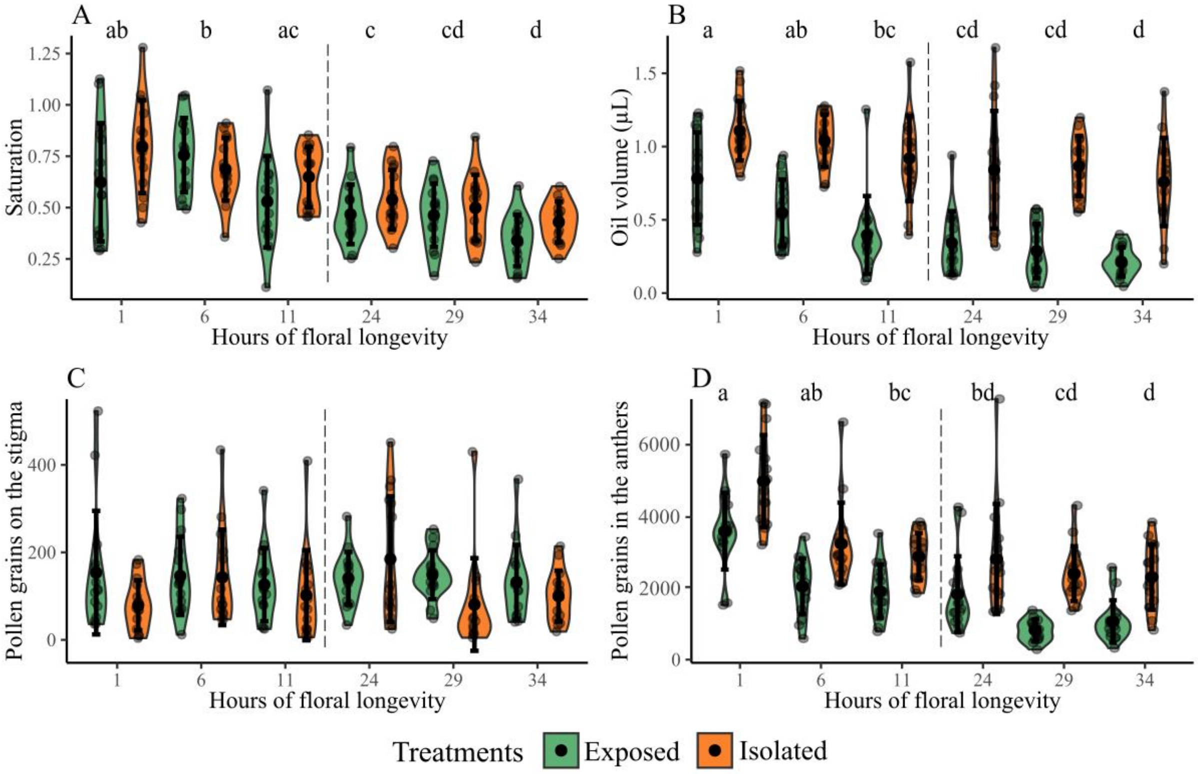

Figure 2: Change in color saturation and reproductive variables of *Stigmaphyllon paralias* throughout floral lifespan and according to exposure to pollinators: A) Saturation; B) Oil volume (μL); C) Number of pollen grains on the stigma; D) Number of pollen grains in the anthers. Significant differences between periods of the floral lifespan are indicated by letters. The dotted line in each graph separates the times of the first (left) and second (right) day of the floral lifespan.

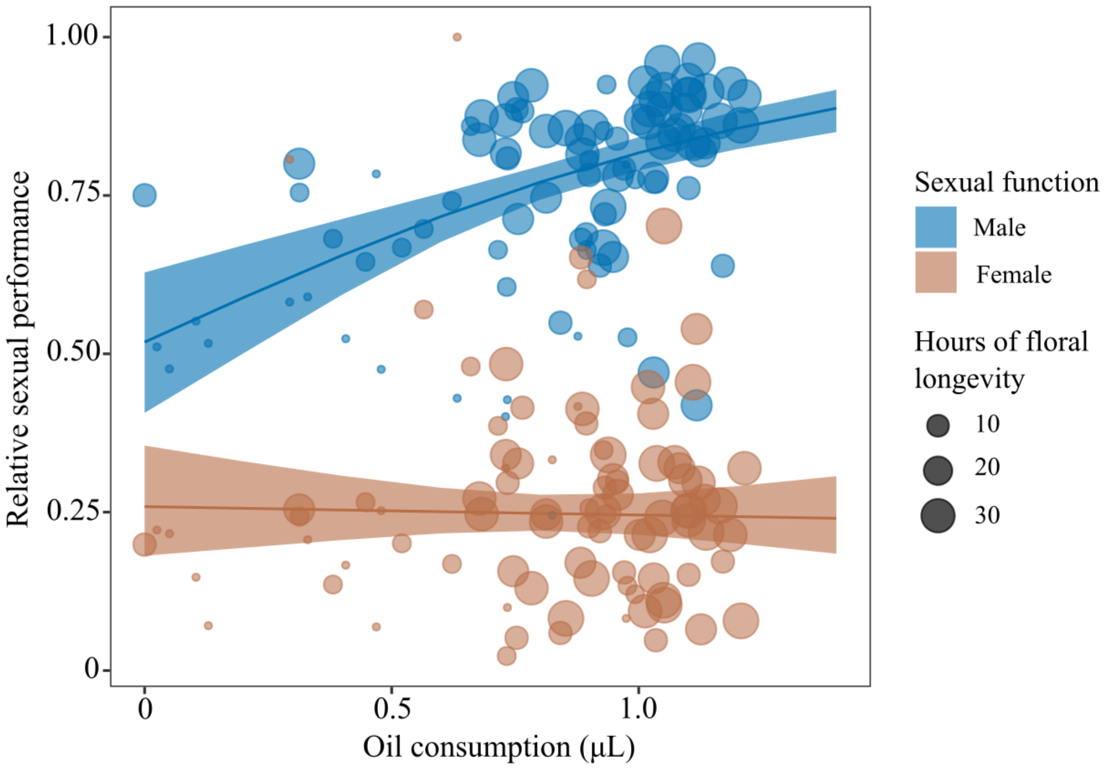

Figure 3: Sexual performance of each reproductive function (male and female) is affected by oil consumption during the floral lifespan of *Stigmaphyllon paralias*. The female function maintains its performance (brown line), while the male function increases performance as oil consumption increases (blue line). Samples are indicated at three periods of anthesis: 10, 20 and 30 hours since floral opening.

Capítulo 3

The niche within the niche: Active exclusion of floral visitors in an oil-producing Melastomataceae flower

Manuscrito a ser submetido ao periódico: Annals of Botany

Fator de impacto: 3.6

The niche within the niche: Active exclusion of floral visitors in an oil-producing Melastomataceae flower

Sabino-Oliveira, A. C.[1*]; Arvelos, C. A.[2]; Matallana-Puerto, C. A. [3]; Magalhães, D. M.[4]; Bento, J. M. [4]; Machado, I. C.[1] & Brito, V. L. G.[2]

1: Biosciences Center, Department of Botany, Postgraduate Program in Plant Biology, Federal University of Pernambuco, Recife, Pernambuco 50670-420, Brazil

2: Institute of Biology, Federal University of Uberlândia, Uberlândia, Minas Gerais 38400-902, Brazil

3: Postgraduate Program in Plant Biology, Institute of Biology, State University of Campinas, Campinas, São Paulo, Brazil

4: University of São Paulo, "Luiz de Queiroz" College of Agriculture, Department of Entomology and Acarology, Piracicaba, São Paulo, Brazil

Abstract

Chemical characteristics are crucial in plant-pollinator communication. Among the many functions they can perform, chemical compounds in flowers can act as floral filters to exclude unwanted visitors. However, knowledge of chemical barriers currently focuses on floral scent and nectar compounds. Therefore, here we investigated whether the floral oil present in secretory structures of *Pleroma candolleanum* (Melastomataceae), a family associated with pollen-bearing flowers, functions as a floral filter in actively excluding visitors. To this end, we collected data from the literature on pollinators of Malpighiacae species, a family with oil flowers, and Melastomataceae species, to understand the sharing of pollinators in the community. We performed controlled experiments to verify the active exclusion of non-oil-collecting bees. Finally, we investigated the possible mechanism causing the exclusion and analyzed the chemical compounds present in the floral oil of *P. candolleanum*. We found that *P. candolleanum* shares pollinators only with oil-bearing species, not with species of its family, Melastomataceae. We also found that non-oil-bearing bees only approach *P. candolleanum*, but do not vibrate the stamens. Finally, we found that the floral oil of *P. candolleanum* contains chemical compounds with repellent properties, which indicates that this may be the cause of the exclusion of a specific group of floral visitors. Therefore, we show that producing floral oil by a Melastomataceae species minimizes the interference of unwanted visitors and potentially increases reproductive success by providing more opportunities for more efficient pollinators.

Keywords: floral filter; *Pleroma candolleanum* chemical compounds; oil flowers; chemical barriers

Introdução

Throughout evolution, angiosperms have developed floral characteristics that guide their pollinators and maximize their reproductive efficiency. These floral traits are crucial for plants to select partners that optimize pollen transfer and ensure reproduction (Faegri and Pijl 1979; Larue *et al.* 2016). However, the floral resources provided do not always meet the demands of floral visitors (Hanna *et al.* 2013), making it necessary to strategically allocate resources that prioritize the most efficient floral visitors, minimizing losses associated with less effective visitors in pollen transfer (Strauss and Whittall 2006; Irwin *et al.* 2010). In this context, plants face adaptive trade-offs in their floral interactions, known as visitor-mediated trade-offs, in which plants balance pollinator attraction with reproductive efficiency, minimizing the costs generated by less efficient visitors, such as resource wastage (Ohashi *et al.* 2021).

Among the types of trade-offs mediated by visitors is the opportunities trade-off, which occurs when different groups of visitors, with different contributions to the plant's reproduction, share the same opportunity for interaction (Ohashi *et al.* 2021). In this case, negative contributions from less efficient visitors can harm the overall efficiency of the interaction, transforming them into "conditional parasites", causing the plant to need to selectively filter the harmful group (Thomson and Thomson 1992; Thomson *et al.* 2000; Thomson 2003; Ohashi *et al.* 2021). As an adaptive response, several species have developed floral filters, which are floral attributes that actively restrict interaction with less efficient groups of visitors, maximizing the plant's reproductive success (Johnson *et al.* 2006; Junker *et al.* 2010; Ohashi *et al.* 2021). However, few studies have been able to demonstrate opportunity trade-offs strong enough to lead to the active exclusion of flower visitors (Thomson and Thomson 1992; Muchhala *et al.* 2010).

Floral filters can act through visual, morphological, or chemical barriers (Raguso 2008a; Junker *et al.* 2013). Visual filters, such as floral colors, can influence pollinator choice by discouraging unwanted visitors, such as nectar robbers (Heinrich and Raven 1972; Johnson *et al.* 2006) or by favoring specific visitors (Lunau *et al.* 2011; de Camargo *et al.* 2019). Morphological barriers include structural adaptations, such as the length of floral tubes, that restrict access by unsuitable visitors (Alexandersson and Johnson 2002; Wester *et al.* 2020). Chemical filters encompass diverse strategies, such as the production of unusual mixtures of compounds (Schiestl *et al.* 1999), variations in compound concentrations (Burger *et al.* 2024), and nectar characteristics, such as dilution (Irwin *et al.* 2004), toxicity or repellent properties for certain groups of visitors (Baker and Baker 1975; Rhoades 1981; Johnson *et al.* 2006; Tiedeken *et al.* 2016). However, despite the undeniable role of chemical characteristics as floral filters (Raguso 2008b; Junker *et al.* 2010), current knowledge is still concentrated on floral scent compounds and widespread resources in angiosperms, such as nectar.

The family Melastomataceae is widely known for characteristic floral attributes, such as poricidal anthers, pollen as the sole floral reward, and vibration pollination, performed by bees that vibrate the stamens to release pollen (Renner 1989; Fracasso 2008; Luo *et al.* 2009), such as bees of the genera *Xylocopa* and *Bombus* (Andena *et al.* 2005; Imperatriz-Fonseca *et al.* 2011). Interestingly, some species of the family produce floral oil, as in the subfamily Memecyloideae (Buchmann and Buchmann 1981) and *Macairea radula* (Marcetieae, Melastomatoideae) (Oliveira *et al.* 2022a). During field observations, we identified glandular structures in the stamens of *Pleroma candolleanum* (Melastomataceae), suggesting the production of floral oil, in addition to exclusive visits by oil-collecting bees of the tribe Centridini, which also perform vibration pollination (Alves-dos-Santos *et al.* 2007; Oliveira *et al.* 2022b). Although the presence of floral oil in Melastomataceae has been previously reported, its ecological function in a family predominantly associated with pollen flowers remains unknown.

Therefore, we investigated whether the secretion in the structures of *Pleroma candolleanum* is composed of floral oil constituents and whether it functions as a floral filter in the active exclusion of non-oil-collecting bees that visit other Melastomataceae species. Specifically, we tested the following hypotheses: 1) *P. candolleanum* shares pollinators only with other oil-collecting flowers, such as species of the Malpighiaceae family, and not with other Melastomataceae species, and 2) Oil-collecting and non-oil-collecting bees similarly approach *P. candolleanum*, but only oil-collecting bees visit and vibrate the stamens. In addition, we investigated the possible mechanisms of this exclusion, testing the hypothesis that: 3) *P. candolleanum* has repellent chemical compounds in its floral oil that restrict interaction with unwanted visitors.

Methods

Study area and model species

We collected data in a rocky field area located in the Serra do Espinhaço, in the municipality of Diamantina, state of Minas Gerais, southeastern Brazil (18°12'06.9"S 43°34'24.9"W). This region has a mesothermal climate (Cwb, according to Köppen 1931), with annual precipitation of approximately 1500 mm and average annual temperatures ranging from 17.4°C to 19.8°C (Giulietti *et al.* 1997). Rocky fields are characterized as a mosaic of mountainous vegetation that grows on rocky outcrops, dominated by herbaceous and shrubby species with xeromorphic adaptations (Alves *et al.* 2014; Silveira *et al.* 2016; Morellato and Silveira 2018).

We conducted the study with *Pleroma candolleanum* (Figure 1-a), a species of the Melastomataceae, a family which comprises approximately 5,500 species distributed predominantly in tropical and subtropical regions (Renner 1993; Bacci *et al.* 2019). Species of this family have arboreal, shrubby, herbaceous, vine or liana habits (Mendonza and Ramírez 2006). Like other species of the genus *Pleroma*, the flowers of *P. candolleanum* are hermaphroditic and characterized by heteranthery, with stamens of different sizes and poricidal anthers that require vibration to release pollen (Luo *et al.* 2009; Brito *et al.* 2021; de Brito *et al.* 2021) (Figure 1-c). A particularity of *P. candolleanum* is the presence of glandular structures in the connective between the anther and the filament of each stamen, which apparently secrete floral oil (Figure 1-d). This differentiates it from most species of Melastomataceae, whose pollen is the only floral resource (Buchmann 1983; Renner 1989; Brito and Sazima 2012).

Data collection

Malpighiaceae and Melastomataceae pollinators in the Cerrado

To evaluate the differences in bee composition in *P. candolleanum*, comparing it with the species communities of Malpighiaceae (oil flowers) and Melastomataceae (pollen flowers), we consulted the pollinators of species of the two families occurring in the Cerrado from the collection of data from the literature. We used Google Scholar and Web of Science as databases, with the following combinations of keywords: “Malpighiaceae + bees + Cerrado”, “Malpighiaceae + pollinators + Cerrado”, “Melastomataceae + bees + Cerrado”, “Melastomataceae + pollinators + Cerrado”. The inclusion criteria established for the articles were: publication in journals, studies conducted in the Cerrado environment, and mention of pollinating bees of at least one species of the families. Studies that did not reach the species level of the pollinating bees were also not included. With the results obtained, we constructed data matrices for each family, in which the rows represented the plant species and the columns the bee species, recording the presence (1) or absence (0) of the pollinator in each species.

In addition, we conducted focal observations in *P. candolleanum* on 29 flowers from three individuals, totaling 12 hours of observation between 10 am and 2 pm, over three days in September 2023. Whenever a bee vibrated the flower stamens, we recorded its identity, which was later confirmed through consultation with experts and specific identification literature.

Active exclusion experiment of flower visitors

To test whether *P. candolleanum* avoids visits from bees that do not collect floral oil, we conducted pollinator preference experiments in the field in September 2023. We used a paired flower presentation system, with a 1 m long stick with a U-shaped end (called a 'pollination stick'), with the flowers separated by 60 cm (adapted from Thomson 1988). At one end, we presented a flower of *P. candolleanum*, and at the other a flower of *Rhycanthera grandiflora*, to be a model species of the family, which has the common characteristics of flowers in Melastomataceae. All flowers used were previously bagged to avoid visits. We performed the experiments in a natural population of *R. grandiflora*, allowing visits from oil-collecting and non-oil-collecting bees, as this species receives both types of visitors. When a pollinator was observed approaching an individual in the population, we carefully moved the pollination rod closer to position the bee in the center, allowing it to choose between the flowers presented. The flowers were switched sides and replaced every 30 minutes to avoid changes in their natural characteristics that could influence visits. The experiments were carried out on three consecutive days, between 10:00 and 13:00, the period corresponding to the peak of bee activity, according to previous observations. Visits were classified into two categories (adapted from Schmitt and Bertsch 1990): “Approach” when the bee approached one of the flowers but did not visit, and “Vibration” when the bee approached, landed, and vibrated the stamens.

Chemical characterization of P. candolleanum oil

We collected the floral oil of *P. candolleanum* in September 2024. For chemical characterization, we previously bagged at least 10 flower buds from 10 individuals with veil bags. After the flowers opened, we collected the oil glands in the laboratory. Using sterilized metal tweezers between collections, we extracted the connective that contains the oil structures, located between the filament and the anther of each stamen. We then stored the connectives from the flowers of one individual in glass jars with 2 ml of dichloromethane solvent for five minutes to extract the oil (adapted from Oliveira *et al.* 2022). After this period, we removed all the connectives from the glass and stored the samples in a freezer at -20 ºC until analysis. In addition, we collected the flower filaments from three individuals to be used as a control group, repeating the same previous process to identify compounds originating from the tissue of the structure, and not specifically from the floral oil.

Subsequently, we analyzed the oil samples on a gas chromatograph (GC, Shimadzu GC-2010) equipped with a flame ionization detector (FID). We injected a 2 µL aliquot of each sample in splitless mode onto a nonpolar Rtx-1 column (30 m × 25 mm id, 0.25 µm film thickness, RESTEK, USA). The temperature was held at 50 °C for 2 min, programmed at 5 °C min–1 to 180 °C, held for 0.1 min, then 10 °C min–1 to 250 °C, and held for 20 min. The FID was at 270 °C and the injector was at 250 °C. We used helium as the carrier gas and collected the data with GC-Solution. We performed the identifications on a Shimadzu GCQP-2010 Ultra quadrupole MS coupled to a GC (Shimadzu GC-2010 Plus). We injected a 2 µL aliquot of selected samples in splitless mode onto an Rxi-1MS column (30 m × 25 mm id, 0.25 µm film, RESTEK, USA). The injector was set at 250 °C using the same temperature program as for GC-FID analysis. Helium was the carrier gas and ionization was by electron impact (70 eV, source temperature at 250 °C). We collected the data with GCMS-Solution. We performed the identifications by comparing spectra with mass spectral library databases (NIST 11) and using retention indices (RIs) and confirmed them by coinjection of headspace samples with authentic standards when available. Finally, we calculated the RIs by comparison with the retention times of a series of linear hydrocarbon alkanes (C7–C30) analyzed with the same separation method.

Data analysis

Malpighiaceae and Melastomataceae pollinators in the Cerrado

To evaluate the differences in bee composition in *P. candolleanum*, comparing it with the species communities of Malpighiaceae (oil flowers) and Melastomataceae (pollen flowers), we performed a PERMANOVA (Permutational Multivariate Analysis of Variance) analysis, a distance-based multivariate analysis of variance. In addition, we conducted a Principal Coordinates Analysis (PCoA) to show the similarities between the species groups.

Active exclusion experiment of flower visitors

To test whether *P. candolleanum* avoids visits from bees that do not collect floral oil, we constructed two generalized linear mixed models (GLMM) with the R package glmmTMB (Brooks *et al.* 2023). In the first model, we used the bee's approach as the response variable (0 - bee approached the flower of *R. grandiflora*, 1 - bee approached the flower of *P.*

candolleanum), the type of bee (oil collector or non-oil collector) as the predictor variable, and the side of the stick on which each flower was positioned as the random factor, to control possible preferences associated with the position. We used the binomial family due to the overdispersion of the data. To verify the effect of the type of bee on the approach, we applied an ANOVA test with the car package (Fox, 2019) and then used the ggpredict function of the ggeffects package (Lüdecke *et al.*, 2025) to obtain values predicted by the model.

For the second model, we previously used the na.omit function to exclude cells with missing data (NA - the bee did not vibrate any flower, it only approached it). We built the model using the bee's vibration as the response variable (0 - the bee vibrated the stamens of *R. grandiflora*, 1 - the bee vibrated the stamens of *P. candolleanum*), the type of bee (oil collector or non-collector) as the predictor variable, and the side on which the flower was positioned as the random factor. We used a binomial distribution again due to overdispersion. We applied an ANOVA test with the car package (Fox 2019) to verify the effect of the type of bee on vibration and used the ggpredict function of the ggeffects package (Lüdecke *et al,*, 2025) to obtain the values predicted by the model.

For both models, we checked the dispersion of the residuals with the simulationResiduals function of the DHARMa package (Hartig and Lohse 2022), as well as the overdispersion of the data (testDispersion function). Finally, we used the ggplot2 package (Wickham and Chang 2016) for graphical constructions. All analyses were performed using the R environment version 4.3.1 (R Development Core Team 2023).

Resultados

Polinizadores de Malpighiaceae e Melastomataceae no Cerrado

We compiled pollinator data for Malpighiaceae species occurring in the Cerrado from 51 papers, covering 44 species of the family and 122 pollinating bees, all known for collecting floral oil, such as the genus *Centris*. For Melastomataceae species, we analyzed 34 papers, identifying 38 species and 61 bees, among which were both oil-collecting and vibration-collecting bees (*Centris*) and pollen-collecting bees (e.g., from the genus *Xylocopa*). In addition, through focal field observations on *P. candolleanum*, we recorded two pollinating bees from the genus *Centris*.

There is a difference between the composition of pollinating bees in the communities of Malpighiaceae (oil flowers), Melastomataceae (pollen flowers), and the species *P. candolleanum* ($F = 9.2561$; $p < 0.001$) (Figure 2), indicating that the variations in distances between species in each group are significantly greater than the variations observed within each group. *P. candolleanum* shares pollinators only with the Malpighiaceae community, and not with species of its family (Melastomataceae). The model explains approximately 18.8% of the total distance variation between species ($R^2 = 0.187$).

Active exclusion experiment

Both oil-collecting and non-oil-collecting bees approached *P. candolleanum* flowers in similar ways ($\chi^2= 0.6745$; df= 1; $p = 0.4115$) (Figure 3-a). However, only oil-collecting bees vibrated the stamens of *P. candolleanum* ($\chi^2= 25.273$; df= 1; $p < 0.001$), while no vibrations were performed by non-oil-collecting bees on these Flowers (Figure 3-b).

Chemical characterization of P. candolleanum oil

The chromatographic analyses performed with floral oil extracted from *P. candolleanum* showed a chemical composition formed by aldehydes, fatty aldehydes, and medium and long-chain fatty acids (Table 1). We identified nine compounds, among which the compound that presented the greatest relative abundance was decanoic acid (32.66%), followed by (E)-2-octen-1-al (23.86%) and dodecanoic acid (19.92%) (Figure 4-a). Some of the compounds have double bonds in their carbon chains ((E)-2-octen-1-al, trans-2-Nonen-1-al, and trans-2-Decenal), which makes them unsaturated. Of the 10 floral oil samples, only one presented problems that prevented identification. The only compound found in the control samples was a small peak of Nonanal, also found in the oil samples (Figure 4-b).

Discussion

The floral oil found in the secretory structures of *P. candolleanum* acts as an efficient chemical filter, promoting the active exclusion of unwanted visitors. *P. candolleanum* shares floral visitors only with other oil-producing flowers, such as species of the Malpighiaceae family, and not with other species belonging to the Melastomataceae family. Bees that do not collect floral oil, such as those of the genera *Xylocopa* and *Bombus*, approach the flowers, but do not vibrate their stamens. In addition, we identified compounds in the floral oil with repellent properties, which appear to act as a deterrent to non-oil-collecting bees. Therefore, we corroborate our hypothesis that there is active exclusion of visitors, and also our hypothesis about a mechanism causing exclusion, based on the identification of repellent compounds.

Among the compounds present in the floral oil of *P. candolleanum*, none were identified in the oil of Malpighiaceae flowers, a family characteristic of oil flowers, nor in Macairea radula, a species of Melastomataceae whose compounds have already been described. The floral oil of Malpighiaceae is characterized by the predominance of long-chain fatty acids, as well as acetylated or methylated esters (Vogel 1974; Buchmann 1987; Cappellari *et al.* 2011; Haleem 2012; Schäffler *et al.* 2015; Barônio *et al.* 2017; Albuquerque 2021; Albuquerque *et al.* 2023). In Macairea radula, fatty acids, and their derivatives are also mainly found, but among them are alkanes (Oliveira *et al.* 2022b). On the other hand, the floral oil of *P. candolleanum* is composed of a unique mixture, with half of the fatty acids absent in the other oils described and half of the aldehydes. This differentiated composition suggests a high degree of chemical specialization in the floral oil of this species.

Among the compounds identified in *P. candolleanum* oil, most have already been described as repellents for some groups of insects (Chamberlain *et al.* 1991; Sivakumar *et al.* 2011; Noge 2015; Sagun and Collins 2016; Cruz-Estrada *et al.* 2019; Temeyer *et al.* 2024). For example, the most abundant aldehyde in *P. candolleanum*, (E)-2-octen-1-al, has moderate toxic potential and a repellent effect against predatory insects (Noge 2015). In addition, medium-chain fatty acids, such as decanoic, undecanoic, and dodecanoic acids, have deterrent effects on insects and, when combined, can act synergistically as efficient repellents (Cruz-Estrada *et al.* 2019). However, the function of these compounds may vary according to the floral visitor. Chemical compounds can act as floral filters, repelling certain groups but selecting the most efficient pollinators (Baker and Baker 1975; Burger *et al.* 2024). Studies have reported that such filters can be particularly effective in excluding less desirable bees and resource robbers while allowing visitation by bees that act as effective pollinators (Tiedeken *et al.* 2016; Barlow *et al.* 2017). Therefore, our findings show that this combination of repellent compounds in the floral oil of *P. candolleanum* functions as a chemical filter, restricting access by non-oil-collecting bees and favoring oil-collecting bees.

Oil-collecting bees of the tribe Centridini have a close relationship with oil-bearing flowers, established throughout coevolutionary processes (Vogel 1990; Vogel and Machado 1991; Renner and Schaefer 2010; Carneiro *et al.* 2015; Carneiro and Machado 2023). The floral oil of the species described to date has fatty acids as its main constituents (Simpson *et al.* 1977; Simpson and Linden 1989; S. Vogel 1990; Barônio *et al.* 2017). These compounds have already been described as essential chemical signals in the communication between flowers and oil-collecting bees, such as the compound diacetin (Dötterl and Schäffler 2007). The repellent effect of the fatty acids present in the floral oil of *P. candolleanum*, however, may not affect Centridini bees, given their long coevolutionary history with oil-producing flowers and their affinity for compounds of this class. On the other hand, non-oil-collecting bees may exhibit avoidance mechanisms of these compounds, through learning processes or genetic predispositions (Després *et al.* 2007).

Furthermore, we observed that bees do not actively collect oil from *P. candolleanum*. During the visit, the oil glands remain around the bee while it vibrates the stamens, allowing only occasional contact of the oil with the bee's body. However, the compounds can be consumed indirectly during grooming behavior, when bees scrape pollen from their bodies, redistributing it to sites less likely to contact the stigmas, such as the scopas (Harder and Wilson 1998). During this process, the oil that adhered to the body can be mixed with the collected pollen. Although the oil consumption is likely accidental, the long coevolutionary history between oil flowers and forager bees may have led to the evolution of metabolic resistance mechanisms. For example, the overproduction of detoxification enzymes capable of metabolizing xenobiotics, chemical compounds that are foreign to the organism and potentially toxic, could neutralize the adverse effects of floral oil (Sandermann 1992; Després *et al.* 2007). However, experimental studies that test the metabolic capacity of oil-collecting bees about the chemical compounds of floral oil are still lacking.

In summary, our findings show that *Pleroma candolleanum* actively excludes non-oil-collecting bees, with repellent compounds found in floral oil being one of the potential mechanisms responsible for this exclusion. However, there is still a gap to be explored: Considering the evolution of floral filters as an adaptive response to the trade-off of opportunities, are bees excluded from this system by floral oil less efficient in the reproduction of the species? Given this, it is essential to investigate the reproductive efficiency associated with different groups of visitors to understand the selective pressures that shaped the evolution of this floral filter.

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Table 1: Compounds found in the floral oil of *Pleroma candolleanum*, with the retention times and relative abundance of each compound

Compound	**Retention time (min)**	**Relative abundance (%)**
(E)-2-octen-1-al	10,88	23,86
Nonanal	12,36	4,28
trans-2-Nonen-1-al	13,90	1,06
trans-2-Decenal	16,79	2,05
Nonanoic acid	17,34	1,59
Internal Standard	18,33	5,78
Decanoic acid	20,26	32,66
Undecanoic acid	22,44	4,62
Dodecanoic acid	24,93	19,92
Tetradecanoic acid	29,13	4,14

Figure 1: Floral structures of *Pleroma candolleanum*. a) Flower of *P. candolleanum*; b) Flower of *P. candolleanum* receiving a visit from a floral oil-collecting bee; c) Stamen of *P. candolleanum*; d) Connective between the filament and anther of *P. candolleanum,* which contains the oil-secreting structures

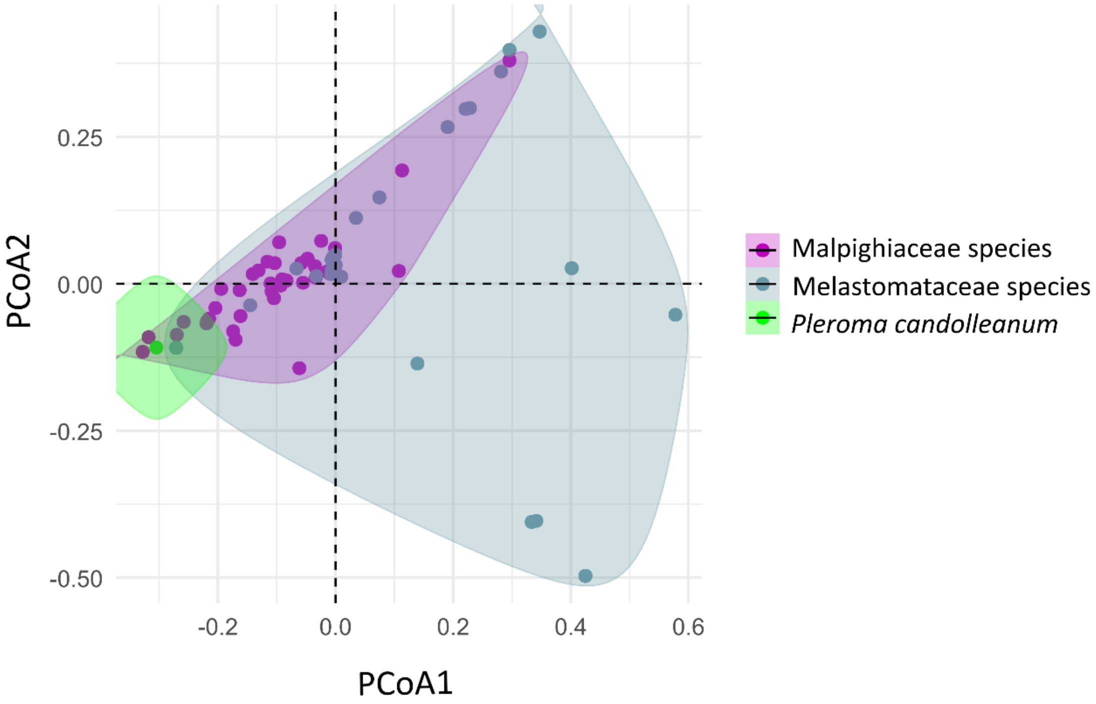

Figure 2: Graphical representation of the PCoA showing the similarity between species of Malpighiaceae (oil flowers), Melastomataceae (pollen flowers) and *Pleroma candolleanum* regarding their pollinators

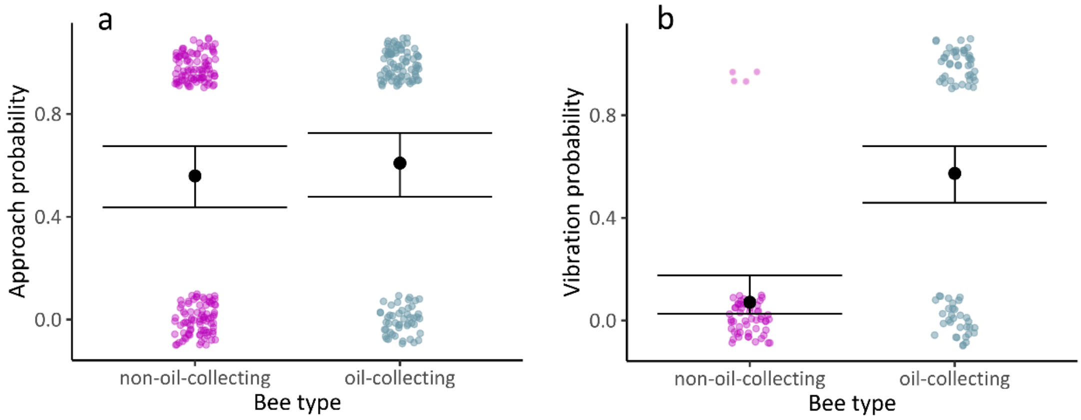

Figure 3: a) Graph showing that both oil-collector and non-oil-collector bees approach *Pleroma candolleanum* flowers in a similar manner; b) Graph showing that only oil-collector bees visit and vibrate their stamens

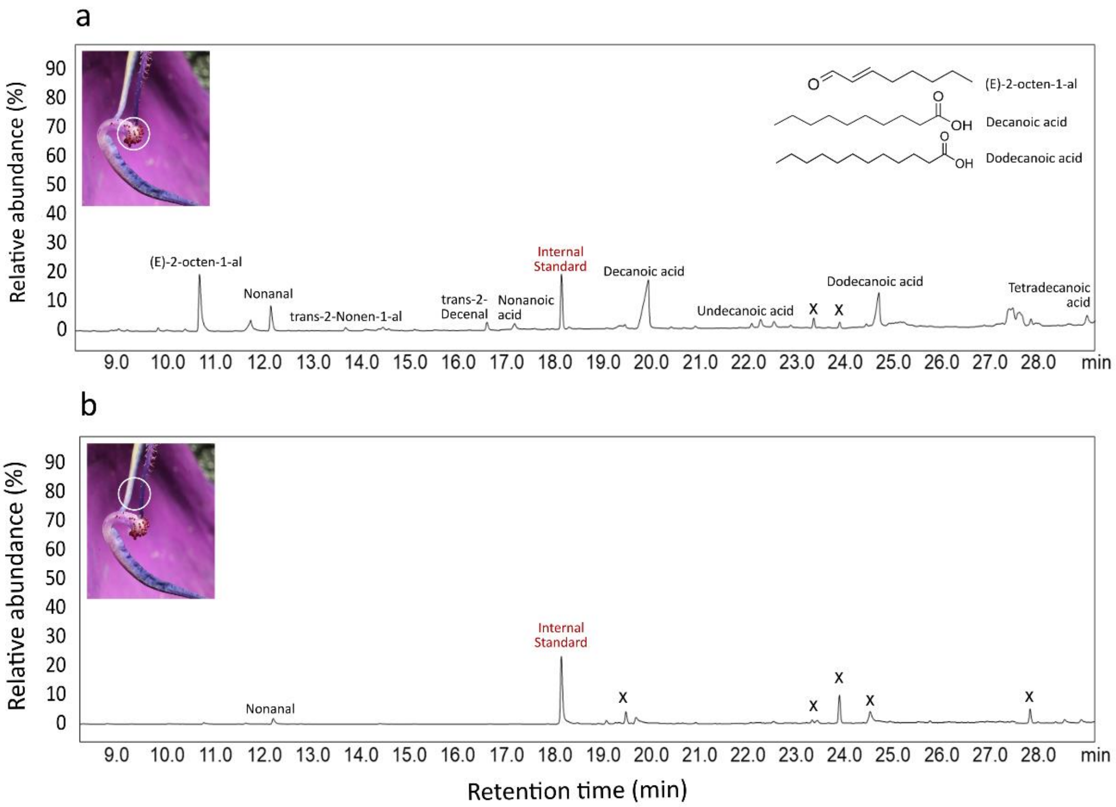

Figure 4: Representative mass spectra of *Pleroma candolleanum* floral oil. a) Compounds found in the floral oil extracted from the conctive present in the stamens of *P. candolleanum*, highlighting the three compounds with the greatest abundance, and b) Compounds found in the filament of *P. candolleanum*, which was used as a control

Capítulo 4

Floral morphology preventing abiotic pollen loss: The case of *Salvia chamelaeagnea* (Lamiaceae)

Manuscrito a ser submetido ao periódico: South African Journal of Botany

Fator de impacto: 2.7

Floral morphology preventing abiotic pollen loss: The case of *Salvia chamelaeagnea* (Lamiaceae)

Sabino-Oliveira, A. C.[1*]; Brito, V. L. G.[2], Machado, I. C. S. [1]; Anderson, B[3]

1: biosciences center, department of botany, postgraduate program in plant biology, federal university of pernambuco, recife, pernambuco 50670-420, brazil

2: institute of biology, federal university of uberlândia, uberlândia, minas gerais 38400-902, brazil

3: Department of botany and zoology, Stellenbosch University, Stellenbosch, South Africa

Abstract

Species of the genus *Salvia* (Lamiaceae) have a staminal lever mechanism. Because pollen is a rare and precious commodity and its loss must be prevented, the location of the upper lip in these flowers allows the anthers to be "hidden", which may prevent pollen grains from being lost. We investigated the function of the upper lip in preventing pollen loss, using the species *S. chamelaeagnea* as a model. We used 30 natural flowers and 30 flowers without the upper lip. We collected one of the anthers immediately after the flower opened and the other after five hours of observation. In unmanipulated flowers, we collected one of the anthers after one visit. Once a flower was visited, we collected one of its anthers, totaling 25 visits. Pollen loss by wind during floral life depends on the presence of the upper lip. Flowers with an upper lip lost only 9.35% of pollen grains during their floral life, while flowers without this structure lost 59.09%. Flowers visited by the carpenter bee *Xylocopa caffra* lost approximately 47.85%. Therefore, the upper lip that constitutes the staminal lever in *S. chamelaeagnea* prevents the flower from losing pollen grains to the wind before it is visited by an effective pollinator.

Keywords: pollen loss by wind; pollen protection; morphological barrier; staminal lever

1 Introduction

It is estimated that only 1% of pollen reach the stigmatic surfaces of conspecific flowers (Harder et al., 2000; Holsinger and Thomson, 1994; Johnson et al., 2005), making pollen export one of the biggest challenges for male fertility in angiosperms (Minnaar and Anderson, 2019). Before fertilization of the ovule can take place, pollen loss can occur at many different stages (Inouye et al., 1994; Minnaar et al., 2019): Pollinators may dislodge pollen from anthers (Timerman and Barrett, 2018), pollinators may consume pollen (Hargreaves et al., 2009), pollen grains can be lost during transport by pollinating animals (Flanagan et al., 2009; Mitchell et al., 2009; Thomson, 2003; Wilson and Morrison, 2000), or they can be displaced or damaged by wind (Reynolds et al., 2009) or rain (Mao and Huang, 2009). Each of these pollen loss avenues is likely to select for different structures that reduce pollen loss, and the more pollen lost through a particular pathway, the stronger the selection to reduce pollen loss is likely to be (Minnaar et al., 2019). Although transporting pollen grains to stigmas is clearly challenging, few studies have attempted to quantify pollen loss to these various pathways (Minnaar et al., 2019).

Several floral traits have been viewed as adaptations to prevent pollen loss or damage from rain or mist: for example, hood-like structures covering the anthers in some tubular flowers can prevent pollen loss resulting from rain (Von Marilaun and Oliver, 1895), as does the closure of the corolla (Bynum and Smith, 2001), pendulous flowers (Aizen, 2003; Huang et al., 2002) or nocturnal floral closure (Von Hase et al., 2006). In addition to rainfall, wind has also been identified as a potentially important agent of abiotic pollen loss (Minnaar et al., 2019) and one study found that wind may remove up to 50% of *Silene* pollen (Reynolds et al., 2009). However, pollen removal by wind is usually studied in the beneficial context of wind pollination (Nadia and Machado, 2014; Roy, 2023; Schultze-Albuquerque et al., 2024) and studies on how animal pollinated plants reduce the loss of pollen to wind are practically non-existent.

Salvia (Lamiaceae) plants display unusual modifications of the male reproductive components and general floral morphology, which may reduce abiotic pollen loss (Thimm, 2008). *Salvia* flowers display two identical stamens, each one of them consists of an upper, fertile lobe and a lower, sterile lobe or plate (Thimm, 2008). Between these two sections is a flexible joint where a filament attaches the entire structure to the corolla (Correns, 1891). The sterile plates of the two anthers block the gullet of the corolla and thus access to nectar within the floral tube (Classen-Bockhoff et al., 2004; Celep et al., 2020). When a pollinator probes the flower, its mouthparts push against the lower plates and the joint acts as a fulcrum around which the fertile stamens swivel, moving forward and placing pollen on the pollinator (Classen-Bockhoff et al., 2004). The corolla tube opens to form a lower lip, which insects often land on in insect pollinated salvia and an upper lip which often encloses the fertile anthers so that they are hidden within the flower (Classen-Bockhoff et al., 2004; Thimm, 2008; Claßen-Bockhoff, 2017; Celep et al., 2020). The anthers only become exposed and emerge from the upper lip when a pollinator pushes against the lower plates (Thimm, 2008).

It is thought that this complex lever structure has played an important role in the diversification of *Salvia* because it allows the flowers to easily adapt to novel pollinators but also results in very precise pollen placement and reproductive isolation when divergent forms do adapt to new pollinators (Celep et al., 2020; Drew, 2020). However, the forces that selected for its evolution are unclear. Some hypotheses include: a) it causes precise pollen placement, which increases the effectiveness of floral-pollinator fit (Thimm, 2008); b) precise pollen placement may reduce pollen wastage through improper pollen transfer to a different species (Reith et al., 2006; Thimm, 2008); c) the mechanism acts as a pollinator filter, allowing only very specific pollinators to access the floral rewards. Having anthers which are hidden until a pollinator visits protects exposed pollen from d) pollen thieves (Hargreaves et al., 2009) and e) the environment that could increase pollen loss.

In this study, we investigate the possibility that the hidden anthers may reduce the loss of pollen to the environment. This is not a hypothesis that has been considered before and in the context of this paper, we only explore pollen loss on non-rainy days, which could be the result of wind or simply passive loss. We expect that: 1) The presence of the upper lip in *S. chamelaeagnea* prevents pollen loss, and that 2) The upper lip allows pollen removal only during effective pollinator visits and lever activation.

2 Methods

2.1 *Study area and model species*

We collected data in a population of *Salvia chamelaeagnea* (Lamiaceae) in the Jan Marais Nature Reserve in the Municipality of Stellenbosch, Western Cape, South Africa (33° 55' 56.27" S and 52' 24.92" E). *S. chamelaeagnea* is a mesophilic, evergreen shrub native to the Northern and Western Cape of South Africa. An effective pollinator of this species is the carpenter bee *Xylocopa caffra* (Will and Claßen-Bockhoff, 2014). The flowers are formed in spirals and are arranged on lateral branches with generally reddish-purple calyxes. The most common color of the flowers is white on the lower lip and purplish blue on the upper lip (Roos, 2020). Like many other *Salvia* species, S. *chamelaeagnea* possesses a staminal lever system (Figure 1a). Prior to visitation by a carpenter bee, the anthers are hidden and enclosed within the upper lip of the corolla. But when the bees probe for nectar, activation of the lower staminal plate pushes the anthers beyond the protection of the upper lip, exposing them to the elements, but at the same time actively placing pollen on the body of the bee (Thimm, 2008) (Figure 1b).

2.2 *Data Collection*

Initially, we measured the wind speed with a digital anemometer as a premise so that the wind would not be a confounding variable in the results found. The average wind speeds were 1.36, 1.5 and 1.56 m/s, respectively, being similar on the three days, considered as "light air" on the Beaufort Scale.

To test if the presence of the upper lip in *S. chamelaeagnea* prevents pollen loss by wind, we used 60 flowers distributed across 10 individuals. This was done over three days (April 2024) so that on each day, we worked with 20 flowers. We divided the flowers into two treatments: Immediately after floral opening, we removed the upper lip from 30 flowers using surgical scissors, thus exposing the anthers. As a control, we left the upper lip intact in another 30 flowers (Figure 1c,e). We then collected one of the two anthers from all flowers using fine forceps. In flowers with intact upper lips, we had to carefully open the lip and insert the forceps to extract the anther. Each collected anther was stored in an Eppendorf tube with 200 µL of a solution of 70% ethanol and 1% glycerin. We then monitored all experimental flowers for a period of five hours, chasing all floral visitors away with a net before they alighted on our flowers. After five hours, using the methods described above we collected the second anther from all experimental flowers and that was also stored in an identical Eppendorf containing ethanol and glycerin. By counting the pollen grains in the first and second anther of each flower, we were able to determine the amount of pollen lost solely due to the abiotic environment.

To test if the upper lip allows pollen removal only during effective pollinator visits and lever activation, we determined how much pollen pollinators removed from flowers during single visits by carpenter bees (Figure 1d). After a flower was visited once, we marked it and after five hours, we collected one of the anthers to compare the pollen quantity between naturally visited flowers and those subjected only to abiotic effects. The anther was also stored in an Eppendorf containing ethanol and glycerin. In total, we observed 25 visits during the three days of experiments.

In the laboratory, we counted the number of pollen grains in each of the anther-containing eppendorfs. Inside the Eppendorf tube, each anther was macerated for one minute using a metal pestle to release all the pollen from the tissue. Subsequently, the tube was placed on a vortex mixer at maximum speed for one minute to agitate the pollen particles and homogenize the solution. Using a pipette, we then placed 25 µL of the solution on a slide, and covered it with a coverslip to count the pollen grains under a microscope. The count was then multiplied by eight, as we counted ⅛ of the total anther solution.

2.3 *Data Analysis*

To test if the upper lip in *S. chamelaeagnea* prevents pollen loss due to wind, we constructed a generalized linear mixed model (GLMM) with the R package glmmTMB (Brooks et al., 2023), using the negative binomial family, due to overdispersion of the data. We used as predictor variables the interaction between treatment (hidden or exposed anthers) and time point in floral lifespan (beginning or after five hours), while the response variable was the number of pollen grains in each anther. Flower identity was used as a random factor, allowing pairwise comparisons of pollen amounts at the beginning of floral lifespan and after five hours at the end of the observation. To verify the influence of the upper lip on pollen loss due to wind, we used likelihood ratio tests with the Anova function from the car package (Fox, 2019). Subsequently, we used the emmeans function from the R package emmeans (Lenth et al., 2022) to perform a Tukey post hoc test and check for differences between time points and treatments. Additionally, we collected the model's predicted values for each sample using the predict function.

To test the difference in the number of pollen grains between flowers with upper lip intact at the beginning of the floral lifespan, after observations, and after a pollinator visit, we also used a generalized linear mixed model (GLMM) with a negative binomial error structure. We used treatments as the predictor variable, the number of pollen grains as the response variable, and flower identity as a random factor. To check for differences between these treatments, we used likelihood ratio tests with the Anova function from the car package (Fox, 2019). Furthermore, we used the emmeans function to perform a Tukey post hoc test and check for differences between treatments, if any.

For all built models, we checked the dispersion of the residuals with the simulateResiduals function from the DHARMa package (Hartig and Lohse, 2022), as well as the overdispersion of the data (testDispersion function). Finally, we used the ggplot2 package (Wickham and Chang, 2016) for graphic constructions. All analyses were performed using the R version 4.3.1 environment (R Development Core Team 2023).

3 Results

The presence on the upper lip prevents pollen loss in *S. chamelaeagnea* (χ^2= 27.684; df= 1; $p < 0.001$). Flowers with the upper lip intact lost only 9.35% of pollen grains from the anthers, while flowers without the upper lip lost 59.09% (Figure 2).

The *S. chamelaeagnea* flowers we allowed to be visited received visits only from the carpenter bee *Xylocopa caffra*. When comparing flowers that remained with the upper lip at the beginning of floral life, after five hours of exposure, and after one visit, we found differences between these groups (χ^2= 42.081; df= 2; $p < 0.001$). Flowers after one visit showed a significant decrease in the number of pollen grains when compared with flowers at the beginning of floral life (Z-value= 6.122; $p < 0.001$). The same pattern was observed when compared with flowers after five hours of exposure (Z-value= 5.742; $p < 0.001$) (Figure 2). Flowers lost approximately 47.85% of the pollen grains from the anthers after a single visit from *X. caffra*.

4 Discussion

As expected, we found that the upper lip that forms the staminal lever in *S. chamelaeagnea* prevents the loss of pollen grains by the wind. Furthermore, we also corroborated our hypothesis that the upper lip allows pollen removal only with the visit of the effective pollinator and activation of the staminal lever. The amounts of pollen produced remained similar in flowers with and without visits, and significant pollen losses occurred only in flowers manipulated with removal of the upper lip and in flowers visited by the carpenter bee *Xylocopa caffra*. These findings demonstrate that the structure of the staminal lever, formed mainly by the upper lip keeping the anthers hidden, has as one of its functions to prevent the wind from dispersing the pollen and to protect them for a first effective visit.

The mechanism of releasing pollen only when a pollinator capable of activating the staminal lever visits the flower ensures an effective first visit with the maximum possible pollen load. Complex flowers that hide their pollen within floral structures limit pollen loss by restricting access to the most effective pollinators (Harder & Barclay 1994; Müller 1996; Westerkamp 1997; Westerkamp and Claßen-Bockhoff 2007.). Since the presence of the upper lip implies the need for greater pollinator forces to release the pollen, being the only protective structure that influences the force required to move the lever (Thimm, 2008), other bee species may lack the necessary strength to move the lever and lower the stamens (Thimm, 2008). The anthers hidden by the upper lip force the bee to come into contact with the stigma (Westerkamp, 1997), ensuring the export of the maximum amount of pollen possible, as there are no pollen loss until the first visit.

Moreover, the reproductive strategy of ensuring the maximum possible pollen release during the first effective visit aligns with the "pollen presentation theory" (PPT) (Percival, 1955), which proposes the existence of an ideal schedule for presenting pollen for pollinators based on the expected number of effective visits. In species with a high likelihood of a single visit and a low probability of subsequent visits, the best strategy would be to release the maximum possible pollen during the first visit, whereas in plants receiving many visits, pollen should be dispersed gradually (Lebuhn and Holsinger, 1998). As the presence of the upper lip reduces the chances of some visitors accessing the pollen, storing it until the necessary force is applied to move the staminal lever (Thimm, 2008). Flowers of *Salvia chamelaeagnea* release a greater amount of pollen during the first visit. After this, the anthers are exposed, which increases the chances of pollen loss to the wind or other visitors, suggesting that retaining pollen until the first effective visit is a key strategy for maximizing pollen transfer. Therefore, protecting pollen from abiotic loss until the first visit is in line with the reproductive strategy that providing as much pollen as possible requires that it be retained in the anthers until this reproductive event during floral longevity.

Thus, our findings highlight the critical role of the upper lip of *S. chamelaeagnea* in protecting against pollen loss due to abiotic factors, especially wind. Without this structure, approximately half of the pollen may be lost within the first five hours after flower opening, even without pollinator visitation. With the presence of the lip, this loss is almost absent. The amount of pollen removed during the first visit of a pollinator is similar to what would have been lost to the wind in the absence of this structure, suggesting that the upper lip acts as an effective barrier controlling pollen release. In this way, the lever mechanism may have evolved with one of its functions being to keep pollen hidden until an efficient pollinator arrives. This adaptation reflects a strong selective pressure for the conservation of pollen until the most opportune moment, reinforcing the relationship between floral morphology and reproductive success in plant-pollinator interactions.

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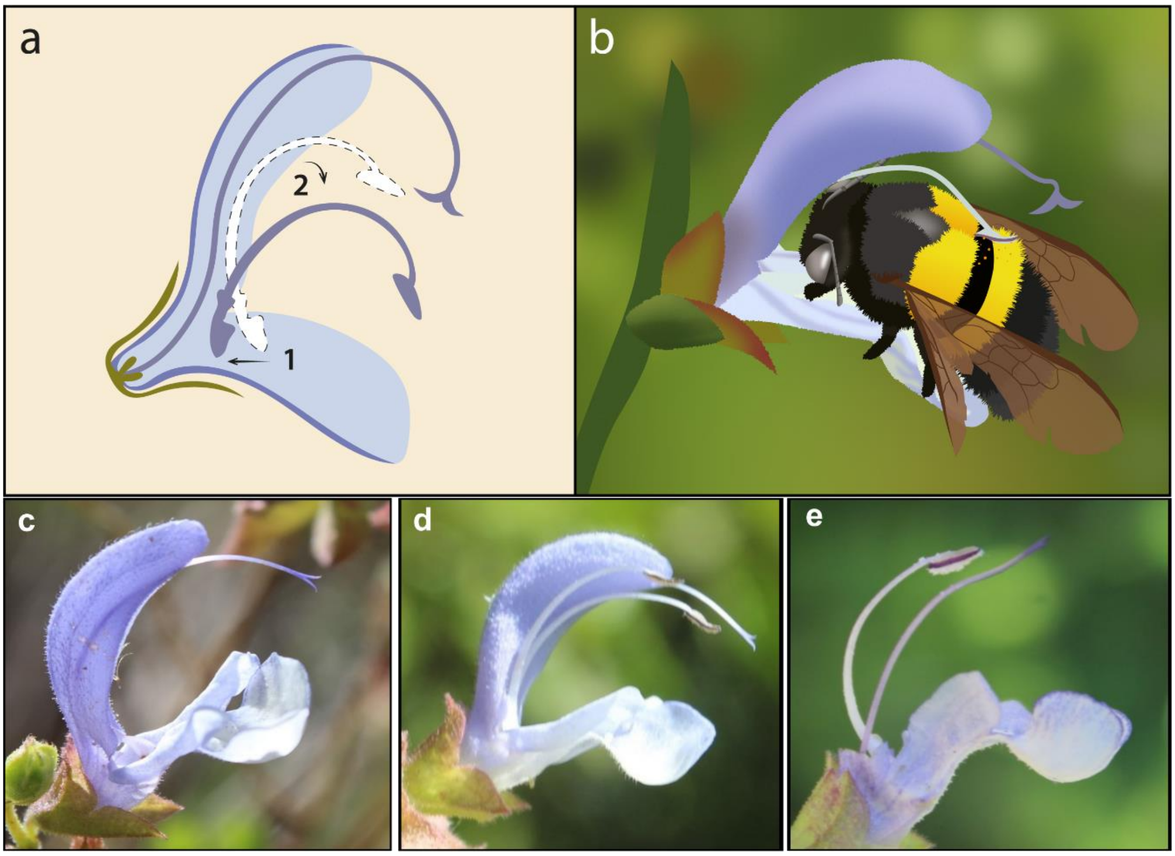

Figure 1: a) Staminal lever mechanism found in *Salvia* species; b) Visit of the bee *Xylocopa caffra* to a flower of *Salvia chamelaeagnea*; c) Flower of *Salvia chamelaeagnea* at the beginning of the floral lifespan; b) After a visit from the carpenter bee *Xylocopa caffra* and c) Without upper lip

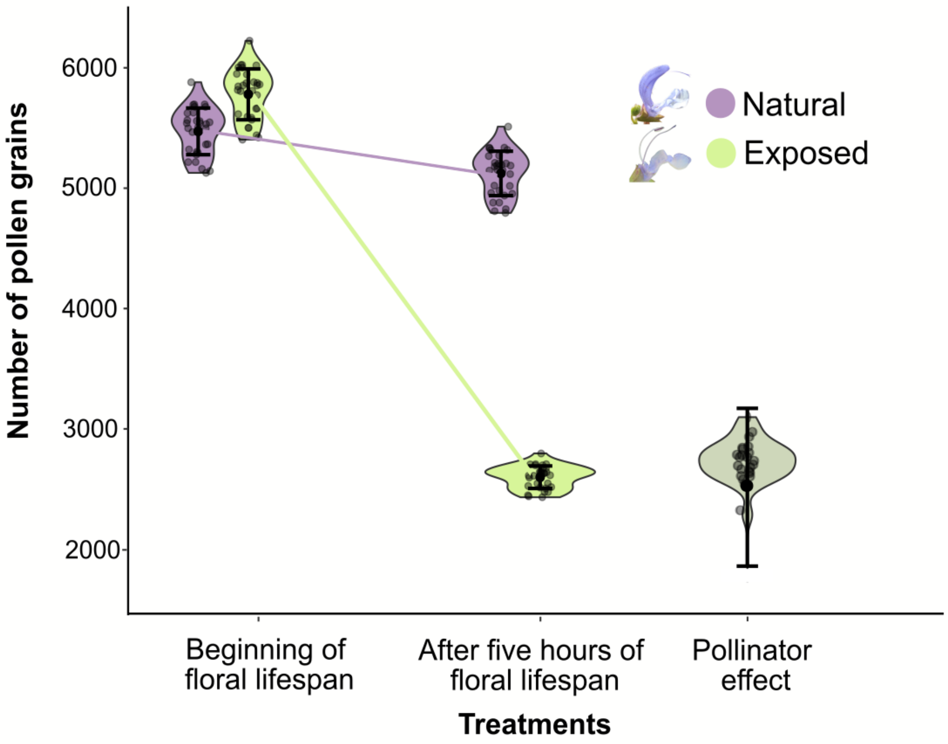

Figure 2. Number of pollen grains in flowers of *Salvia chamelaeagnea* at the beginning of the floral lifespan and after five hours, with and without the upper lip. Also, the number of pollen grains in flowers after a single visit by the carpenter bee *Xylocopa caffra*

3 CONSIDERAÇÕES FINAIS

Esta Tese fornece informações importantes sobre o papel dos atributos florais na mediação das interações entre plantas e polinizadores. Mostramos que características como cor, longevidade e morfologia das flores não apenas influenciam a atração de polinizadores, mas também impactam a eficiência reprodutiva e a proteção de recursos florais. Destacamos a complexidade e a relevância dos atributos florais no contexto das interações ecológicas. Em conjunto, os achados sugerem que as plantas desenvolveram múltiplas estratégias para otimizar a reprodução e a eficiência no uso de recursos florais, adaptando suas características para maximizar o sucesso reprodutivo nas condições de seu ambiente.