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Orientador:
Prof. Dr. Paulo Roberto Guimarães Júnior
Co-orientadora:
Profa. Dra. Ana Paula Aprígio Assis

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Comissão Julgadora:

_____   _____

Prof(a). Dr(a).   Prof(a). Dr(a).

_____   _____

Prof(a). Dr(a).   Prof. Dr. Paulo Roberto Guimarães Jr.

                                                 Orientador

DEDICATÓRIA

DEDICO ÀQUELES QUE PERDERAM SUAS VIDAS EM PROL DA CONSERVAÇÃO DA NATUREZA.

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Sumário

Resumo

Interações mutualistas compõem processos ecológicos importantes na estruturação de ecossistemas, influenciando padrões de variação e adaptações nos organismos. As espécies que estão interagindo irão interferir no crescimento, sobrevivência ou reprodução dos indivíduos de suas espécies parceiras, de forma que podem ser fontes de pressão de seleção e gerarem mudanças evolutivas. Quando essas mudanças são recíprocas, as espécies envolvidas em um mutualismo estão em um processo coevolutivo. Contudo, ambas espécies estarem impondo pressões seletivas uma à outra não significa que tais pressões são de igual intensidade. Desta maneira, os padrões fenotípicos associados a interações ecológicas na natureza podem ser consequência de contribuições diferenciais da trajetória evolutiva das espécies que interagem. A assimetria nas pressões seletivas pode ter muitas fontes. Em geral, mutualismos têm consequências para diferentes componentes da aptidão média da espécie. Por exemplo, uma espécie de polinizador, ao visitar uma flor, obtém alimento, seja ele pólen ou néctar, influenciando um componente de viabilidade da aptidão. Já o benefício da interação para a planta é reprodução, uma vez que o polinizador irá dispersar os gametas masculinos (pólen) ou trazê-los até o gameta feminino (óvulo), influenciando o componente de fecundidade da aptidão média da espécie. Além disso, as interações mutualistas estão embebidas em redes, o que também pode representar uma fonte de assimetria para a dinâmica coevolutiva entre as espécies envolvidas. Por exemplo, uma dada planta cujos frutos são dispersos por uma única ave possui alta dependência dessa ave, de forma que a ave pode representar uma importante fonte de pressão seletiva em traços ecologicamente relevantes para a interação da planta. Contudo, se essa planta dispersa suas sementes por meio de várias outras aves, a pressão seletiva de uma ave sobre a planta pode ser muito menor e assimétrica. Minha tese de doutorado buscou investigar as consequências das assimetrias descritas acima e definidas por nós como *assimetria de seleção* e *de especialização*, respectivamente, para a dinâmica coevolutiva em mutualismos. Nós combinamos revisão da literatura com modelos matemáticos, teoria de redes e dados empíricos para compreender o papel de tais assimetrias nos padrões fenotípicos que observamos em comunidades ecológicas. Esta tese é formada por três capítulos. O primeiro é composto por uma revisão de literatura, onde eu busquei identificar condições que gerariam assimetria de seleção, em especial para polinização, e estudar as consequências de tais nos processos coevolutivos. No segundo capítulo eu utilizei modelagem matemática para explorar as consequências da assimetria para a propagação de efeitos evolutivos em redes de mutualismo. Por fim, no terceiro capítulo realizei experimentos testando duas estratégias de deposição de pólen e discuti suas consequências para a ecologia reprodutiva da planta e evolução floral. Interações mutualistas são mediadas por múltiplas

características, estas, podem estar coevoluindo sob forças de seleção que varia em simetria de acordo com o quanto afetam a eficiência da interação. Nós também verificamos que a estrutura da rede que as espécies estão evoluindo pode ser alterada em casos que a assimetria de seleção esteja estruturada por guilda trófica, o que afeta como efeitos indiretos podem contribuir para a evolução de uma característica. Por fim, como plantas são hermafroditas, as rotas de coevolução com os polinizadores também podem contribuir assimetricamente. Nós observamos que há evidência para a formação de camadas de grãos de pólen em polinizadores, demonstrando que o próprio polinizador pode ser uma arena onde ocorre a competição das flores por espaço.

Abstract

Mutualistic interactions are key processes structuring ecosystems, affecting patterns of trait variation and adaptation in organisms. Interacting species affect growth, survivorship and reproduction of individuals from the interacting species, being important source of selective pressure and generate evolutionary changes. When the evolutionary changes are reciprocal, species involved on the mutualism are in a coevolutionary process. However, even though species are imposing reciprocal selective pressures, it does not mean selection has equal strength. Therefore, phenotypic patterns linked to ecological interactions in nature may be consequence of differential contribution to the evolutionary trajectories that interacting species are facing. Asymmetries in selective pressure may have several sources. In general, mutualism have distinct consequences for fitness components. For instance, one pollinator species, which visits a flower, obtain food, been nectar or pollen, affecting the survivorship and growth. On the other side, the benefit for the plant is reproduction, once the pollinator will disperse the male gametes (pollen) or bring then to the female gamete (ovule), affecting the reproduction for the plants. Moreover, mutualistic interactions are embedded in networks of interactions, where another source of asymmetry may play. For instance, one species which fruits are are dispersed by only one bird have a high degree of dependence on this disperser. In this way, this bird represents an important source of selective pressure for the plant traits involved on the interaction. However, if this plant dispersers its seeds through several birds, the selective pressure of just one bird on the plant will be smaller and asymmetric. In this thesis, I have investigated the consequences of the described asymmetries, respectively selective asymmetry and specialization asymmetry to the coevolutionary dynamic in mutualisms. I used literature review with mathematical modeling, network theory and empirical data to build up about how asymmetries in coevolutionary processes may affect phenotypic patterns. This thesis is composed by three chapters. The first is composed by a literature review, in which I looked for sources of selective asymmetries on traits and individuals, specifically for pollination. In the second chapter I used mathematical coevolutionary models to explore structured asymmetry on the interacting guilds and how evolutionary effects would cascade. Finally, the third chapter was developed in South Africa and we investigated the one fitness pathway from hermaphrodite plants. To test two competing hypotheses, I realized experiments tracking pollen deposition on pollinator body. In conclusion, mutualistic interactions are mediated by several traits, which can be in a gradient from highly asymmetrical to symmetrical selection. Each trait contributes distinctly to the efficiency of the interaction and some of them may be evolving more symmetrical than others. We also found that the evolutionary effects network structure changed in cases of asymmetry, affecting directly trait convergence for each guild. Lastly, we observed evidence of pollen layering on

pollinator body, indicating that flowers may be competing for space in pollinator and that male fitness can have an important impact in coevolution plant-pollinator.

Introdução geral

A natureza está repleta de padrões encantadores. Em especial, acoplamento de características em espécies interagentes, que integram um mesmo sistema, têm atraído a atenção de naturalistas há séculos (Schemske, 2010). Este padrão, conhecido como acoplamento fenotípico, é bastante recorrente em mutualismos, *i.e.* interações que resultam em benefício mútuo para os indivíduos de espécies diferentes que interagem (Jordano, 1987). Exemplos empíricos são a correlação entre o comprimento de tubos de flores e de probóscides de moscas (Anderson and Johnson, 2008); ou entre tamanhos de frutos e abertura de bicos de aves que os consomem (Jordano, 1995). O reconhecimento destes padrões instiga muitas perguntas na ciência, que busca uma maior compreensão sobre como processos ecológicos e evolutivos moldam características das espécies, levando à convergência ou divergência fenotípicas em determinados contextos (Guimarães et al., 2011; Thompson, 2009). Para compreender a ecologia e a evolução de características de espécies interagentes é preciso combinar conhecimento sobre história natural e sobre os processos que atuam conjuntamente moldando os fenótipos das espécies. Nesta tese me propus a estudar como mutualismos, geram mudanças fenotípicas evolutivas e influenciam padrões e processos ecológicos. Para tal, realizei uma revisão de literatura não sistematizada focando em interações de polinização para compreender como variações no benefício obtido pela interação podem alterar a dinâmica coevolutiva. Ainda, investiguei se características que possuem funções distintas podem variar na simetria da força de seleção. Posteriormente, com o auxílio de um modelo matemático coevolutivo (Guimarães et al., 2017), simulei a evolução de características que mediam interações mutualistas sob diferentes cenários de assimetria. Por fim, realizei um experimento a fim de contribuir no entendimento de como a aptidão masculina pode influenciar a ecologia e a evolução de sistemas de polinização.

Como mutualismos aumentam o valor adaptativo de ambos os indivíduos que interagem, estas interações permitem que diferentes espécies possam impor pressão de seleção recíprocas entre si (Janzen, 1980). Para que os organismos possam evoluir por seleção natural é essencial que essa variação em seu valor adaptativo seja genética, ou ao menos parte dessa variação precisa ser herdável (Pujol et al., 2018). A reciprocidade na pressão de seleção pode desencadear co-adaptação e como consequência co-evolução se as características envolvidas na interação possuírem variação genética herdável (Thompson, 1989). Contudo, é necessário olhar para a interação e o benefício obtido através dela (Bronstein et al., 2006). Especificamente é importante verificar como o que é obtido com a interação influencia o valor adaptativo final daqueles indivíduos. Por exemplo, em uma relação de polinização, a planta se beneficia por meio da interação pois os animais carregam os gametas das plantas entre indivíduos facilitando a reprodução, enquanto o animal se beneficia pois obtém recursos das plantas ao consumir pólen ou néctar (Kiers et al., 2010). Uma compreensão mais profunda sobre o impacto da interação para o valor adaptativo de cada espécie nos permitirá verificar a magnitude e a taxa de mudança evolutiva das populações. De fato, é sabido que interações mutualistas podem variar geograficamente, de maneira que em alguns lugares ocorre um benefício mútuo mais simétrico do que em outros lugares (Medeiros et al., 2018). Thompson (2005) propôs que a variação geográfica nesta reciprocidade das interações é possivelmente um dos principais motores da tamanha diversidade na natureza. Porém, quais são os fatores que influenciam a simetria (em força) da seleção natural em mutualismos? Como tal assimetria pode estar estruturada on species traits? Quais são as consequências de tal assimetria para o processo coevolutivo? Neste trabalho, exploramos as fontes de variação na simetria de interações mutualistas em três escalas: i) dentro dos indivíduos - diferentes características que participam da interação sofrem diferentes forças de seleção; ii) no nível de especialização da espécie na rede, que definem a probabilidade de pressões seletivas estarem alinhadas ou conflitantes;

iii) nas interações entre guildas, onde a simetria é fortemente influenciada pelo componente do valor adaptativo influenciado na interação (*i.e.* sobrevivência x reprodução) e, para muitas redes de interação, está estruturado nas guildas tróficas (Ollerton, 2006).

A variação na simetria dentro dos indivíduos, ocorre entre diferentes caracteres, uma vez que muitas das características que mediam interações mutualísticas são características quantitativas (também chamados de características complexos). Essas características são complexas pois são influenciadas por muitos genes e também pelo ambiente (Assis et al., 2016). Soma-se a isso o fato de que, em muitos casos, mais de um dessas características mediam a interação (Thompson et al., 2013). Diferentes características irão contribuir para o incremento no valor adaptativo por meio da interação de maneiras distintas (Opedal, 2021). Por exemplo, as características reprodutivas da flor influenciam o benefício, de uma interação com um polinizador mais do que suas características atrativas, estando sob força de seleção distinta. Contudo, estas características não são livres para evoluírem independentemente entre si (Berg, 1960). Estas características possuem função múltiplas e/ou desenvolvimento compartilhado com outras características, e portanto, apresentam muitos genes compartilhados, com efeitos pleiotrópicos o que leva a algum grau de correlação entre as características (Caruso, 2004). A equação multivariada de resposta à seleção descreve como poderíamos esperar que tais características evoluam diante de uma determinada pressão evolutiva, dada essa não independência dos diferentes caracteres (Lande, 1979). Como diferentes características estão sob pressão seletiva distinta (Opedal, 2021), mesmo que elas participem afetem a mesma interação, podemos esperar contribuições distintas da interação para a trajetória evolutiva das espécies. A variação no gradiente de seleção em cada característica e, portanto, na força evolutiva fará com que se gere uma assimetria na reciprocidade da interação. Um exemplo desta assimetria é o caso de cores em flores, as quais têm a função primária de atração de

polinizadores (van der Kooi et al., 2021). Enquanto o oposto não é verdade para a visão em polinizadores, que utilizam a visão para inúmeras outras funções essenciais para sua sobrevivência e reprodução, como encontro de parceiros, retorno aos ninhos e fuga de predadores (Van Der Kooi et al., 2021). Por outro lado, características vinculadas ao acoplamento do polinizador com a flor e o acesso ao recurso, como comprimento de um tubo floral e a probóscide de um polinizador possivelmente estão sobre seleção muito mais simétrica (Anderson and Johnson, 2008). Tanto o comprimento do tubo floral, quanto o comprimento da probóscide são características com função primária de mediar a interação e ambas afetam a eficiência da interação, com potencial de influenciar o valor adaptativo dos indivíduos da mesma maneira. Assim, algumas características envolvidas na interação são passíveis de um processo coevolutivo simétrico, enquanto outras características, também envolvidas na interação, estarão sobre um processo coevolutivo muito mais assimétrico. Contudo, vale lembrar que nenhuma destas características está livre para evoluir independentemente (Armbruster et al., 2014). De tal modo que pressão seletiva para aumentar uma característica que permite o acesso ao recurso pode gerar mudanças evolutivas em outras características também, como as reprodutivas. A função de diferentes características das espécies que participam da interação e o potencial delas em coevoluir de forma simétrica foi explorado no primeiro capítulo desta tese.

O segundo ponto foca na interação entre as espécies e como a estrutura da rede de interações pode influenciar o processo evolutivo, com especial atenção para as assimetrias na quantidade de espécies parceiras (*i.e.* especialização) (Jordano et al., 2003). A estrutura de redes mutualistas varia entre diferentes tipos de mutualismos e também pode variar espacialmente e temporalmente (Bascompte and Jordano, 2007). Contudo, alguns padrões são observados e quantificados para os diferentes tipos de mutualismos. Por exemplo, redes de polinização com frequência apresentam espécies que interagem com múltiplos parceiros, enquanto estes parceiros interagem com poucas espécies (Delmas et

al., 2019). Outro padrão estrutural observado em redes de polinização empírica são grupos de espécies que interagem frequentemente entre si, formando módulos (Dupont and Olesen, 2009). Estas estruturas das redes de interação exercem grande influência no processo coevolutivo, podendo representar a partição do gradiente de seleção mutualístico para cada espécie, bem como caminhos que podem permitir a propagação de efeitos evolutivos indiretos (Guimarães, 2020). Efeitos evolutivos indiretos aqui se referem a mudanças evolutivas geradas em uma espécie através de uma interação indireta (Guimarães et al., 2017; Ohgushi, 2005). Além disso, sabemos que as interações entre as espécies não ocorrem de forma aleatória. Na verdade, determinadas características exercem um papel muito importante no estabelecimento da interação, por exemplo, atuando como barreiras ou como acopladoras na interação (Santamaría and Rodríguez-Gironés, 2007). As características que atuam como barreira são aquelas que selecionam quais espécies poderão interagir. Por exemplo, algumas plantas produzem metabólitos secundários que repelem alguns animais que visitariam a flor (Stevenson, 2020). Já as características que geram acoplamento são aquelas que acabam por aumentar o benefício da interação para aquele par de espécies. Por exemplo, espécies que possuem tubo de flor longo tendem a acumular mais néctar e, apesar de moscas de probóscide longa poderem visitar flores com tubos curtos, elas visitam as flores de tubo longo por obterem mais recurso por visita (Minnaar et al., 2019b). Estes padrões são gerados por processos (co)evolutivos e ecológicos e influenciam a estrutura da rede (Santamaría and Rodríguez-Gironés, 2007). Para redes de polinização, especificamente, a variação na riqueza de espécies para animais e plantas pode afetar os padrões de interação e quais seriam as potenciais espécies a propagar os efeitos indiretos em uma comunidade. Este ponto também foi explorado no primeiro capítulo desta tese.

O terceiro ponto foca na escala das interações estruturadas por meio de guildas, onde a simetria é fortemente influenciada pelo benefício obtido na

interação e que afetará quão forte a seleção natural pode ser para cada espécie envolvida (Kiers et al., 2010). O serviço/recurso trocado em muitos mutualismos não tem necessariamente a mesma importância para todos os organismos envolvidos, ou seja, alguns organismos recebem serviços/recursos muito mais valiosos dos que executam/entregam a seus parceiros (Kingsolver et al., 2012). Dois exemplos são as interações de dispersão de sementes e de polinização, que consistem na troca de um serviço (e.g. dispersão de embriões para lugares com maior potencial de sobrevivência ou dispersão de gametas) por recursos (e. g. alimentos, como néctar ou polpa). Em ambos os tipos de mutualismo, para a planta a interação representa o incremento da prole (reprodução direta). Para os animais, no entanto, a interação aumentará a chance de sobrevivência, o que, indiretamente aumenta a chance de reprodução, que pode ou não se concretizar. Esta assimetria está estruturada entre os diferentes níveis tróficos envolvidos na interação e pode ser um fator determinante para a emergência dos padrões de convergência e divergência nos traços de uma comunidade, dependendo do papel que essa característica desempenha na interação(Guimarães et al., 2011; Ollerton, 2006). O fato dessa assimetria estar estruturada em redes de interação bipartida (*i.e.* espécies dentro de um mesmo nível trófico não interagem entre si) altera a propagação de efeitos evolutivos na rede mutualista, explorado no segundo capítulo desta tese.

Por fim, no caso de coevolução em sistemas de polinização onde as plantas são hermafroditas, existe uma expectativa de que os diferentes componentes da aptidão (feminino e masculino) contribuam de maneira assimétrica para a dinâmica coevolutiva entre planta e polinizador (Moore and Pannell, 2011). A razão de tal assimetria é o fato de que a produção de gametas masculinos é muito maior do que a produção de óvulos. Desta maneira, o número de sementes totais geradas pela função feminina seria saturado rapidamente, uma vez que o número de óvulos é sempre baixo. Contudo, grãos de pólen tem o potencial de fertilizar inúmeros óvulos(Minnaar et al., 2019a). Por tal motivo, espera-se que

plantas capazes de evoluir estratégias para uma dispersão mais eficiente do pólen, conseguiriam produzir mais descendentes o que representaria um incremento importante para o valor adaptativo total de um indivíduo (Moore and Pannell, 2011). Por esta razão, inclusive, observa-se associação entre características atrativas e investimentos maiores na produção de gametas masculinos (Paterno et al., 2020). Contudo, sabe-se muito pouco sobre a movimentação do pólen e seu sucesso em fertilizar novos óvulos(Anderson and Minnaar, 2020). Por este motivo, realizei experimentos com pássaros polinizadores e flores que são visitadas por eles a fim de verificar se a competição por espaço para deposição de pólen no polinizador contribuiu para a evolução de estratégias de fertilização (*e.g.* impedimento de deposição de novo pólen e cobertura do pólen prévio). Sabe-se que existência de competição por espaço no polinizador pode atuar como força evolutiva para a evolução de características específicas das flores (Muchhala and Thomson, 2012). Porém nosso conhecimento atual é voltado à competição interespecífica. No terceiro capítulo eu virei a ocorrência de competição intra-específica.

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Coevolution in traits mediating pollination: the relative roles of asymmetries in traits roles, reciprocal selection strength and network structure

Pamela C. Santana[1]; Ana Paula Aprígio Assis[1,2]; Paulo Roberto Guimarães Junior[1];

1 Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil
2 Departamento de Genética, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

Abstract

Pollination interactions are mediated by a suite of species traits that affect individual fitness, having the potential to impose reciprocal selection and generate coevolutionary dynamics. The relative strengths of selection can vary depending on the trait, the degree of benefit from the interaction and the specialization. In this review, we focus on three sources of selection asymmetry in pollination interactions that may impact coevolutionary dynamics: *i)* asymmetries in some traits involved on the interaction and with asymmetric contribution to interaction efficiency; *ii)* asymmetries in selection strength, as a consequence on how much benefit an individual gains with the interaction; *iii)* asymmetries in specialization, as individuals might interact with a different number of mutualistic partners from different species, which may lead to differences in the partitioning of the total selective pressures a given species receives. Selection asymmetries may change the rate and magnitude of trait evolution for each interacting species and affect trait matching among species. In this review we propose that the coevolution in pollination interaction occurs as a two-step process. First, the flower recognition by the pollinator and its attraction to the flower, which might be an asymmetrical selective pressure. Second, the matching between the morphology of the flower and the morphology of the digestive apparatus of the pollinator, which may be under a symmetrical selective pressure. In a broader scale, asymmetries in selection strength may be structured in the trophic guilds as distinct fitness components are impacted by the interaction. Because evolutionary effects may cascade through the network of interacting species, the phenotypic patterns at the community level might be influenced by these asymmetries. Lastly, because pollination networks are often nested and highly connected, indirect effects might play an important role in trait evolution. Therefore, selection asymmetries may determine which traits undergo stronger coevolutionary change and generate trait convergence for specific functional traits in each guild of species.

Introduction

Plant-pollinator interactions are mediated by several evolutionary and ecological processes (Bronstein 2015). These processes determine species' phenotypes and affect species diversification (Bascompte, 2019; Bronstein et al., 2006; Cook and Rasplus, 2003; Schemske, 2010). For over a century, scientists have hypothesized and studied patterns of variation in traits mediating these interactions in ecological communities. Two patterns are very common: (1) trait matching between interacting species (*e.g.* Dalsgaard et al., 2021; Newman et al., 2014) and (2) trait convergence within a trophic level (Anderson et al., 2014; Anderson and Johnson, 2009). Trait convergence is generated as a response to selective pressures favoring traits to be similar between unrelated species. Patterns of trait similarity are indeed a key aspect of pollination systems, leading to the syndrome hypothesis, in which trait similarity emerges as a consequence of selection favoring convergence of traits among plants that share similar pollinators (Dellinger, 2020). For instance, phylogenetically unrelated plants that are visited by long proboscis flies often present flower tubes (Anderson and Johnson, 2008). Trait matching between plants and animals, in turn, may be generated by the reciprocal selective pressures between interacting species. Patterns of trait matching are widely spread in nature (Figure 1). For instance, the length of a hummingbird's bill often matches the corolla length of the plant (Dalsgaard et al., 2021). Our understanding of the ecological and evolutionary processes shaping traits that mediate pollination interactions has greatly advanced, however, the role of asymmetries in the ecological and

(co)evolutionary effects of the interaction for each partner in generating these patterns is still unclear.

Mutualistic interactions affect fitness of both interacting individuals, leading to the notion of reciprocity in ecological and evolutionary consequences of mutualisms (Bronstein 2015). The reciprocity in selection may generate coevolution, reciprocal evolutionary changes in the interacting species (Janzen, 1980, 1966; Thompson, 2009). Coevolution is considered a key process in shaping trait matching in mutualisms (Baker et al., 2010; Benkman et al., 2010; Pellmyr, 2003; Thompson et al., 2013; Zhang et al., 2013). Trait matching is a pattern in which traits from different species are tightly aligned (Anderson and Johnson, 2009; Pauw et al., 2017 - Figure 1). Coevolutionary models of mutualisms suggest that evolution, through trait matching as selective source, generates trait convergence in the same guild (Guimarães et al., 2011). Trait convergence within a trophic level may be a consequence of been embedded in networks (Guimarães et al., 2017). Mutualistic coevolution can also generate coevolutionary arms race dynamics, as in the classic example of Darwin's (1862) between the longspurs in Madagascan star orchids (*Angraecum sesquipedale*) and the long tongues of their hawkmoth pollinators (*Xanthopan morgani praedicta*). Here, moths with longer proboscides than the average spur length in the population will access the most nectar. However, plants with spur lengths longer than the average moth proboscis will have increased pollen receipt. The escalating dynamics generate traits match because the evolution of one trait triggers the evolution of the other and *vice-versa* (Wallace, 1867; Nilsson, 1988; Anderson and Johnson, 2008). However, it is often difficult to distinguish if trait matching is a consequence of a mutual co-adaptation between the mutualistic partners or just one species adapting unilaterally to its interacting partner or either a gradient between these two processes (Anderson 2015, Nuismer et al., 2022).

The multivariate response to selection equation explicitly states that evolution through natural selection is dependent both on the: 1) the ability of

traits to evolve and 2) strength and direction of selection - the linear selection gradient vector (Lande, 1979). The first component, the genetic structure of the population, is responsible for allowing evolution by natural selection to happen, while the second one is responsible for generating coevolution. Selection has indeed been observed acting on traits mediating mutualistic interactions in natural populations. There are many examples where the focus of selection has been on one trait such as narrow and deep corollas of flowers, which are matched by the proboscis lengths of long tongued fly pollinators (Anderson and Johnson, 2009); the bill lengths of nectar-feeding birds (Dalsgaard et al., 2021); or the tongues of bees (Darwin, 1862). However, for coevolution to occur, selective pressures must be reciprocal, but they need not have symmetrical (equal) in strength. When reciprocal selection is strong enough for populations of both species to respond, we should expect reciprocal evolutionary changes, but not necessarily at the same rates and magnitude (Herrera, 1985, Figure 2).

Figure 2 - Examples of trait matching in pollination interactions: a) A sword-billed hummingbird (Ensifera: Trochilidae) feeding from a passionflower (Passiflora mixta: Passifloraceae) in Ecuador. Photo from (Pauw, 2019 - https://doi.org/10.1146/annurev-ecolsys-110218-024845. b) A long tongued fly (*Moegistorhynchus longirostris*) feeding on *Lapeirousia anceps.* Photo from (Minnaar et al., 2019 - https://doi.org/10.1111/nph.15971. c) Female bee with long legs (*Rediviva longimanus*) collecting oil on long spurs from *Diascia whiteheadii.* Photo from (Pauw et al., 2017 - http://dx.doi.org/10.1098/rspb.2017.1707

Mutualistic interactions involve the exploitation of resources or services that often markedly differ between interacting partners (Ollerton, 2006). In pollination, plants benefit by acquiring a service, namely the dispersal of gametes

(*i.e.* pollen), while animals usually benefit by acquiring resources that provide nutritional gain (*e.g.* carbohydrates, inorganic nutrients, and water) (Ollerton, 2006) (Figure 2a). Therefore, in this type of mutualism (called services-by-resources mutualism), the interaction impacts different life-history components, and consequently, the fitness gain is likely to differ for each interacting species (Baker et al., 2017; Kingsolver et al., 2012, 2001). In addition, plant-pollinator interactions are the consequence of conflicting interests between plants and pollinators (van der Kooi et al., 2021). Plants attract pollinators to obtain sexual reproduction. On the other side, pollinators are searching for rewards important for their survivorship. Therefore, the interaction itself can result in a diverse gradient of outcomes ranging from mutualistic to parasitic interactions (Bronstein, 1994). In addition, asymmetries related to how many functions a given trait has to perform for one organism will ultimately lead to a lower potential for symmetrical selective pressure on these traits, due to conflicting selective pressures. In this review, we focus on the section of such gradient in which the fitness effects are positive for both species (*i.e.* the interaction is a mutualism), but in which the fitness benefits may translate into strongly asymmetric to symmetric strength of selective pressures for the traits mediating mutualistic interactions. The differences in the strength of selection may affect coevolutionary process (Benkman, 2013).
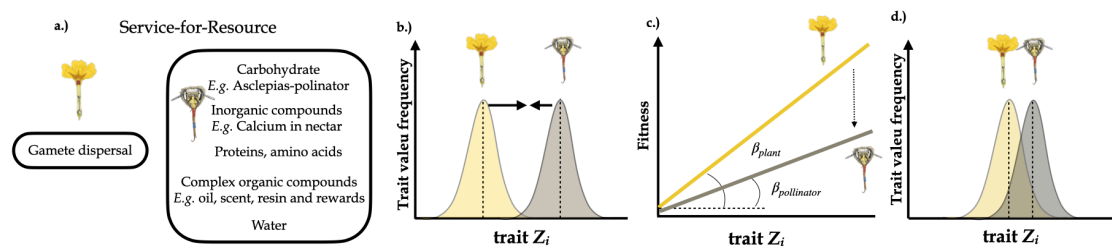
Figure 1 - Pollination is a mutualistic interaction in which each partner may impose selection on the other, as the interaction impacts fitness for both species. a) Those fitness gains might differ from one interacting partner to the other, as the plants gain a service (gamete dispersal) and animals gain resources. b) The graph shows the expectation of a population under directional selection for trait matching. The normal distribution represents the distribution of a quantitative

trait in plants (yellow) and animals (grey). Pollination may lead to coevolution, in which trait values for both populations will evolve in response to reciprocal selective pressures. Arrows indicate the direction of selection and arrow's length indicate the strength of selection. Because fitness benefits for plants are higher (plant traits impact on the interaction are directly connected to reproduction), the selection will be stronger in plants (longer arrows). c) The link between fitness function and trait values will directly generate differences in the selective pressures, demonstrated as distinct selection coefficients (β) for plants and pollinators. The strength of selection β is represented by the angle formed in the linear relationship between the trait and fitness. Selection may generate changes in trait evolution for each species but the selection asymmetry may generate differences in the amount of evolutionary change for each species. d) In this way, we expect that both species will undergo reciprocal phenotypic changes leading to trait matching (increasing the overlap between trait distribution), but plants may undergo more evolutionary change than animals in each generation.

Coevolution of plant and pollinator traits assumes that directional selection is the main driver of phenotypic evolution for traits involved on the interaction for both species in a more or less synchronous and reciprocal way (Janzen, 1980, Thompson 2005). However, after over a century of looking for evidence of coevolution, we still are on the infancy of comprehending how much the strength of the selection varies between interactors and how it affects multiple traits involved in the interaction. In this study, we provide an overview of the current understanding of trait (co)evolution in pollination networks and propose hypotheses for where coevolution may be playing an important role in shaping traits. We will focus on the role of asymmetry in selection reciprocity for the coevolution of a set of traits mediating the interactions of pollination mutualisms. We can decompose the reciprocity in two main components (Figure 3): *i*) the symmetry in the strength of selection, which will be largely affected by trait function in each interacting partner and the fitness benefits from the interaction and may represent a partial component of the selection differential; *ii*) the symmetry in the combined selection generated by all interacting species. This can be best described through the network of interactions and will compose the total biotic selection differential for each species. We will discuss the effects of the variation in the asymmetry in both of these aspects.
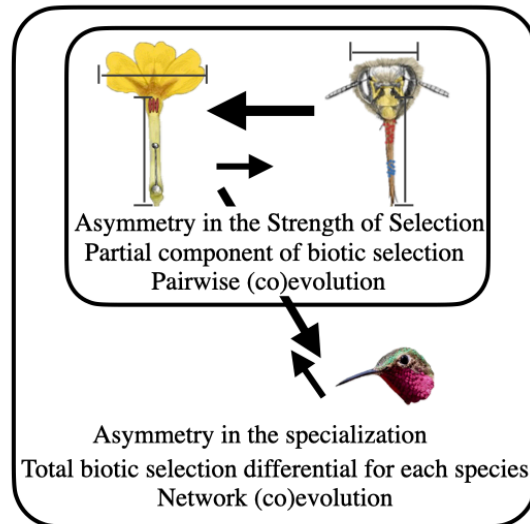
Figure 3 - Asymmetry in selective strength in pollination interactions decomposed into two main components. The first one is the fitness gains from the interaction, which might differ from one interacting partner to the other (smaller box). Arrows sizes represent the strength of selection. The second component integrates the whole set of interactors a species has. In this example, the flower interacts with the hummingbird and the bee. So, the total biotic selection differential for the flower will be composed by the selection imposed by both pollinators. However, the biotic differential of selection will be only the flower for the bee and the hummingbird.

Impacts of selective asymmetry on traits mediating the interaction

Pollination interaction is mediated by a functional module, composed of a set of traits that together perform a function in the organism (*e.g.* flower and the birds beak - (Dehling et al., 2016). A functional module, in turn, may be an evolutionary module, *i.e.* sets of phenotypic traits evolving in a coordinated way, because they are under joint selection and share an underlying genetic architecture (the case of most pollination systems) (Assis et al., 2016). Even though several traits are involved in the interaction, it does not mean that they are experiencing the same selective strengths from the mutualistic partner (Opedal, 2021). Studying coevolution focusing on the role of different traits will allow us to disentangle the role of different evolutionary processes in shaping the

traits that mediate the interaction. In the last decade, coevolution and unilateral evolution (*i.e.* only one species is under directional selection) were used as competing explanations for phenotypic evolution of interacting organisms (*see* van der Kooi et al., 2021). However, a multi-trait perspective may allow us to comprehend which traits are under symmetrical selective pressure (and may be coevolving), which ones are under asymmetrical selective pressure (and may be under unilateral evolutionary changes). Under this perspective, coevolution and unilateral evolution may jointly occur on the same species, in the same interaction, but in different traits involved in the interaction. Through the community perspective, we could expect that characteristics which are under more symmetrical selective pressure may show a distinct phenotypic pattern than the characteristic that are under an asymmetrical selective pressure. Moreover, at a broader scale, the differences in fitness benefits may generate structured evolutionary changes among interacting (*2nd chapter of this thesis*).

(Co)evolution of traits mediating pollination in plants

The functional module that mediates the pollination interaction on the plant side is the flower, the reproductive structure of Angiosperms (Sauquet & Magallón, 2018). Its biological role is to facilitate sexual reproduction and, for multiple species, to promote outcrossing (Ollerton et al., 2011). Most flowers are composed of three whorls (sets of traits) involved in different functions. From the periphery to the center, a flower has: 1) the perianth, composed by one or two sets of sterile organs, which contains sepals that commonly protect younger organs or may, together with the petals, attract and guide pollinators; 2) the androecium, composed by one set of male reproductive organs, which produces and exports pollen (male gamete); 3) the gynoecium, a set of female reproductive organs which produces the ovule (female gamete), receives the pollen, and protect the seed while developing (Endress, 2011). Seed production depends on

a successful transfer of pollen from anthers to stigmas, which will depend on pollinator attraction and fitting (Minnar & Anderson, 2019), except in the cases of apomixis (*i.e.* asexual reproduction without fertilization). In some cases, self-pollination will occur, when the pollen is deposited on the stigma of the same flower or in a flower of the same individual, but seed quality is much higher with outcrossing.

Pollinators are recognized to be the primary drivers of floral trait evolution (Bradshaw, H. D.; Schemske, 2003; Harder, L.D.; Johnson, 2009; Schemske and Bradshaw, 1999). The selection imposed by pollinators may explain the astonishing diversity of floral structures, even among closely related species (Schiestl and Johnson, 2013). Pollinators may exert selective pressure on flower traits via two main mechanisms: *i)* **Attractiveness:** traits such as display size, colour, scent, nectar and shape (Aguiar et al., 2020; Whitney and Glover, 2007; Wright and Meagher, 2004), which may evolve to attract more pollinators (Newman et al., 2012); *ii)* **Matching** (flower-pollinator fit)**:** traits such as floral orientation, floral depth (Anders Nilsson, 1988; Campbell et al., 1996), style length (Armbruster, 1991; Newman et al., 2014)and corolla flare (Aigner, 2004) which may evolve to enhance the efficiency of pollen transfer between reproductive parts of plants and the specific morphological traits of their pollinators. However, some of these traits might play a role both in pollen transfer efficiency and attractiveness, like petal elaborations which may also mechanically enhance pollen transfer by providing grip or points of leverage (Whitney and Glover, 2007). A third, but rarely investigated mechanism of pollinator filtering, is the possibility that floral traits may have evolved as barriers to filter out less efficient pollinators, like some plant secondary metabolites which prevent some species of visiting a flower (Stevenson, 2020). It is important to keep in mind that in addition to the effect of pollinators, floral traits are subjected to an array of biotic (*e.g.* herbivory) and abiotic selective pressures (*e.g.* water loss, wind damage and nutritional constraints) (Campbell and Powers, 2015; Caruso

et al., 2017). The mechanistic understanding of the extent and causes of variation in natural selection can help us to disentangle suit of traits that may be under more symmetrical reciprocal selective pressure and which ones might be evolving under an asymmetrical selective pressure in the interaction, evolving mainly under other non-coevolutionary processes or abiotic sources of selection.

Opedal (2021) compiled selection estimates on floral and inflorescence traits and summary statistics from selection studies with temporal and spatial replication. He observed that selection on pollination traits is often strong and that the magnitude of variation in selection among functional traits varies. From the variation in selection strength magnitude in multiple traits involved in pollination, we may expect that plant traits for pollinator attraction traits are likely to be under highly asymmetrical selective pressure from pollinator. We may expect that because pollinator sensory traits should be strongly constrained by the many additional roles played by those traits (*e.g.* mate finding and predator avoidance (van der Kooi et al., 2021). Consequently, plant traits such as: color, brightness, contrast, size, pattern, iridescence, flower symmetry, tactile stimuli, scent composition (Kevan and Lane, 1985; Lunau and Maier, 1995) and pollinator traits such as visual system are all likely to be under highly asymmetric selective pressure, which may generate asymmetric coevolutionary dynamics. The functional mechanisms of each trait involved on the interaction may open venues for conflicting selective pressure and increase the chance of selective asymmetry in coevolutionary process. Considerable signal complexity exists within each of these sensory modalities and their importance for the interaction also varies, generating also variation in the degree of asymmetrical selection. The opposite should be true for flower-pollinator fit traits, which may be under a symmetrical selective pressure scenario, once the main function of the traits to both sides is related to an efficient interaction. Indeed, Opedal (2021) found selection to be stronger on flower-pollinator fit traits, which may be under higher symmetrical selective pressure and may vary more locally. This could explain

why we see patterns of geographical covariation so commonly between feeding attributes of pollinators and morphological fit traits on flowers (*e.g.* Anderson et al., 2014; Anderson & Johnson, 2008).

Another possible important source of variation in selection strength and direction, which may contribute to generate asymmetries, is the fact that plants are hermaphrodites in general, while animals are dioecious, implying that the total fitness of the plant will be composed by male and female components. Some flower traits may evolve mainly under male or female components of the fitness (Figure 4 - Anderson & Minnaar, 2020; Minnaar, Anderson, et al., 2019; Willson, 1979; *third chapter of this thesis*). For instance, the evolution of showy and attractive flowers to pollinators is connected to a higher investment in male function (Paterno et al., 2020). The reason for that is because the male component of fitness is harder to be saturated compared to the female one (Bateman, 1948). Therefore, by attracting more pollinators, plants may increase its fitness through fertilization of other flowers, while the female component can be saturated more easily (Willson, 1979). Because of this reason, Moore & Pannell (2011) proposed that the male component of the fitness contributes disproportionally to the coevolutionary process. The divergence in the contribution for total fitness is directly connected with pollen x ovule ratio (Harder & Barret, 2006). It is also connected with mating systems (selfers vs outcrossers) (Pannell, 2015). How much a plant depends on a pollinator for reproduction and may imply in asymmetries of selective pressure between species. While, in animals, selection due to mutualism will be mainly affecting only one pathway in fitness (male or female) (Minnaar et al., 2019a).
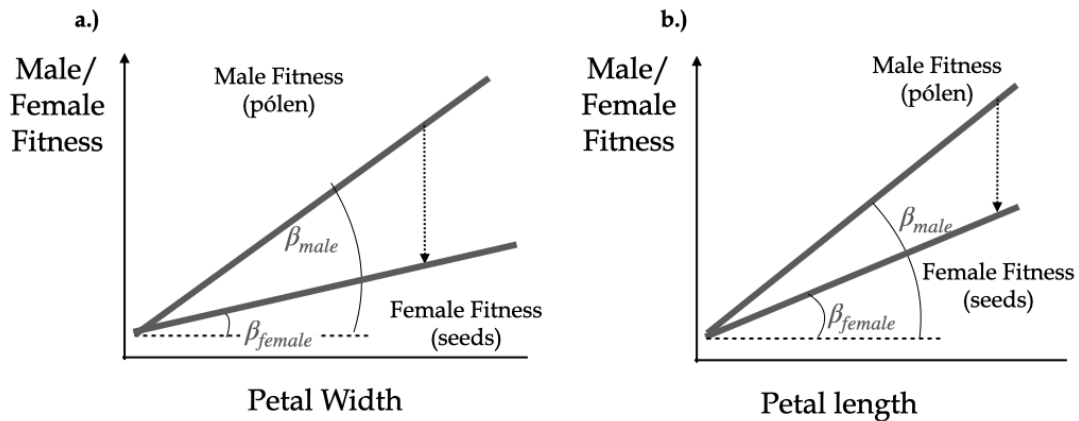
Figure 4. Graph showing how trait evolution might be impacted by the two components of species fitness. For instance, petal length and petal width might influence male fitness equally, with the increase of the trait resulting in more pollen deposition on pollinators (male fitness line in a and b). However, petal width may influence female fitness in a different way that petal length does, if petal length affects pollen deposition from pollinator on stigmas differently than petal width (female fitness line in a and b). β strength is represented by the slope.

(Co)evolution of traits mediating pollination in animals

From the animal perspective, several functional traits are involved in pollination interactions, specifically sensory capabilities related to cognition and behavior (nervous system and visual, olfactory, and tactile mechanisms) and feeding attributes (morphology and physiology) (Chittka and Thomson, 2001). Flowers present stimuli to attract pollinators while animals have to detect them (Chittka and Raine, 2006). Pollinators rarely use a single cue but rather use multiple signals to find suitable resources. Again, we can see the interaction as a two-step process: first, the animal has to find the flower, secondly, it has to obtain the resources found within the flower. To find the flower, pollinators use floral signals (Wester and Lunau, 2017). However, their sensory capabilities are also under selective pressure of many other stimuli that also need processing (*e.g.* noise, stimuli transmitted by predators, potential mates, conspecifics, host plants, and the physical environment). Animal cognitive systems are under several

different and conflicting selective pressures other than the mutualistic interaction, which makes the relative contribution of mutualistic selection smaller for animals than for plants. This, ultimately leads to selective strength asymmetry related to the interaction. Therefore, the selection on the sensory traits of pollinators differ importantly from their attractive counterparts in plants (*i.e.* floral signals), which in most cases evolved with pollinators as the primary selective pressure (van der Kooi and Ollerton, 2020). The unilateral exploitation by plants of pollinator sensory bias may explain why some trait patterns observed in flowers are paralleled in the unrelated ecology of their pollinators (Schiestl, 2017). For instance, some female hummingbirds species look for red plumage in males, the color that is also common on hummingbirds' flowers (*e.g.* Herrera et al., 2008). Similarly, many bee-pollinated flowers have dark center patterns similar to the dark entrances to bee colonies (Biesmeijer et al., 2005). Chemically, many flowers emit compounds to attract pollinators which are also used in intraspecific communication: geraniol is both a component of floral scent and is also found in the Nasanov pheromone that honey bees use to mark flowers and colony locations (Schiestl, 2010). The potential highly asymmetrical selection on plant attractive traits may have evolved to adapt to pre-existing pollinator traits. Indeed, when we look into species' evolutionary history, it has been documented that important traits in mediating interactions for each species probably evolved in distinct times, as pollinators' sensory systems (van der Kooi and Ollerton, 2020). Reinforcing the adaptation by plants on pollinator sensory bias in an asymmetrical selective pressure scenario.

In contrast, plant selection on pollinator's feeding morphology and digestive physiology is likely much stronger than sensory capabilities (Shimizu et al., 2014), as many pollinators depend on pollen, nectar and/or floral oils for nutrition and survivorship (Roy et al., 2017). For *Bombus terrestris* specifically, it has been shown that preferences for specific nutrient contents and ratios impact its reproductive success, demonstrating a direct impact on fitness (Kraus et al.,

2019; Ruedenauer et al., 2020). In addition, *Euglossini* bees, *Ficcus wasps* and *Yuccas* moths*, which plants directly affect pollinator reproduction, may be under more symmetrical selection between partners. The more symmetrical the selective pressure between species in the interaction, the higher the chance of a stronger coevolutionary dynamics. However, an important consideration is that pollinator learning may allow them to bypass the coevolutionary dynamics associated with mechanical fit traits. For example, bees may learn to switch to a new species rather than have to adapt to their physical traits .

Asymmetric selective pressure due to multiple interactions

We have discussed so far, the underlying causes and consequences of asymmetry in the selection strength in multiple traits involved in the interaction and how it may generate asymmetry in the coevolutionary outcome. However, in the same way that interactions are mediated by multiple traits, traits are mediating interaction with several partners (Vizentin-Bugoni et al., 2014). Therefore, traits involved in the success of the mutualistic interaction are usually under several selective pressures from different species as most species interact with multiple mutualistic partners. Consequently, diverse selective pressures may sometimes be conflicting as a consequence of the multiple interactions. One way of comprehending the potential effects of the selection gradient partition is by analyzing the network of interactions (Guimarães et al., 2011). The network structure exemplifies how the degree of specialization varies and then we can predict how each interaction might affect selection (Phillips et al., 2020). Pollination networks are nested and strongly asymmetric (*i.e.* a species that interacts with a few partners often interact with partners that have several other interactions, implying an asymmetry on the dependence between partners) (Bascompte et al., 2006; P. Vázquez, Diego; Aizen, 2003). Thompson et al. (2017)

suggest that natural selection can shape coevolving species traits in ways that are fine-tuned to their local network of interaction. Coevolution within local networks can have both direct and indirect effects on trait evolution of the species involved. We will discuss these two paths below.

Direct effects from the interaction

The architecture of plant - pollinator interactions have been studied for a few decades now and some patterns have been consistent (Bascompte and Jordano, 2007; Olesen et al., 2006). Plant - pollinators networks are bipartite, meaning that species only directly interact with species from another guild and species within the guild cannot interact through direct interactions (Ollerton, 2006). The first asymmetry we can observe in the network structure is that each guild presents distinct species richness. For example, in plant-pollinator networks the species richness of pollinators are often almost three times higher than the species richness of plants (73 +/- 148 SD) against (26 +/- 43,5 SD) (Figure 5a). We could expect that the asymmetry (*i.e.* difference) in richness between the trophic guild would affect directly to the potential number of interactors partners, but not necessarily. Looking at empirical plant-pollinators network (N=148) we found some highly connected pollinator species (Figure 5b), but the main number of interactions for both trophic groups is very similar (Figure 5c) and much smaller than the maximum number of potential partners, which is the species richness of the partner's guild. The smaller species richness of plants compared to animals increases the chances of several plants interacting with the same pollinator species, turning some pollinators into a highly connected species (Jordano et al., 2003). Highly connected or super-generalist species are the ones that interact with several partners, as honeybees (Olesen et al., 2007), and they may intensify trait convergence in a community through direct interactions (Guimarães et al., 2011). Is possible that generalist pollinators interact with a guild of plant species spaced temporally across their lifespan which may

generate a pattern of interacting with multiple individual partners throughout their lives (Maruyama et al., 2014). As a consequence, fitness effects from interaction will be likely very asymmetric between the pairs of interacting partners. Indeed, plant - pollinator networks often are nested, as specialists tend to interact with subset of species that generalists interact with (Bascompte et al., 2006). As a consequence, most plant species become heavily dependent on a pollinator, while the pollinator does not depend as much on the plant, characterizing strongly asymmetric relationships (Bascompte et al., 2006; P. Vázquez, Diego; Aizen, 2003). Highly connected species of pollinators generates a source of specialization asymmetry between the trophic groups and splits the gradient of selection differently for the interacting partners, implying that selection strength from each pairwise interaction may be very asymmetric: the highly connected species is a strong source of selection to multiple partners but each of its partners is a weak source of selection on the highly connected species. In the case of pollinator guilds, in which a group of plants is pollinated by the same species, it is common to observe convergence in floral traits among the plant assemblage (Anderson et al., 2014; Fenster et al., 2004; Newman et al., 2014).
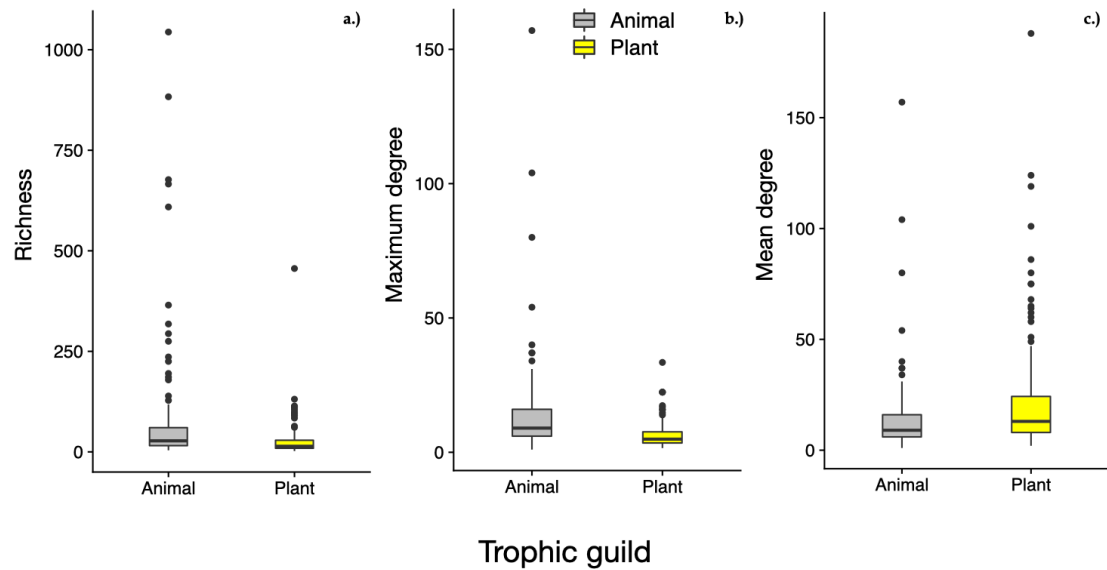
Figure 5. Patterns of interaction for species in each trophic guild. Data from 148 pollination networks available at https://www.web-of-life.es/. a) Boxplot showing the variation in species richness for each trophic group. b) Maximum number of interacting partners (degree) for each trophic guild. c) Number of interacting partners in average (Mean degree).

The architecture of mutualistic network is better known than the underlying mechanisms structuring it (Maruyama et al., 2014). An underlying explanation for plant-pollinator network architecture may be because interactions require a certain level of trait matching to be effective (Santamaría and Rodríguez-Gironés, 2007). Morphological and/or biochemical specialization effectively restricts the number of interaction partners (Vizentin-Bugoni et al., 2014). Olfactory signaling between a flower and a pollinator (Kite et al., 1998) and nectar composition and pollinator preference (Perret et al., 2001; Pyke et al., 2020) has been shown to play a functional role in determining plant-pollinator networks structure. Species that interact more frequently with each other might be under stronger coevolutionary dynamics, generating stronger coevolutionary changes between a few species than with the others (Watts et al., 2016). How specialized species are (*i.e.* number of partners) can be correlated with the level of biological intimacy (*i.e.* the degree of physical proximity or integration of partner taxa during their life cycles), but this relationship is not linear (Ollerton,

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2006). Intimacy and network specialization may be connected, implying a more symmetrical contribution to the biotic selection on the trait (Hembry et al., 2018). Therefore, the degree of specialization (*e.g.* number of partners) may strongly affect the coevolutionary dynamics of co-occurring species (Pauw et al., 2017; Shimizu et al., 2014). For instance, Garcia et al. (2022) found that the presence of bee pollination in addition to fly pollination generated plant communities with colour dispersion characteristics. Adding evidence that the pattern of selection may change by the presence or absence of some pollinator species (Caruso, 2000; Eisen et al., 2020). In addition to the number of partners, the efficacy of the pairwise interaction may be important in quantifying the potential for coevolution, because selection on flower phenotypes might differ depending on visitation rates or flower-pollinator fit (Pauw et al., 2017). For example, species that possess highly specialized flowers may be visited by more than one pollinator, but only one pollinator species be important for effective reproduction. Consequently, this unique pollinator may be the most important source of selection upon floral traits (*but see* Benkman, 2013). Even taking in count the efficiency of the interaction for the plant, asymmetry may still take place. If that pollinator visits several plant species for nectar, it is unlikely to strongly depend on any unique plant species. Consequently, selection by one plant species on a generalist pollinator is likely to be comparatively weak. For this pollinator, the adaptive landscape and consequently the favored phenotype will be determined by a combination of the selective pressures exerted by the several different plants it interacts with.

Another potentially important factor structuring networks is the occurrence of "barrier traits" which may create "forbidden links" in the network(Vizentin-Bugoni et al., 2014). For example, pollinators with short tongues may be unable to acquire nectar from long tubed flowers, preventing the interaction from occurring (Nuismer et al., 2013). While complementary traits would (co)evolve under the most effective pollinator selective pressure, the

barrier traits would evolve to avoid the least effective pollinator, under antagonistic selection and competition between the pollinators. For instance, one hypothesis behind the evolution of red flowers is that bees cannot see them well (Chittka, 2017). Consequently, hummingbirds find more nectar when they visit this flower because of less competition from bees (Bergamo et al., 2016). Red is also an important sexual recognition color used by female birds, perhaps explaining why flower color did not evolve to a different wavelength that bees perceive poorly (de Camargo et al., 2019). (Co)evolution between the hummingbird and the flowers appear to play a role in floral color evolution but the presence of bees (*i.e.* the network interaction) also appears to be important. Diverse selective pressures do not necessarily mean they will cancel each other and prevent coevolution from occurring. Instead, they could in fact form complementary selection components resulting in stronger net selection on a trait (Campbell et al., 2014; Caruso, 2004, 2000; Caruso et al., 2019; Muchhala and Thomson, 2009). These selection forces may in fact make some pollinator combinations (*e.g.* hummingbirds and bats, Dellinger et al., 2019; Lagomarsino and Muchhala, 2019) more likely to evolve into stable bimodal systems (*i.e.* systems with intermediate adaptations to two distinct functional pollinator groups *sensu* Goldblatt & Manning, 2006) than others (*e.g.* hummingbirds and bees, Dellinger, 2020). The only situation in which a selective pressure is likely to cancel another completely is when the selection imposed by a mutualistic partner and an antagonist is similar, which may be the case of nectar robbery.

The network structure may allow us to disentangle which pairs of species have higher potential for (co)evolution and how the evolutionary effects may propagate to others species (Guimarães et al., 2011, 2017 - Figure 6a-b). The specialization asymmetry in networks effects can be summarized in a matrix nxn (where n is the total number of species interacting) where each column represent the total selective effect a species has on the species in the rows (Guimarães et al., 2017) (Figure 6c). By summarizing the selective pressure each species is imposing

and receiving, we can summarize the effects of the specialization asymmetry. And look at the network structure as the possible pathways for evolutionary effects to propagate. Pollination networks are characterized as a connected network, where some highly connected species make that through a direct or an indirect pathway, all nodes in the network are connected (Guimarães, 2020, Figure 7a). The fact that pollinator-plant networks are connected implies the indirect effects very important for plant-pollinator (co)evolution (Guimarães et al., 2017).
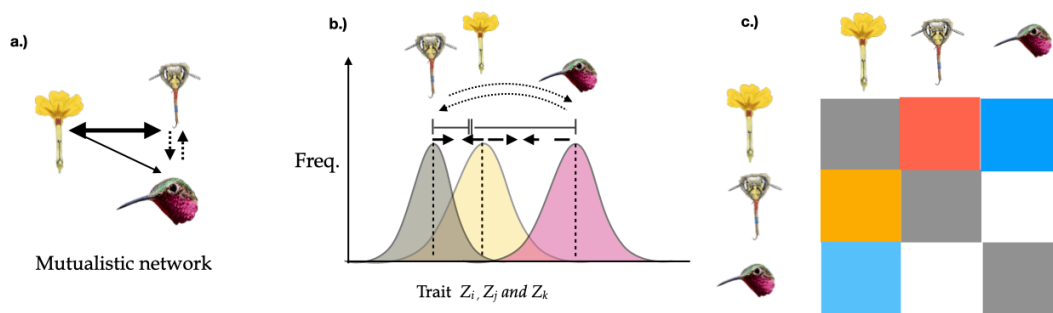
Figure 6. Hypothetical figure showing how network patterns of interaction could influence selection on interacting species and possible pathways to propagate: a) bee imposes stronger selection on floral traits than the hummingbird, indicated by the arrow sizes. In addition, the plant will exert a higher selective pressure on the bee than on the hummingbird; The arrows represent pairwise interaction. b) selection will lead to evolution of traits, however the magnitude of evolution will differ depending on the selective pressure. c) specialization asymmetries can be computed in a matrix (heatmap in c) in which each element represents the selection imposed by the species in the columns on the species in the rows, above the diagonal is the impact of the pollinators on the plants and below the diagonal the impact of the flower on the pollinators. In this example, plants are experiencing stronger selective pressure because they depend on animals to achieve reproductive success, and the opposite is not true. White regions in the heatmap represent no selection imposed, due to the absence of direct interaction between the species. The difference in colors on the matrix represents the strength of selection between plants and pollinators (warm colors represent higher values of selective pressure and cold colors represent smaller values). In this example, a second form of asymmetry is generated by the differences in how many species each species interacts with (specialization asymmetry). The ones that interact with more species will depend less on each individual interacting partner than the ones that interact with fewer species. As the flower interact with both pollinators, selection on it is imposed by both species. The opposite is true for bees and hummingbirds.

Indirect effects from an interaction

An important aspect of coevolution in networks is that trait evolution is influenced not only by the direct interactions, but it will also be affected by indirect selective pressures (Ohgushi, 2005). It means that the evolution of a species will also be affected by the interactions that the partners establish with other species that may create a cascade of evolutionary changes. For example, in a three-species network between a nectarless mimetic orchid (*Disa nivea*) and a highly specialized pairwise pollination mutualism (*Zaluzianskya microsiphon* pollinated by *Prosoeca ganglbaueri*), the spur length of the orchid is very similar to the tube length of the model and also to the proboscis length of its fly pollinator (Anderson et al., 2005). While the tube of the nectar producing model and the tongue length of the fly might be coevolving through symmetrical reciprocal selection, the mimic orchid cannot possibly exert selection for fly proboscis to match its spur length. Instead the trait matching represents a case of coevolution between pollinator and orchid, with indirect selection being imposed on the mimetic orchid by the nectar producing model (Anderson et al., 2005). This shows how indirect effects can represent important selective pressures that drive trait evolution in communities, highlighting the complexity of coevolutionary dynamics (Bergamo et al., 2017; Guimarães et al., 2017). Numerical simulations using coevolutionary mathematical models have shown that the higher the importance of mutualistic interaction to trait evolution, the higher the contribution of indirect effects (Guimarães et al., 2017). We also found that asymmetry in selective pressure can concentrate the contribution of indirect effects in one guild, generating trait convergence in only one guild (*second chapter of this thesis*).

Importantly, indirect effects could change the evolutionary trajectory of interactions or even buffer the effect of the asymmetry. To understand the complexity of this dynamic evolutionary scenario, we need to investigate the

impact of indirect effects on the coevolutionary process (Guimarães et al., 2017; Pires et al., 2020). The interaction linkages in a network will rearrange the adaptive landscape through the indirect effects (*see* Ohgushi, 2005). We can predict that species with few interactions, that are more dependent on the species they interact with, will be more affected by indirect effects than species which interact with more species (Guimarães et al., 2017). As the evolutionary outcome of species will be dependent of individual contribution of each species selection, species that interact with more species can cancel or potentialize the effect of a pairwise interaction. For instance, using Arroyo et al (1982) pollination network, we estimated the contribution of indirect effects each species have using Guimarães et al (2017) coevolutionary model. We found that species with less evolutionary change contributed more to the propagation of indirect effects (Figure 7b). Therefore, trait evolution will be affected by the outcome of adapting to different interactors and a cascade of indirect effects, but species do not contribute equally to the propagation of indirect effects.
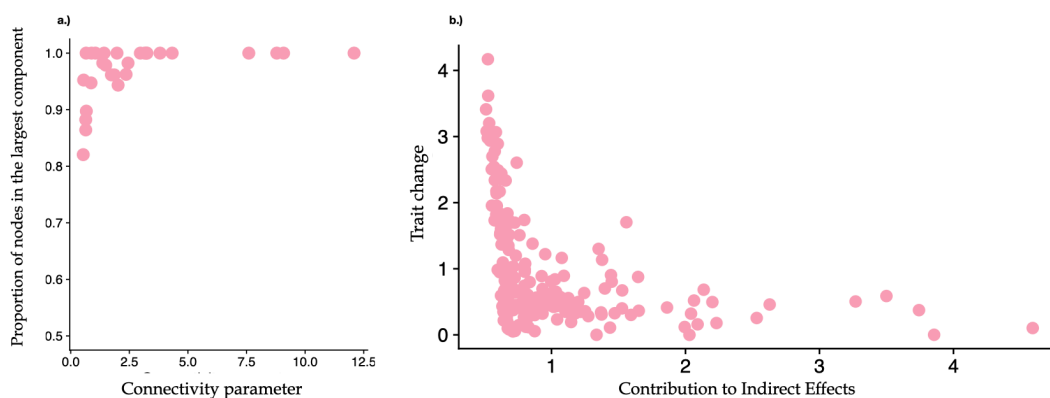
Figure 7. a) Structural phase of plant-pollinator networks. The x-axis represents the connectivity parameter, which is dependent on the distribution of interactions per species, and the y-axis represents the proportion of species connected by either direct or indirect pathways in the network. Pollination networks are at the supercritical phase, in which they are part of a giant component. The data includes 28 empirical networks. Adapted from Guimarães (2020). b) Using Arroyo et al 1982 pollination network and Guimarães et al. (2017) coevolutionary model, we estimated the contribution species have through indirect effects to the trait evolution of other species. We find that species that change less contribute more to the propagation of indirect effects.

Asymmetry in the coevolutionary process

The evolution of independent traits is better comprehended in pollination systems (Dellinger, 2020). However, pollination interactions are usually mediated by multiple quantitative traits, *i.e* traits genetically connected, and they are interacting with a suite of similarly genetically connected traits in a different organism (Benitez-Vieyra et al., 2019). The comprehension of the coevolutionary process in phenotypically integrated traits is still unclear (Thompson et al., 2017). Genetic correlations between traits impact populations' evolutionary trajectories in response to varying selective gradients, affecting coevolutionary dynamics (Assis et al., 2020; Assis and Guimarães, 2022; Nuismer and Doebeli, 2004). Theory predicts that life history traits tend to exhibit stronger genetic correlation and smaller genetic variances between traits than morphological traits (Hansen et al., 2011), Fisher 1930). As pollination interaction affects directly life-history traits in plants (*e.g.* number of seeds produced, timing of reproduction) but morphological traits for animals, it is likely to have drastic effects on coevolutionary dynamics and distinctly for each guild. Genetic correlations between traits and lower variance in life-history traits may lead to smaller evolutionary changes for those traits, even with stronger selection than the observed for morphological traits. The genetic architecture, trait correlations and the additive genetic (co)variance behind each trait, will define the potential response to selective pressures imposed by mutualistic partners, and whether selective pressures from mutualism will result in coevolution or not (Assis and Guimarães, 2022; Pujol et al., 2018). Therefore, even though we know selection may vary a lot, the coevolutionary process will only happen if species were able to respond to selection (Opedal, 2019).

An important aspect of the coevolutionary potential for pollination systems is the diversity of pollinators group, which varies greatly in their genetic (co)variance, directly affecting their potential for coevolution in the traits involved in the interaction. Pollinators can be: rodents, birds, moths, butterflies, flies, beetles, wasps and bees. For each of these groups patterns of phenotypic integration, varies a lot and we know it can impact coevolutionary dynamics and outcome (Assis et al., 2020). On the other side, we know that morphological traits in flowers are less tightly integrated than morphological traits in animals (Conner et al., 2014), opening an avenue for asymmetry in the rate and magnitude of coevolutionary process.

Lastly, pollination is directly connected to reproductive isolation for plants but is usually not for animals (Ramsey et al., 2003). Although it is well known that coevolution does not imply cospeciation (Thompson 2005), even though plant and pollinator speciation have been interpreted as an outcome of coevolution in the past (Cardinal and Danforth, 2013). Coevolution has been suggested as potential mechanism for co-speciation due to the existence of strong reciprocal specialization in some interactions, which would generate a co-radiation or co-diversification, resulting in mirrored phylogeny (Kiester et al., 1984). However, mirrored phylogenies may be generated due to other processes (*e.g.* geographical barriers that generate speciation concomitantly) and not necessarily due to coevolution (Cook and Rasplus, 2003; Pellmyr, 2003). Additionally, differential consequences of asymmetry in selective pressure may allow species to change at different rates and open opportunities for diversification in plants. We have evidence that pollinators can promote plant speciation (Gervasi and Schiestl, 2017) but the opposite is not true (Ramírez et al., 2011). Lagomarsino et al. (2016) found that key differences in biotic traits, including fruit type and pollination syndrome, confer enhanced diversification capacity among closely related species co-occurring in a rapidly changing environment. They also found an association with vertebrate pollinators and

increased rates of speciation via floral isolation indicating that distinct evolutionary processes may play in each side of a pollination system. For instance, some specialized orchids and their bee pollinators result in a one-sided evolution pattern (unilateral evolutionary process) where plants adapt to insects but not *vice versa* (Ramírez et al., 2011).

A full comprehension of (co)evolution needs to take into account jointly the roles of species' additive genetic (co)variance (phenotypic integration and modularity) and the strength, direction, reciprocity and symmetry of selection shaping complex traits in networks of species interacting (Figure 8 - Assis & Guimarães, 2022).
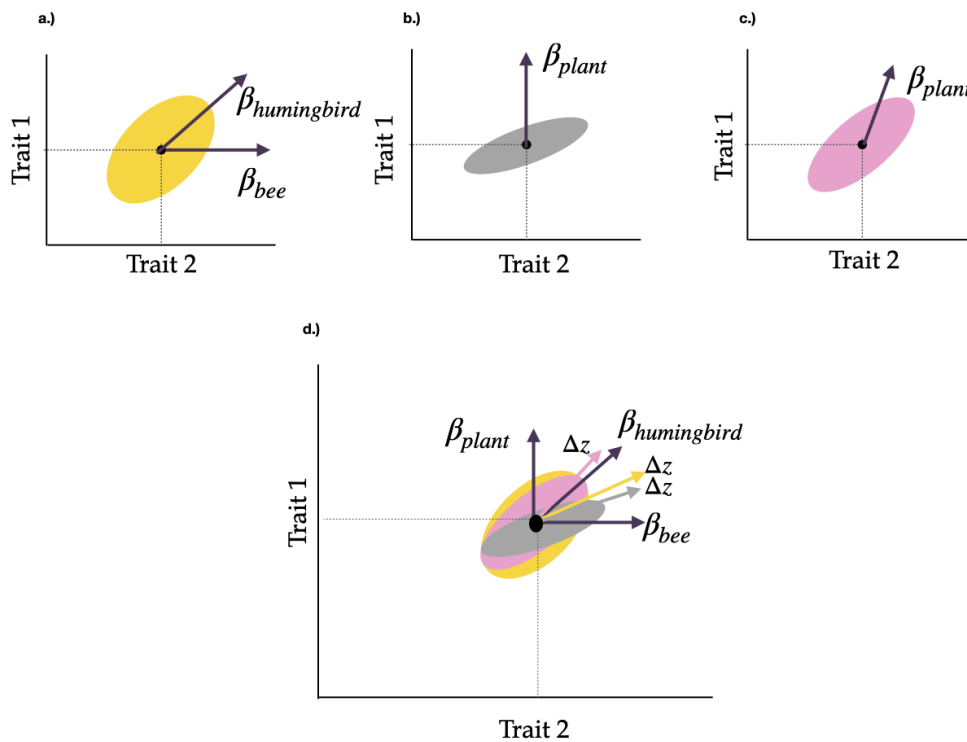
Figure 8. a), b) and c) represents the phenotypic covariance for two traits involved on the interaction for a plant (yellow), bee (gray) and hummingbird (light pink). β represents the selective vector and selection favors different directions for each species. d) Co-evolution (ΔZ for both species) will be an interplay of the multiple traits involved on the interaction and on the multivariate selection and is represented by the arrow direction and color.

Conclusion

Asymmetries are wide spread in mutualistic pollination interactions, from the selective strength to the asymmetric specialization in the network. These asymmetries may generate asymmetries in the coevolutionary process, but only for specific traits involved in the interaction. For some traits involved in the interaction, their evolutionary process will be mainly adapting to the interacting trait on the partner. However, any of these traits are independent to evolve and their genetic architecture behind the traits maybe affect the coevolutionary dynamics.

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Selection asymmetries affect coevolutionary paths in mutualistic networks

Pamela Cristina Santana[1*]; Ana Paula Aprígio Assis[1,2]; Paulo R. Guimarães Jr.[1]

*pcsantana@ib.usp.br

[1] Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, 05508-900, São Paulo, SP, Brazil.

[2] Departamento de Genética, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

Abstract

Mutualistic interactions affect individual fitness and species trait evolution. As a consequence, mutualisms may foster reciprocal selection and drive coevolutionary dynamics. Fitness consequences of mutualism often vary across interacting individuals, generating fitness asymmetries, on most mutualistic interactions. Fitness asymmetries translate to differences in the strength of selection imposed by pairwise interactions. Because mutualisms often form ecological networks, these asymmetries may not only affect directly interacting species but its evolutionary effects may cascade through the network of interacting species, potentially influencing phenotypic patterns at the community level. Here, using a mathematical model, we investigated the potential consequences of selection asymmetries to coevolutionary dynamics of mutualistic networks. Our results suggest that selection asymmetries reorganize the network structure of evolutionary effects, constraining evolution due to indirect cascading effects to the guild of species that receives higher selective pressure from the mutualism. As a consequence, the amount of trait evolution species in each interacting guild experience during the coevolutionary process changed. Species under stronger selective pressures from the interaction had a greater rate of evolutionary change. At the community level, selection asymmetries fueled trait disparity in the network, having a conspicuous effect on the outcome of coevolution. In highly asymmetrical interactions, we observed trait convergence in only one guild of species, the one where mutualistic selection is stronger. As a consequence, matching in pairwise traits between interacting partners varied more in asymmetrical scenarios. Jointly, our results highlight how patterns observed in species that interacts in networks may be shaped by asymmetries in selective pressure.

Keywords: coevolution, co-adaptation, indirect effects, phenotypic evolution, trait evolution

Introduction

Mutualisms are ecological interactions that provide fitness benefits to interacting individuals of different species (Bronstein et al., 2006; Bronstein 2015). As a consequence, mutualisms might represent an important source of selection, shaping the evolution of species' traits (Cook and Rasplus 2003, Benkman 2013). Mutualisms as a source of selection and trait evolution have been documented in several different types of mutualistic interactions, such as pollination by animals (Muchhala and Thomson 2009, Campbell et al. 2014, Caruso et al. 2019), protection of plants by ants (Rutter and Rausher 2004), and seed dispersal by birds (Galetti et al. 2013). Therefore, mutualistic interactions are an important aspect that might shape species phenotypes in ecological communities (Schemske 2010).

In mutualisms, the process of adaptation can be reciprocal, as partners can be concomitantly adapting to each other, giving rise to a coevolutionary process (Janzen 1966, 1980, Thompson 1989, Kauffman and Johnsen 1991, Thompson 2005). Coevolution is considered a key process in shaping trait evolution, potentially leading to the widespread trait matching observed in nature between mutualistic partners (Pellmyr 2003, Thompson 2009, Thompson et al. 2013, *but see* Nuismer et al. 2013). Trait matching is a pattern where the traits of the interacting species are coupled, such as a similar length of a flower's corolla tube and the proboscis length of its mutualistic interacting insect (Anderson and Johnson 2008, 2009, Pauw et al. 2017). Even though coevolution is a mechanism associated to trait matching, it is difficult to distinguish the pattern observed from the process of mutual co-adaptation between the mutualistic partners or the process of just one species adapting to its interacting partner (unilateral evolution, *i.e.* only one species evolves due to mutualistic selection) (Caruso 2000, Anderson and Johnson 2008, 2009, Porter and Simms 2014, Sazatornil et al. 2016). Indeed, in nature, it is expected that mutualistic interactions are at some point in

a continuum between perfect reciprocal consequences to the fitness of interacting individuals and interactions that are perfect asymmetrical in its fitness consequences, *e.g.* strong fitness benefits to one partner, whereas the other partner show a negligible effect on fitness due to the interaction. This variation in fitness, in turn, may impact pairwise trait matching patterns (Schemske 1983, Baker et al. 2017).

For instance, in pollination and seed dispersal mutualisms, the interaction impacts different life history attributes of the interacting individuals, often resulting in different fitness benefits to interacting partners (Chapter 1 *in this thesis*). In general, the interacting animals obtain resources from the plants (pollen and nectar or fruit and seed pulp, needed for survival and growth) while the interacting plants obtain mainly sexual reproduction through the animals (Jordano et al. 2003, Ollerton et al. 2011, Baker et al. 2017). Therefore, due to the differences in fitness effects, asymmetries in the strength of selective pressure should arise not only in pairwise interactions, but be structured in networks among interacting guilds (*e.g.* plants and pollinators), ranging from symmetrical selective pressures to highly asymmetrical selection strength (Anderson, 2015; Caruso, 2000; Porter and Simms, 2014). Asymmetries in selective pressures may be common in mutualistic interactions in nature, and may shape different rates of trait evolution, trait convergence and trait match patterns observed in networks, such as seed dispersal (Valenta and Nevo 2020) and pollination syndromes (Johnson and Steiner 2000, Fenster et al. 2004). Indeed, Clark et al (2019) found asymmetric bargaining between mutualistic plants and microbes, where the host plant had a high degree of control over the carbon-for-nitrogen exchange ratio under some conditions of soil nutrient concentration. In this case, through asymmetric selection, the coevolutionary dynamics could lead one side of the interaction to have a dominant role in guiding trait evolution (*e.g.* Ramírez et al., 2011).

Most mutualistic interactions form large networks of interacting species (Dáttilo et al. 2016, Delmas et al. 2019). Adding a complexity layer into the coevolutionary process, as the effects of selection pressures could cascade and influence species trait evolution not only through their direct interactions but also through indirect effects (Ohgushi 2005, Bergamo et al. 2017, Guimarães et al. 2017). For instance, in a co-flowering plant community, the pattern of selection by pollinators on corolla length was influenced by the community species composition (Caruso 2000, Eisen et al. 2020). However, it is hard to distinguish the consequence of multiple selective pressure and indirect effects contribution to trait evolution. In mutualistic networks, selection asymmetries may reduce the relative importance of direct effects on coevolution to trait dynamics (Jordano 2010). In addition, the prevalence of selection asymmetries may influence the magnitude of indirect effects, shaping trait evolution differently through networks of interacting species (Guimarães et al. 2011, 2017).

Here, we investigated how selective pressures asymmetries affect coevolutionary dynamics and species trait evolution within mutualistic networks. To explore the potential consequences of selective asymmetries to coevolution of interacting assemblages we used a combination of evolutionary models and network theory (Guimarães et al. 2017). Specifically, we used a trait-based coevolutionary mathematical model to explore the impacts of direct and indirect effects to coevolutionary dynamics. In order to investigate how selection asymmetry influences direct and indirect evolutionary change we varied the relative selective pressure imposed between the interacting guilds (Fig 1a-d). We then evaluated the impacts of selection asymmetry to the structure of the networks emerging as a result of the coevolutionary process. We show that selection asymmetries between interacting partners affect coevolutionary dynamics, leading to changes: *i)* in the network structure of the evolutionary effects; *ii)* in the overall contribution of indirect effects to trait evolution, as indirect effects concentrates in one set of the partners in high asymmetric

scenarios; *iii)* in the rate of trait evolution for each interacting guild; and *iv)* changes in the trait matching in the network and final trait values distributions at the community level.
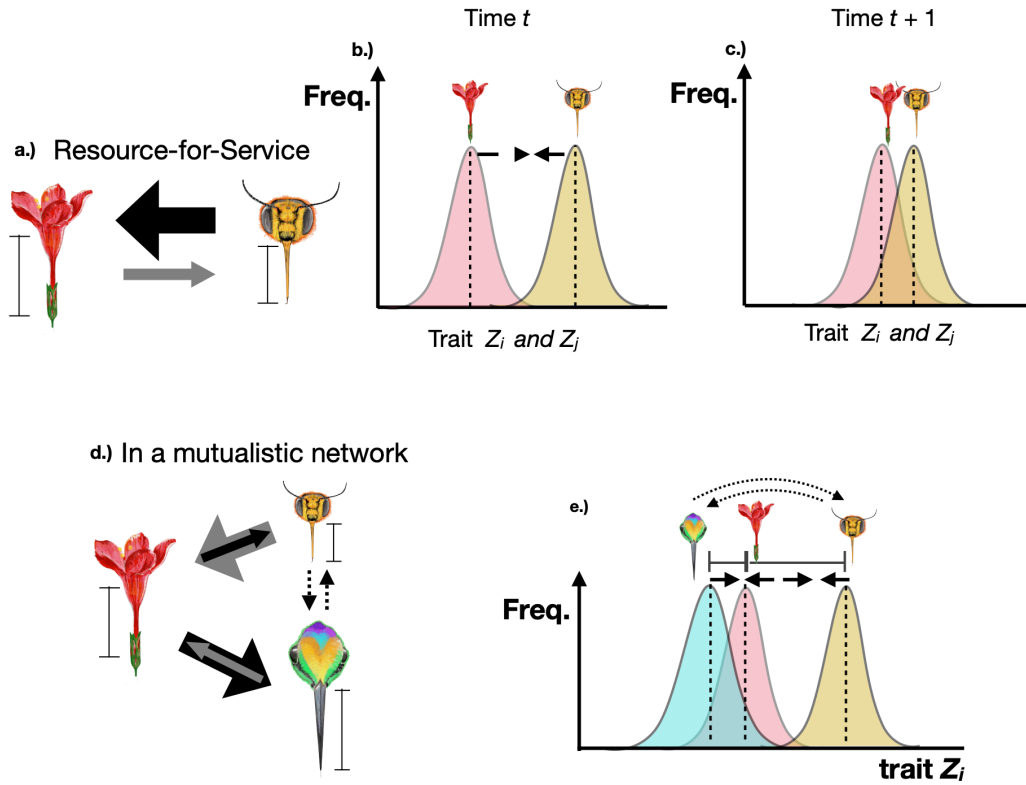
Figure 1. Selective pressure asymmetry may impact the evolutionary change and the adaptive landscape of mutualistic interacting species: a) two organisms interact, but the selective pressures they exert in one another has different strength, indicated by the arrow sizes. We assume that selection imposed by the mutualistic interactions is favoring trait matching. The plant is more dependent on the interaction than the bee, so the selective pressure imposed by the bee is stronger than the amount of selective pressure imposed by the plant on the bee; b) The curves represent the distribution of a quantitative trait in one population of interacting species. Selection differential is indicated by the arrows and is stronger on flowers. c) The adaptive landscape for both species. Plants that have traits values closer to bee's trait distribution have higher fitness. In contrast, the selective pressures plants exert in bees is weaker. Therefore, the amount of evolutionary change in each species will be different. In this way, we expect that both species will achieve phenotypic matching but flower traits will experience higher evolutionary change than the bees traits; d) When another species is added in the network, trait evolution trajectory become more complex. The initial selective pressures exerted between the partners will present different strengths, indicated by the alterations in arrow sizes, and also indirect effects between non-interacting species will emerge, affecting trait evolution, indicated by the dashed arrows; e) The quantitative trait curves will be a result of vectors influencing the same point summed to indirect effects (dashed arrows).

Methods

Model description

We used a coevolutionary model (Guimarães et al. 2017, Medeiros et al. 2018) to explore the consequences of selection asymmetry between sets of mutualistic partners. We evaluated how selection asymmetry between set of partners affect: *i)* the network structure of evolutionary effects; *ii)* the contribution of indirect effects to trait evolution in each set of species; *iii)* the magnitude of evolutionary change among species within each set of species. In our simulations we assumed that directional selection was the only evolutionary process governing species evolution, and therefore we considered that population sizes were large enough in order to genetic drift to be negligible. We also assumed no gene flow and that trait genetic variance was fixed and high enough to allow the populations to respond to natural selection.

Our model describes the evolution of *N* species, each characterized by a single quantitative trait ($z_i^{(t)}$) interacting in a mutualistic network. The quantitative trait $z_i^{(t)}$ represents the population mean trait value for species *i*. The trait $z_i^{(t)}$ mediates the mutualistic interaction, such as the length of a hummingbird beak for a pollinator and the tube length of a corolla flower for the plants. In our model, $z_i^{(t)}$ will evolve under the selective pressure exerted by its interacting partners (mutualistic selection) and by other abiotic and biotic factors (environmental selection) in each time step. Selection imposed by the mutualistic partners favors trait matching and favored trait value is a consequence of a multi-specific selection gradient in the interacting network. Because selection is a result of both biotic (due to the mutualistic interactions) and abiotic selective pressures, we are able to investigate the effects of differential dependence of the mutualistic interaction. Initial trait values ($z_i^{(t=0)}$) were sampled from a uniform distribution, ranging from 0 to 10. Furthermore, we assumed that genetic variance ($\sigma_{z_i}^2$) is fixed

and equal for all species (Table 1). In each generation, the mean trait of each species can evolve to a new trait value in response to selection according to:

$$z_i^{(t+1)} = z_i^{(t)} + \varphi_i \left[\sum_{j,j\neq i}^{N} q_{ij}^{(t)} \left(z_j^{(t)} - z_i^{(t)} \right) + p_i \left(\theta_i - z_i^{(t)} \right) \right] \qquad \text{(eq. 1)}$$

in which φ_i is a composed parameter encompassing the additive genetic variance of the trait and the fundamental slope of the adaptive landscape (Guimarães et al. 2017). The selection differential is then defined as $\sum_{j,j\neq i}^{N} q_{ij}^{(t)} (Z_j^t - Z_i^t) + p_i(\theta_i - Z_i^t)$ and can be decomposed in: *i)* its biotic component $\sum_{j,j\neq i}^{N} q_{ij}^{(t)} (Z_j^t - Z_i^t)$, in which $q_{ij}^{(t)}$ is the contribution to selection imposed by species *j* in species *i* and its value is a function of trait matching (*see* equation 2); $\sum_{j,j\neq i}^{N} q_{ij}^{(t)}$ represents the total contribution of mutualistic interactions to the differential selection of species *i*, and can range from 0 (no contribution of mutualism to trait evolution) to 1 (only mutualistic selection determines trait evolution); and *ii)* the contribution of other sources of selection not related to mutualism to trait evolution, $p_i(\theta_i - Z_i^t)$, hereafter referred to as environmental selection, in which $p_i = 1 - \sum_{j,j\neq i}^{N} q_{ij}^{(t)}$ and θ_i is the trait value favored by the environment and it is fixed over time. The mutualistic contribution of each distinct pairwise interactions to selection (q_{ij}) varies, depending on trait matching as:

$$q_{ij}^t = m_i a_{ij}^{(t)} \frac{e^{-\alpha\left(Z_j^{(t)}-Z_i^{(t)}\right)^2}}{\sum_{k,i\neq k}^{N} a_{ik}^{(t)} e^{-\alpha\left(Z_k^{(t)}-Z_i^{(t)}\right)^2}} \qquad \text{(eq. 2)}$$

in which, m_i is the parameter that modulates the intensity of mutualistic selection, $\sum_{j,j\neq i}^{N} q_{ij}^{(t)} = m_i$, and a_{ij}^t describes if species *i* interacts with *j*. Initially,

we set $a_{ij}^{t=0}=1$ for all potential partners of species i, which means that species i initially interacts with all species in the other set. The term $\dfrac{e^{-\alpha\left(z_j^{(t)}-z_i^{(t)}\right)^2}}{\sum_{k,i\neq k}^N a_{ik}^{(t)} e^{-\alpha\left(z_k^{(t)}-z_i^{(t)}\right)^2}}$ controls the potential selective pressure species j can exert in i. Note that the strength of mutualistic selection is associated to the level of trait matching between interacting species, so that functional interactions (high trait matching) are associated to higher directional selection on interacting partners. When the phenotypic distance between the two species ($e^{-\alpha\left(z_j^{(t)}-z_i^{(t)}\right)^2}$) is high this term tends to zero, therefore, partners that show strong trait matching to species i have a higher contribution to the mutualistic selection. However, when species achieve a perfect phenotypic matching ($z_i^{(t)}=z_j^{(t)}$), there is no longer act as a source of selection, because $q_{ij}^{(t)}(Z_j^t - Z_i^t)=0$. The parameter α describes the sensitivity of phenotypic distance to the selection imposed by species j.

We explored selection asymmetry using theoretical bipartite networks, in which interactions only occur between species of distinct sets (*e.g.* plants and pollinators), which is the case for most mutualistic interactions (Ollerton 2006). We initiated the simulations with all species connected and equal numbers of species in each set ($k_i = \dfrac{N}{2} - 1$, in which k_i represent the number of links any given species has in the network and N is the total species richness of the network and fixing the species richness of each set of the species as $N/2$).

In our simulations, we varied the parameter m_i in order to investigate how selection asymmetry in reciprocal selection between mutualistic partners affect coevolutionary dynamics when structured in sets. We decided to structure asymmetry in sets because of the intrinsic condition of mutualistic interactions in which the fitness benefits for each species in different sets are often distinct (*i.e.* survivorship of pollinators x reproduction of plants), representing the first layer of differences in fitness benefits across species. For every species i of a given set

A (*e.g.,* plants) and for every species *j* of a given set B (*e.g.,* pollinators), we varied values for m_i for each set from a highly asymmetrical ($m_i \gg m_j$ or $m_j \gg m_i$) to a symmetrical scenario ($m_i = m_j$) with m_i mean values combinations ranging from 0.1 to 0.9 of mutualistic selection for a set (trophic group) and standard deviation of 0.01. We assumed mi for a given species as fixed in a given simulation. Therefore, asymmetry was represented by the difference between the mutualistic selective pressure that species in each trophic group imposes to species in the other trophic group. All other parameter values and initial values of variables were sampled from the same distributions (Table 1), except the mutualistic selection parameter (m_i) and therefore the unique differences among scenarios are the degree of mutualistic selection and the asymmetry in selection between sets. To investigate the impact of asymmetries in selective pressure imposed by interactions on coevolution, we explored the following descriptors of evolutionary dynamics:

i)   *How does selection asymmetry influence network structure of evolutionary effects?*

    In our simulations, species interactions lead to the reorganization of the species adaptive landscapes and our network represents the evolutionary effects species are imposing on each other. During the course of simulations, if one species evolves and became phenotypically distant from some partners, its evolutionary effect on these partners will decrease, until the point in which the species will have a negligible evolutionary effect on the interacting partner (q_{ij} values close to zero). However, in our networks it is possible that *i* has an evolutionary effect on *j,* but *j* has a small evolutionary effect on *i*. This occurs because the asymmetry in selective pressure allows one species to impose strong effects while the partner does not. We described the network as a directed

network in which, if the evolutionary effect of i on j became close to zero (q_{ij} <0.01), we removed the link, but keeping the link between j to i, if $q_{ji} > 0.01$. Using this approach, we were able to construct the network of evolutionary effects for each set of species (Donnat and Holmes 2018). We separated the **Q** matrix into the two sets of interacting guilds and turned each matrix into a Laplacian matrix (**L**), by subtracting the adjacency matrix (**A**) from the degree matrix (**D**). After that, we extracted eigenvalues from **L** to study dynamics in the network. Eigenvalues from **L** allow to evaluate changes in the overall network structure associated to the cascading effects. To track the changes in the eigenvalues from **L,** we evaluated the distribution of eigenvalues, *i.e.* matrix spectrum, as we were interested in understand any change in structure at the local scale. To verify how the eigenvalues from **L** changed for each set of interacting guilds, we ordered the eigenvalues and subtracted them, going from a symmetrical scenario to a highly asymmetric one.

ii)   *How does selection asymmetry affects the contribution of direct and indirect effects to the patterns of trait distribution observed in the network?*

Species embed in networks of interactions have the potential to influence even species that they do not directly interact (Guimarães et al. 2011, 2017). However, those indirect effects are not equally distributed, but some species are more prone to receive more indirect effects due to the network structure. Guimarães et al. (2017) proposed an analytical method to study reciprocal direct and indirect evolutionary effects in networks of species interacting, by computing the coevolutionary matrix (**T** matrix). In this matrix, each element indicates the relative contribution of the interacting and non-interacting species in the network to the selection gradient of species i, which allows the calculation of the contribution of direct and indirect effects to trait evolution. We used this

framework to estimate the amount of evolutionary change due to direct and indirect effects in the equilibrium simulated networks. The indirect effects were defined as the proportional effect of non-interacting species on trait evolution of species *i.* The contribution of indirect effects to trait evolution in a network is defined as:

$$I_e = \frac{\sum_i^N \sum_{j,i \neq j}^N (1-a_{ij})t_{ij}}{\sum_i^N \sum_{j,i \neq j}^N t_{ij}}$$ (eq. 6)

in which t_{ij} are elements of matrix **T**. After we estimated the relative contribution of indirect effects, we compared in the different scenarios of symmetry in selective pressure.

iii) *Does selection asymmetry influence the amount of evolutionary change, leading to distinct coevolutionary change in each set?*

As we varied m_i from a symmetrical to an asymmetrical scenario, we were able to investigate if and how asymmetry influences the equilibrium phenotypic values. In each simulation, we estimated the amount of evolutionary change (E_c) for each set (*i.e.* plants and animals) until achieving equilibrium trait values as:

$$E_{ci} = (z_i^\tau - \theta_i)$$ (eq. 4)

in which z_i^τ is the equilibrium trait values for each species, θ_i is the environmental optimum for each species in the network, which also represent the initial trait values for each species, as we simulated situation where species begin the coevolutionary dynamics in their environmental optimum. Having estimated E_c for each species, we then computed the variance in E_c for each set of

species. The variance in E_c gives us an indication of how asymmetry in mutualistic selective pressure would impact the amount of evolutionary change within each set.

iv)   *How does selection asymmetry between mutualistic partners affect trait matching in networks?*

To address this question, we computed the mean network trait-matching between interacting partners. This was estimated as:

$$\langle D \rangle = \frac{\sum_{j,j \neq i}^{N} |z_i^{\tau} - z_j^{\tau}| a_{ij}^{\tau}}{N(N-1)} \qquad \text{(eq. 5)}$$

in which a_{ij}^{t} is equal to 1 if the interaction is still functional at equilibrium. Conversely, $a_{ij}^{(t)} = 0$ if the difference in traits of species i and j was high enough that interaction was deemed non-functional; z_i^{τ} is a *Nx1* vector of trait values for each species in the network. Therefore, $\langle D \rangle$ represents the mean trait matching for interacting species in the equilibrium network. We then computed the variance in trait matching $\langle D \rangle$ between functional interacting partners. This quantity gave us how variable was trait-matching among interacting species. Finally, we estimated for each species in the network its best fitting interacting partner, *i.e.* the species that presented the highest trait-matching partner (lowest trait distance).

All simulations were performed in R 3.6.1 (R Core Team, 2019) and codes will be made available in https://github.com/SantanaPC. We performed 100 numerical simulations for each combination of m_i and m_j starting with totally connected network with 50 species in each set. All species initial trait's values were equal to the environmentally favored trait value for a given species (Θ). Each simulation ended after all species achieved asymptotic trait values.

Table 1. Description of key parameters, variables, and initial conditions of the coevolutionary network model used.
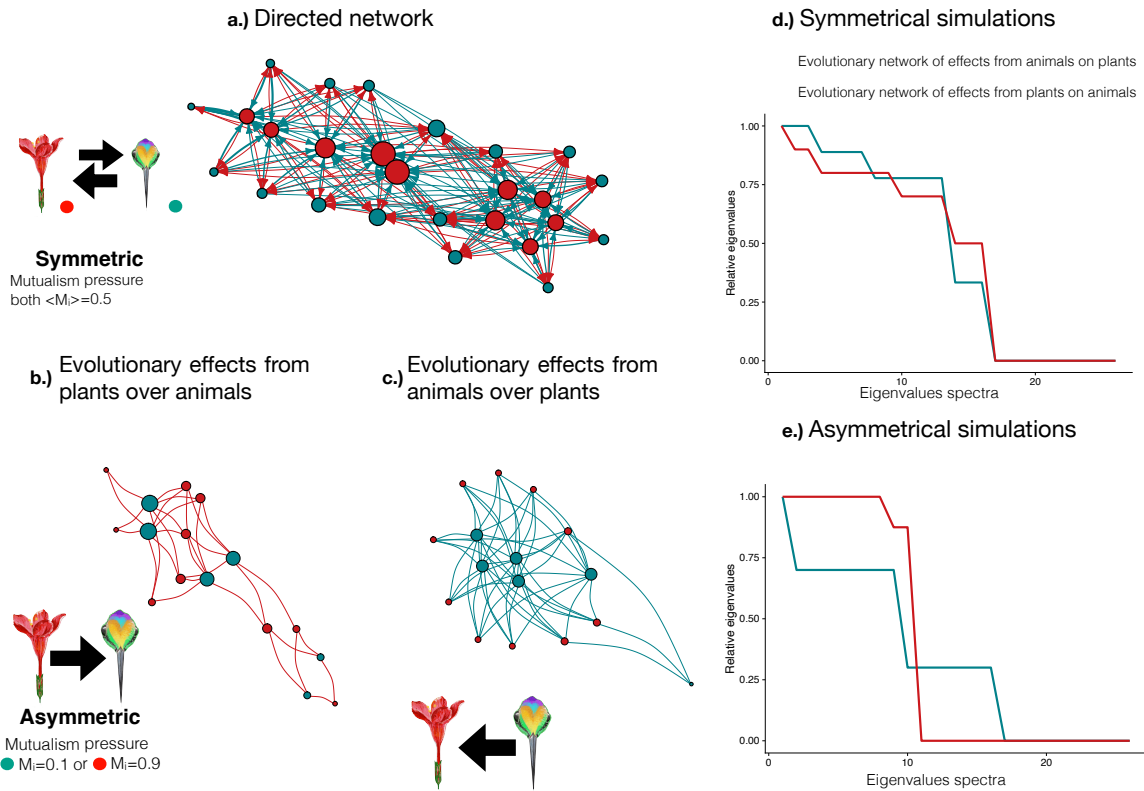
Parameter or Variable	Description	Sampling distribution
$z_i^{(t)}$	Initial mean trait value of species i	$z_i^{(t)} \sim U(0,10)$
ϕ_i	Parameter composed by the heritability of the trait and the slope of the adaptive landscape	fixed as 0.4
θ_i	Optimum trait value favored by environmental selection	$\theta_i \sim U(0,10)$
α	Sensibility of evolutionary effect due to the trait matching between interacting species	fixed as 0.2
m_i	Strength of mutualistic selective pressure	Range from 0.1 to 0.9

Results

Selection asymmetry changed network structure affecting the cascading of evolutionary effects

In our simulations, species interactions led to the reorganization of the species adaptive landscapes and the network of evolutionary effects were different in each symmetry scenario (Fig 2a). Higher selection asymmetry led to distinct network structure between each interacting guild and affecting directly the cascade of evolutionary effects (Fig 2b-c). The matrix spectra differed with the increase in asymmetry, indicating that the connectivity patterns inside each set differs markedly comparing symmetrical to highly asymmetrical scenarios (Fig 2 d-e). The potential for evolutionary effects cascading is concentrated in the first few eigenvalues for the plants, and more equally distributed for the animals.

Network of evolutionary effects and Eigenvalues distribution

a.) Directed network

d.) Symmetrical simulations

b.) Evolutionary effects from plants over animals

c.) Evolutionary effects from animals over plants

e.) Asymmetrical simulations

Symmetric
Mutualism pressure both <M$_i$>=0.5

Asymmetric
Mutualism pressure
M$_i$=0.1 or M$_i$=0.9

Figure 2. Effects of selection asymmetry reshape the network topology for each set of species in a bipartite network. a) A directed network generated by symmetrical simulations. b) Evolutionary effects measured as with **Q** matrix from plants over animals' network generated in highly asymmetrical scenarios. c) Evolutionary effects from animals over plants' network generated in highly asymmetrical scenarios. Eigenvalues distribution in *d)* and *e)* X-axis shows the eigenvalues spectra and Y-axis shows the relative eigenvalues. d) From symmetrical simulations. e) From asymmetrical simulations. The increase in selection asymmetry generates a change in the structure of the evolutionary effects network where most of the variance is concentrated in the first few eigenvalues for the plants, and more equally distributed for the animals. The effects from animals on plants are all connected while the effects from plants on animals are disconnected. Therefore, as the asymmetry makes that *i* had a functional interaction with *j*, but *j* does not have a functional interaction with *i* the propagation of their effects will be distinct, seen by the network structure.

Selection asymmetry diminished the overall contribution of indirect effects to trait evolution, but concentrated its contribution on the set of species that rely heavily on mutualism

Higher selection asymmetry strongly decreased the contribution of indirect effects to trait evolution (Fig. 3a), as we observed higher contribution of indirect effects to trait evolution when selection was symmetric among species (Fig. 3a). Additionally, selection asymmetry concentrated the indirect effects into one set, the one that mutualism represented a higher proportion of overall selection (represented by plant species in the figure, Fig. 3b). In symmetrical scenarios, the relative contribution of indirect effects to trait evolution was dispersed (Fig. 3c). In a highly asymmetrical scenario, contribution of indirect effects was concentrated in just one set of species (Fig. 3d). However, the dispersion of indirect effects were not equal in the set of species that relies more heavily in the mutualism, but concentrated in some species (possible to observe by color variation in the heatmap - Fig. 3 c,d).
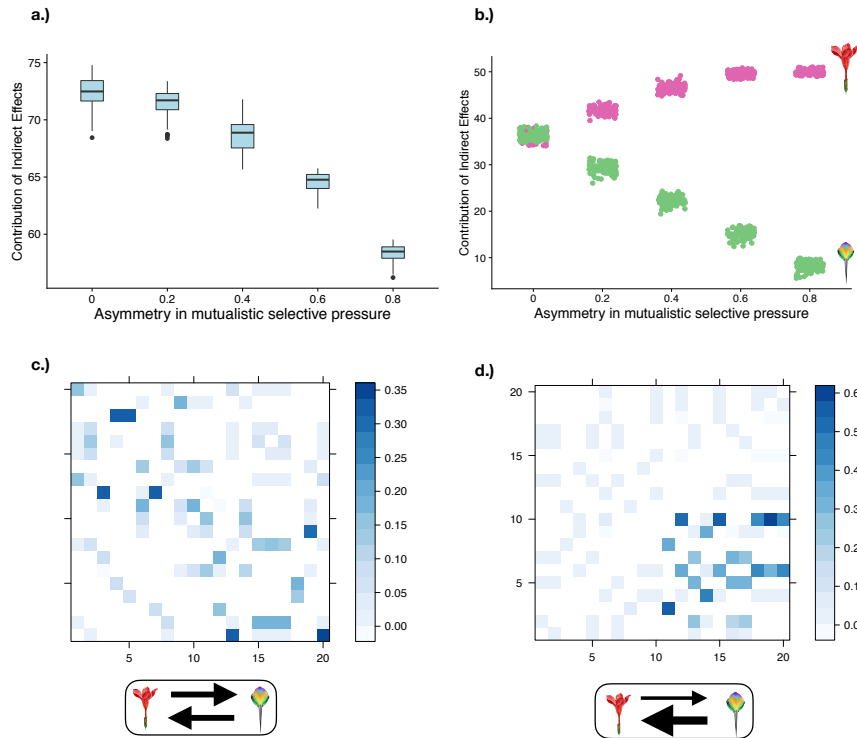
Figure 3. Higher selection asymmetry decreased the contribution of indirect effects to trait evolution and had driven indirect effects contribution to one set of species. Simulations ranged from a totally symmetrical scenario ($m_i = m_j = 0.5$) to a highly asymmetrical ($m_i = 0.1$; $m_j = 0.9$). a) Total contribution of indirect effect to trait evolution in different scenarios of asymmetry. The higher importance of indirect effects in trait evolution was in a symmetrical scenario ($m_i = m_j = 0.5$) where contributions of indirect effects are nearly 70; b) Total contribution of indirect effect to trait evolution in different scenarios of asymmetry in each set of species. The graph demonstrates that the species that relied more heavily in the mutualism (demonstrated here in pink) were more affected by the indirect effects than the species that to which mutualistic selective pressure were low (demonstrated here in green); c) and d) are heat maps representing the coevolutionary matrix with the indirect effects imposed to each species. All species are represented in all lines and columns. Stronger colors indicate stronger evolutionary effects of a species (column) on the trait evolution of another species (row). c) is the *T-matrix* of a symmetrical scenario ($m_i = m_j = 0.5$) in which it is possible to observe the indirect effects were spreading to all the species and d) is in an asymmetrical scenario where it is possible to see that effects were concentrated in just a trophic group, the one that rely heavily on mutualism ($m_i = 0.1$; $m_j = 0.9$). Note that indirect effects were not equally distributed even among species of the same trophic group, with some presenting a higher contribution of indirect effects than others.

Selection asymmetry changes the variance in the amount of evolutionary changes observed in each set of interacting partners

The outcome of coevolution was influenced by the degree of selection asymmetry (Fig. 4a-d). Simulations in which only the strength of mutualism selection varied (with all other parameters equal) achieved different equilibrium trait values (Fig. 4a-b). As expected, the set of species in which the mutualistic interaction had a higher relative contribution to the overall selection gradient undergone higher evolutionary change (higher values of m_i – represented as plants species in Fig. 4b). On the other hand, we observed small evolutionary changes in species to which mutualism had a small contribution to the overall selection gradient (smaller values of m_i), making these species final trait-values to be in the vicinity of its environmental favored trait-value (indicated as points in Fig. 4b). In this sense, the equilibrium trait value of species that mutualism contributed more to the selection gradient (plants in our simulations) was closer to its partners' environmental favored trait values (animal species in our simulations). We also observed a decrease in community trait variation or trait convergence, but only among the species that relies more heavily in the mutualism. Furthermore, the higher the selection asymmetry in our simulations the greater the variance in the amount of evolutionary change experienced by each set of species (Fig. 4c-d). The set of species more independent on mutualism (smaller values of m_i) changed more in symmetrical scenarios then in highly asymmetrical scenarios (Fig. 4c). In contrast, in asymmetrical conditions, these set of species (animals in the Fig. 4c), presented a lower magnitude of evolutionary change than the set of species that heavily dependent on mutualism (plants in the Fig. 4d).
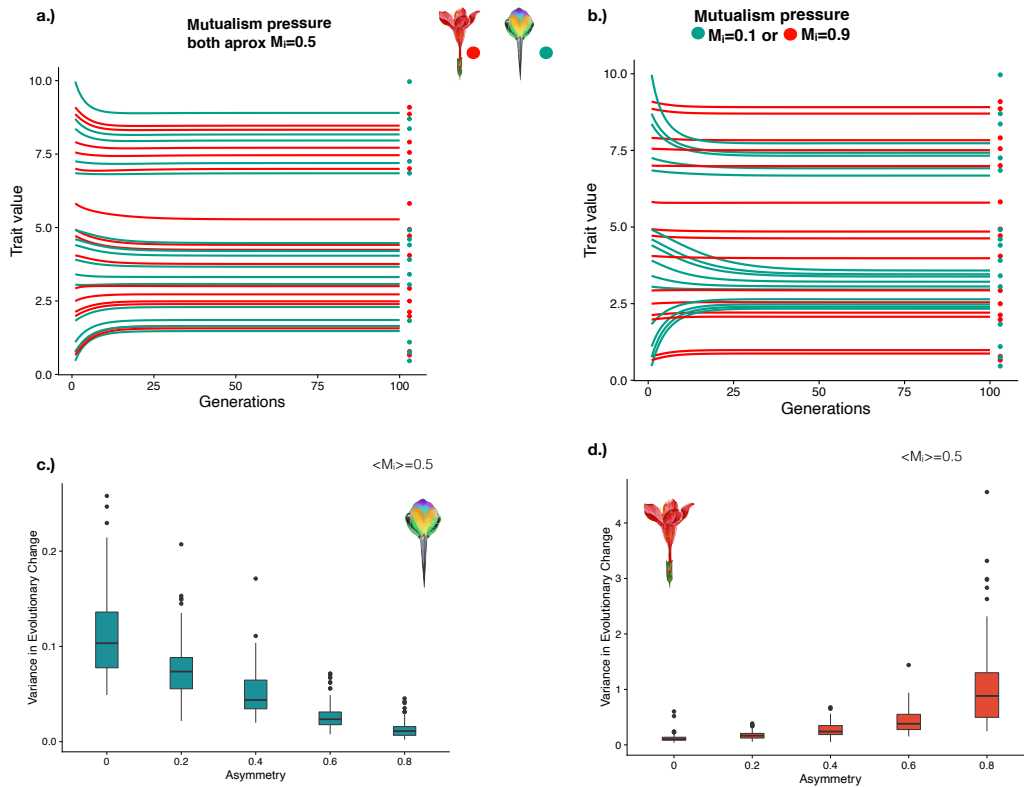
Figure 4. Selection asymmetry changed evolutionary trajectories and led to a variation in the amount of evolutionary change experienced by species. In all graphs presented, $\langle m_i \rangle = 0.5$. a) and b) represents species trait values trajectories along the coevolutionary dynamics, starting at the environmental optimum (Θ). Each set of species (N= 15; trophic group: plants or animals) are represented by a different color and dots represent each species environmental peak (Θ). Each line represents one species (n_animals= 15 n_plants=15); a) When the strength of mutualistic selection is symmetric ($m_i = m_j = 0.5$), the evolutionary trajectory for both sets of interacting species was similar, with a similar amount of evolutionary change in each trophic group; b) in a highly asymmetrical scenario ($m_i = 0.9$ pink lines; $m_j = 0.1$ green lines), we observed a higher disparity in the amount of evolutionary change observed in each species sets. It is possible to observe that the green set of species have its final trait values closer to its environmental peaks (Θ) than the pink lines. The pink species have, therefore, tracked some green species, but also converged to groups. It is also possible to observe some green species isolated in the end of the simulation. This species established their trait value near to their environmental peak, but far from the other species trait values. Possibly, the reason for this, is that since mutualistic selection represented a small fraction of total selection for these species, mutualism did not effectively change its final trait values. Therefore, in the end, they were isolated in the community. The other set of species, as they occurred in the network and mutualistic pressure were composed by all interacting partners, species were adapting to a group of mutualistic partners and converging their trait. Comparing *a.* and *b.* we can see that, at the end of the simulation, equilibrium trait values were different. c) and d) shows the variance in Evolutionary Change (E_c) and demonstrate that the pattern showed in the *a.* and *b.* are consistent through the simulations. c) Shows that with the increase in asymmetry of selective pressure between mutualistic partners, variance in the amount of change in one set of mutualistic partners decreased, even though they did not reach high values. In these simulations, for this group, we varied m_i from 0.1 to 0.5. d) Shows the

opposite for the other trophic group (bees), which the increase in asymmetry of selective pressure also increased the variance in E_c. For this group (plants), m_i varied from 0.5 to 0.9. However, note that for this group, the magnitude of change in much higher.

Selection asymmetry decreased overall trait matching between interacting species

We then analyzed how selection asymmetry influenced trait matching in the network. Our findings indicate that higher trait-matching was found only in a symmetrical scenario where both partners impose high mutualistic selective pressures (Fig. 5a, $m_i=m_j\cong0.9$, smaller values of mean pairwise trait distance). When the interaction was highly asymmetrical, the mean pairwise trait distance increased, indicating a decrease in trait matching between interacting species in the network. However, the variance in trait matching was higher in more asymmetrical scenarios (Fig. 5b). This indicates that asymmetrical scenarios were characterized by distinct groups of species with some pairwise interactions with high trait matching while other presented a low degree of trait matching. The network-mean partners minimum distance increased with selection asymmetry, reinforcing that selection asymmetry led to a decrease in the overall network trait matching of pairwise interactions. Taken together, these results indicated that selection asymmetry reshape the topology of evolutionary effects network, affecting how species can propagate evolutionary effects and promoting trait divergence, decreasing overall trait matching between interacting partners and concentrating convergence in only one guild.
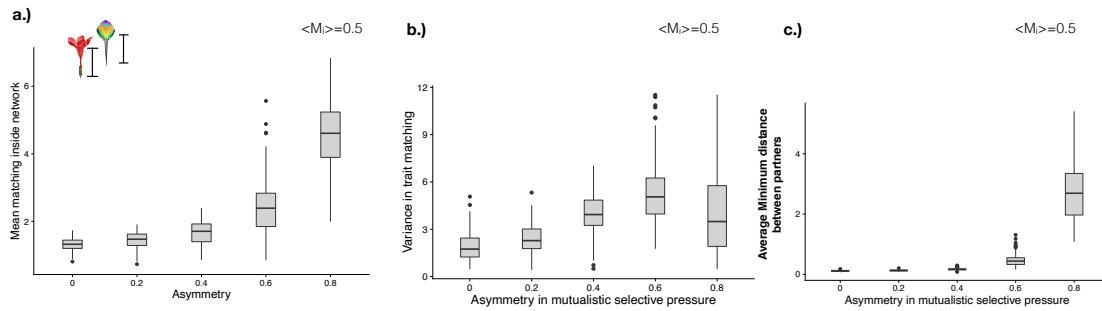
Figure 5. Trait matching in the network and distribution of phenotypic patterns in the network. a) Mean pairwise distance between the traits of interacting species was higher when selective pressures were more asymmetrical, but also in symmetrical scenarios where mutualistic pressure imposed are small ($m_i = m_j \cong 0.1$), indicating that a high trait matching inside components appears only in high symmetrical selective pressure ($m_i = m_j \cong 0.9$); b) The variance in trait matching ($Z_j^\tau - Z_i^\tau$) was higher with the increase in selection asymmetry, meaning that some pairwise interaction were coupled while other are not with more frequency in asymmetrical scenarios. c) The average minimum distance between mutualistic partners increased with selection asymmetry, which indicate that in asymmetric scenarios species tended to be more phenotypic distant to any other potential partners.

Discussion

Mutualistic interactions play a major role in shaping species traits in ecological communities (Bronstein et al. 2006, Thompson 2009). Most mutualistic interactions are characterized by the exchange of distinct benefits (*e.g.* service-to-resource), thereby, the interaction affects different components of fitness in each interacting partner (Baker et al. 2017). In this way, selection asymmetry, resulting from these different fitness effects, might be widespread and play an important role in coevolution (Schemske 1983). Using a coevolutionary mathematical model and network theory, we analyzed how selection asymmetry affects coevolutionary dynamics. Our results indicated that selection asymmetry reshaped interactions among species generating a distinct network of evolutionary effects for each set. Selection asymmetry also changed the dynamics

of indirect effects on coevolution, decreasing its total contribution to trait evolution and concentration of indirect effects in only one set. Additionally, selection asymmetry also led to differences in the final coevolutionary outcome, where one set experienced higher rates of trait change than the other. Ultimately, selection asymmetry decreased trait matching inside networks components (subgroups), and increased trait disparity in overall network, leading to trait convergence in just one trophic group.

First, in highly asymmetrical scenarios, we observed a distinction between the topological structure from the evolutionary effects networks of each set, plants and animals. Our results indicate that structured asymmetry could reshape the network structure for coevolving species. For one set, the network of interactions is perceived as a sparse and non-modular structure with the presence of at least a few highly connected species (Jordano 1987, Bascompte et al. 2003, Dáttilo et al. 2016, Dormann et al. 2017, Delmas et al. 2019). For the other set, small groups of species that interact more frequently, associated with a small average number of interactions per species and with low variance in the number of interactions across species (Guimarães 2020). The eigenvalues structure provides a better comprehension of the dynamics of the system and our results indicate that evolutionary effects propagates differently for each set of interacting partners. In addition, our results indicate that, some species are tracking partners trait evolution, rather than equally affecting each other on the network. More than that, our results indicate that it can occur differently inside each set of species.

Second, selection asymmetry decreased the overall contribution of indirect effects to trait evolution, but canalized this contribution to just one trophic group (set of species). We expected that in interactions under symmetrical selective pressure, direct effects would have a higher importance in driving trait evolution. In this case, selection asymmetry would create a dispersion of indirect effects, making them more relevant. However, our results

show the opposite. Our simulations shows that even in asymmetrical scenarios indirect effects are important (Guimarães et al. 2017). Species that relies more heavily in the mutualism are more likely to evolve in response to changes in its interacting partners, which allows more ways of indirect effects to contribute to partners trait evolution. On the other hand, species that do not depend much on mutualistic interaction often will be affected just by directed interactions or even not respond to any evolutionary shift from their mutualistic partners in a way that even direct effects will not drive their evolutionary trajectory. The change in network topology of evolutionary effects in asymmetrical scenarios probably had an important role in driving indirect effects. Isolated subgroups have profound consequences for coevolutionary processes in networks, as the direct effects and even the indirect effects cannot cascade through some pathways, presenting each component an independent coevolutionary dynamic (Guimarães et al. 2007).

Third, our results indicated that the more asymmetric the selective pressure between mutualistic partners, the higher was the amount of evolutionary changes, mainly for the set of species that depended heavily on the mutualism. This result implies that species that relies heavily on mutualism endure a longer coevolutionary dynamics. Selection asymmetry also affected the distribution of traits across species. In our simulations, we observed that high levels of trait matching were only generated under high symmetrical mutualistic selective pressure. The overall community-level trait matching decreased with the increase in asymmetry selection, but the higher variance in trait matching in asymmetry scenarios indicated that still some pairwise interactions were coupled while others not. Mutualistic interactions often are taken as an outcome of trait complementarity evolution, in which there is a high degree of trait matching between interacting species, such as: *i)* similar functionality of extrafloral nectaries in phylogenetically nonrelated plants (Marazzi et al. 2013); *ii)* seed size and body mass of frugivores (Jordano 1995); *iii)* corolla depth and the length of hummingbird bills (Dalsgaard et al. 2008, Maglianesi et al. 2014, Maruyama et al.

2014, 2015, Vizentin-Bugoni et al. 2014); *iv)* tongue length of insects and the corolla depth of the flowers (Anderson and Johnson 2009, Sazatornil et al. 2016, Klumpers et al. 2019). But often these studies find that trait phenotypic pattern are not perfect coupled between all interacting partners in empirical asymmetrical mutualistic networks. Most of them reveals that some species are quite precisely matched while others not, what is in agreement with our simulations. We also observed the occurrence of phenotypic subgroups in one set of species in our bipartite simulated networks. Coevolution is known to generate trait convergence among species (Guimarães et al. 2011), but the novelty our results bring is that, through asymmetrical selection, coevolution could generate different degrees of convergence between distinct sets of mutualistic partners (Thompson 2009).

Combining our results on indirect effects contribution to trait evolution and a stronger selection imposed on plant traits, it might contribute to the understanding on why: *i)* floral traits tend to rapidly evolve and; *ii)* flowers and fruits often show evidence of trait convergence, leading pollination and seed dispersal syndromes, whereas similar trait patterns are less common in animals (Howe and Smallwood 1982, Schiestl and Johnson 2013, Schiestl 2017). In this context, indirect effects would be concentrated among plant species in plant-animal mutualisms, giving rise to trait convergence only in this set of species (Papiorek et al. 2016, LoPresti et al. 2020). For instance, in plant-hummingbird interactions in the tropics, convergent evolution is known to play a major role in shaping floral traits (Martín González et al. 2015, Wolowski et al. 2017). Bergamo et al. (2017) showed that phenotypic-matching pattern could structure the potential of indirect effects through shared pollinators and they discuss that plants may have evolved under the pressure of well-fitted pollen vectors (Castellanos et al. 2004). In mutualistic networks, it is expected frequent reciprocal changes even when it involves just few partners and not all species in the network (Guimarães et al. 2007). Here, we show that differences between the

selective pressure imposed by mutualistic partners would constrain coevolutionary dynamics. In that way, networks would rather evolve with some species being the drivers and others tracking the coevolutionary process but, mainly, inside the same trophic group. Therefore some species would constrain coevolution and assume a more important role in defining species traits in the community acting as a coevolutionary vortex (Thompson 2009, Guimarães et al. 2011). Together with the structured asymmetry in selective pressure, degrees of specialization of species to one another may contribute to the pattern of trait distribution observed in networks (Vázquez and Aizen 2004, Bascompte et al. 2006, Guimarães et al. 2006, Vázquez et al. 2007, Lomáscolo et al. 2019). Asymmetric specialization is widespread in nature, for instance, in ant-plant interactions, ants species had fewer partners than plant species, and ants were more dependent on the plants than the reverse, comprising a highly asymmetrical interaction (Fonseca and Ganade 1996). Asymmetric specialization could impact trait evolution by changing the selection differential for trait-matching in each species. Future work should investigate the role of asymmetric specialization in coevolutionary dynamics, which could change trait patterns.

Author Contribution

PCS, APAA and PRG conceived the study. PCS and APAA performed the simulations. PCS analyzed the data and wrote the first draft. All authors contributed to the final manuscript and gave final approval for publication.

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Male-male interference competition for space on pollinators

Pamela C. Santana[1]; Jake Mulvaney[2]; Monika Moir[2]; Erika M. Santana[1]; Bruce Anderson[2]

[1] Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil
[2] Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa

*corresponding author, e-mail: banderso.bruce@gmail.com

Abstract

Many plants have precise pollen placement strategies so that large amounts of pollen can be found over very small and discrete areas located on pollinators. This may lead to male-male competition if pre-existing pollen (1) is smothered by pollen from subsequent male flowers or (2) prevent subsequent pollen from attaching to pollinators. We investigated these alternative hypotheses using caged sunbirds (*Cinnyris chalybeus*) and sunbird pollinated flowers (*Tritoniosis antholyza).* Pollen from two different flowers were labelled with quantum dots so that the pollen grains could be distinguished. Two male phase flowers were offered in succession to sunbirds before the sunbird was allowed to visit a female phase flower. Secondly, we offered sunbirds a quantum dot-labelled flower followed by a flower without reproductive structures, so we could control the effect of time, before allowing the bird access to the female phase flower. We found that low numbers of labelled grains reached the stigmas of flowers. Moreover, we found that pollen from the second male flowers was better represented on the stigmas of subsequently visited female flowers. Our results suggest that pollen from earlier visited flowers is smothered by pollen from subsequently visited flowers. Smothering may act as male-male competition strategy in flowers that present stamp pollen.

 Keywords - gamete competition; male fitness; pollen movement; pollen precedence; pollen smothering.

Introduction

Pollen movement plays a key role in flower evolution and plant ecology. Since most plants are hermaphroditic, fitness is determined by two pathways: the female fitness pathway (seed production) and the male fitness pathway (siring success) (Minnaar et al., 2019). Success in the male fitness pathway will be given through mass export of pollen grains (Anderson and Minnaar, 2020). However, male fitness is expected to be highly variable: most pollen grains will not sire seeds and will be lost along a complex pollen journey, but it can potentially fertilize several ovules (Bateman, 1948; Minnaar et al., 2019). Therefore, male pathway, in general, is harder to be saturated comparable to the female pathway, because the number of male gametes is much higher than the female gametes (*i.e.* pollen/ovule ratio), so the potential to fertilize ovules is much higher than the potential to be fertilized. The bigger the amount of investment in male gametes, the more flowers have invested in attractive traits. Therefore it has been pointed out that the male fitness pathway is the main responsible for the evolution of showy flowers to pollinators (Bateman, 1948; Paterno et al., 2020). Despite the importance of processes that affect pollen movement and siring success for floral traits evolution, very little is known about the fates of the grains which are deposited onto pollinators (Ashman and Morgan, 2004; Opedal, 2021). A truly comprehension of the effect of the male pathway in reproductive ecology and flower evolution will require a combination of tracking the fate of pollen (Ellis and Johnson, 2010; Minnaar et al., 2019) and the paternity through genotyping the offspring.

For most angiosperms, animal vectors move pollen from one flower to another (Ollerton et al., 2011). Flowers have different strategies to place pollen on the pollinator's body (*i.e.* diffuse, stamp, stroke) (Minnaar et al., 2019). These different pollen placement strategies are likely to generate distinct pollen landscapes on the bodies of pollinators and this may generate male-male competition where males compete for a good placement (Stanton, 1994). Flowers with very precise pollen

placement strategies (*e.g.* stroke or stamp) may generate three-dimensional, layered pollen landscapes on the pollinator's body (Minnaar et al., 2019). This may result in intrasexual competition even before pollen has been deposited on another stigma (Armbruster et al., 2009; Minnaar et al., 2019). Muchhala & Thomson (2012) demonstrated that plants of different species compete for space on the pollinators' body and that different species can displace or smother granular pollen from previous visits. Intraspecifically, competition between pollen grains may be equally or even more intense because there is likely to be more overlap of pollen placement between plants of the same species than there would be between plants of different species. Anderson & Minnaar (2020) suggested that competition between rival pollen grains may occur on the bodies of pollinators through smothering or pollen preclusion.

Pollen smothering may occur when flowers are able to cover pre-existing pollen from previous visits, so their pollen has a higher probability of reaching the stigma of subsequently visited flowers (Minnaar et al., 2019). Pollen preclusion may occur when pre-existing pollen loads prevent or preclude the deposition of new pollen grains onto pollinators (Harder et al., 2021). In pollen preclusion, we could expect that the amount of pollen deposited onto a pollinator will be inversely proportional to the amount of pre-existing pollen on that pollinator (Figure 1). Furthermore, we may expect that earlier-visited flowers may deposit more pollen than later-visited flowers. To the best of our knowledge, only one study has to date tried to document such competition in plants with granular pollen. Moir et al (*In review*) found evidence of pollen layering on fly pollinators and that this may cause pollen preclusion. There are also a few examples of both pollen preclusion and smothering in plants that package their pollen in pollinaria (Cocucci et al., 2014; Duffy and Johnson, 2014; Harder et al., 2021). Cocucci et al. (2014) found evidence for both preclusion and smothering exerted by milkweed pollinaria: some species possess pollinarias with horns that prevent the attachment of pollinaria from the subsequent visits. Furthermore, other species possess pollinaria which attach to and smother pollinaria which were previously

placed on pollinators (Cocucci et al., 2014). Which conditions or traits determine which strategies will take place, smothering or preclusion, is still unkonwn.

To explore the effects on the success of reaching the stigma through pollen deposition into the pollinator body, we tested two alternative hypotheses (Figure 1): *i) pollen preclusion* – where pollen from the first-visited male flower has a higher probability of being deposited on the stigma of a subsequently visited flower; *ii) pollen smothering* – where pollen from the last flower visited has a higher probability of been deposited on the next flower's stigma. To test these hypotheses, we did experiments using caged sunbirds (*Cinnyris chalybeus,* Nectariniidae) and sunbird pollinated flowers (*Tritoniosis antholyza,* Iridaceae). We chose *T. antholyza* as a study system, which is visited by sunbirds and has hermaphroditic flowers, but the sexes are separated in time. We were able to track deposition success of different flowers after their pollen was labelled with different colors using Quantum dots. We found evidence suggesting that pollen grains may smother the rival grains that were previously deposited on pollinators (Arnold, 1994; Delph and Ashman, 2006; Willson, 1979).
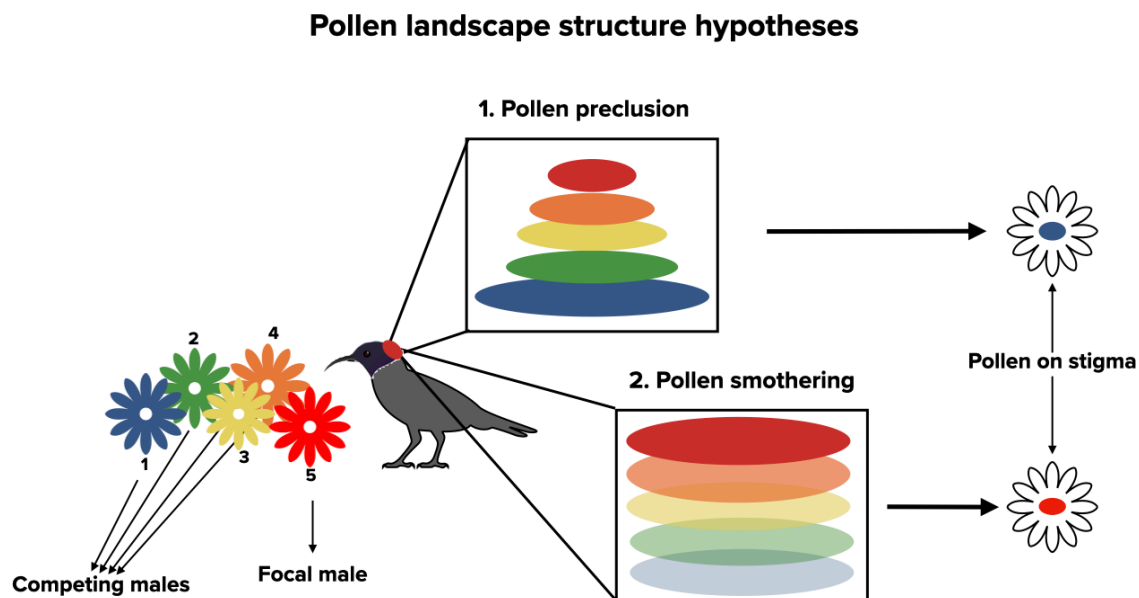

Figure 1: Two alternative pollen landscape structure hypotheses. Flowers are visited sequentially by the sunbird pollinator, represented by numbers at the flowers. The same sequence of visits may

generate two different structures of pollen landscape and affect the probability of being deposited on the stigma of a subsequently visited flower: *i) pollen preclusion* – Where pollen from the first male is better represented on pollinators and has a proportionately higher probability of being deposited on the next flower's stigma; *ii) pollen smothering* – where pollen from the last flower smothers and prevents the removal of previously deposited pollen.

Materials and methods

Study site and species

This study was conducted in the Fynbos biome, on private properties (with landowner permission) in Betty's Bay and in Stellenbosch, Western Cape, South Africa, under the CapeNature permit number CN41-28-16214 and SAFRING ringer number 1622. We conducted experiments between October and December/2021. *Tritoniosis antholyza* is a summer flowering species from the Iridaceae family, endemic to South Africa, which presents spirally arranged flowers that mature from the bottom upwards (Manning and Goldblatt, 2005). Individual flowers open sequentially within the inflorescence. Flowers from *T. antholyza* are hermaphrodites and protandrous, they first open in male-phase and after two days the stamens reflex backwards and the style elongates as the flower becomes female-phase (Figure 2). *T. antholyza* flowers have three anthers positioned under the upper tepal. Pollen is deposited on the dorsal surface of the sunbird pollinator's head as it inserts its bill into the deep corolla (Manning & Goldblatt, 2005 - Figure 2).
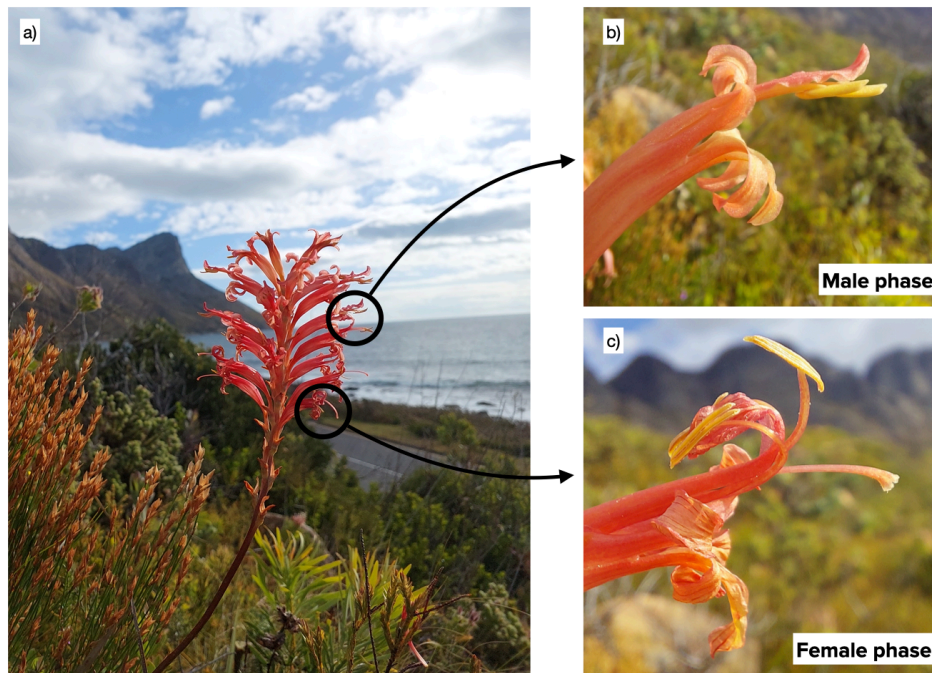
Figure 2: *Tritonipsos antholyza* inflorescence, showing flowers spirally arranged, and maturing so that male flowers are on the top and female flowers are at the bottom. Photos by Bruce Anderson.

Sunbirds are frequent visitors and effective pollinators of *T. antholyza*. In general, sunbirds visit several flowers in the inflorescence. They probe each flower directly from the front. Sunbirds cannot easily groom pollen from where it is deposited on their heads, so pollen layers may build up without significant disturbance. We captured specimens of the sunbird (*C. chalybeus*) with mist nets at sunrise and near sunset, to avoid heat stress on the birds. The nets were continuously monitored for catches and birds were removed immediately. All sunbird individuals we caught during the study period were fitted with a ring for identification. We released fledglings, birds with brood patches, and birds in molt immediately after capture. Once caught, the sunbirds were placed individually in birdcages (between 100x60x60 cm and 80x60x60 cm in size) that were covered to reduce stress on the birds. A 20% w/w sucrose solution with the addition of a nutritional supplement (Ensure®, Abbot Laboratories, Johannesburg, South Africa) for vitamins, minerals, and protein (Fleming et al., 2004; Lerch-Henning and Nicolson, 2013; Peaker et al., 1990) was

always available in the birdcages. The cages were kept protected from rain, wind, and cold temperatures to avoid physiologically stressing the experimental birds. The feeders were Eppendorfs with a small hole on top and a flower with an open bottom placed in the Eppendorf hole. We added an invisible tape at the feeder's side to prevent feeding directly from the Eppendorfs, without probing the flower entrance. The feeders were constantly filled throughout the day. Perching branches and water baths were provided inside the cages. After sunbird acclimation (approximately between 3 and 8 hours), we started the experimental procedures by offering labelled flowers to them. We conducted the experiments with eight individuals. Each bird was used, on average, for five experimental trials, retained for a maximum of four days, and released at the site of capture.

Male-phase flower presentation experiments

To investigate the consequences of pollen layering, we conducted 43 pairwise experiments, in which sunbirds were allowed to visit a sequence of two male-phase flowers. We used Quantum dots to label pollen grains and conducted experiments to investigate the possibility of pollen layering (*for details* Minnaar & Anderson, 2019). We labelled all three newly dehisced anthers per flower with the same color of Quantum dot solution (from now called as Qdot). Between 50 µl – 60 µl was required to visibly saturate each anther. We used three colors of Qdot solution (green – 523 nm, pink – 628 nm, yellow 590 nm) to label the flowers and differentiate the pollen grains. All flowers were collected between three to one day before experiments, to assure they were not visited by pollinators and had their complete pollen load at the anthers. For the male - phase flowers, only those with all of their anthers dehisced were used in the experiments. For the female - phase, we removed the anthers from the flower, allowing the stigma to be without obstacles in front.

Each experimental run consisted of the following steps: 1) allowing a sunbird to make three probes into a single male-phase flower with all anthers saturated with Qdot; 2) removing the first flower and replacing it with another male-phase flower, but with pollen grains labelled a different color using Qdots; 3) allowing the pollinator to probe the new flower three times before removing the flower and replacing it with a flower in the female phase; 4) allowing the pollinator to probe the female flower three times; 5) The stigma was harvested and viewed under a Leica M125 dissecting microscope using a Qdot excitation box (*see* Minnaar & Anderson, 2019); 6) by tracking the pollen grain allowed us to quantify the number of pollen deposited by the first and second male flower. Because pollen grains are expected to be lost simply as a time-effect where pollinators may carry more pollen from the last flowers because there less time for it to fall off, we implemented a control to distinguish this effect from pollen competition effects. Here, instead of visiting a second flower in male phase, we allowed the sunbird to visit a second male-phase flower that had been emasculated. Experiments were always run in pairs (one control and one treatment presentation) but in random order with a 30 min interval. Qdots colors used to mark the first and second flower were also randomized to prevent us that any effect of color fixation would be connected to one treatment. Between the experimental and control run, we fed sunbirds using flowers in Eppendorfs. However, these flowers had their reproductive parts removed and replaced with clear adhesive tape attached to the upper tepal. When the birds fed from these flowers, any remaining pollen grains on the head of the bird were removed by the tape. We waited one-hour before starting a pair of presentations and during this hour, pollinators were also allowed to feed from a flower with adhesive tape to remove pollen grains from the bird. We ran the experiments during the daytime, presenting on average three pairwise treatments per day per bird. In total, we conducted 86 experimental runs, 43 controls, and 43 treatments.

Data analyses

In these experiments, we tested two alternative predictions compared to our control (Figure 3), one for each hypothesis: 1) pollen preclusion, in which there is a positive relationship between sequence position and the number of pollen grains on the pollinator, possibly due to a pollen interaction effect; 2) pollen smothering, in which pollen from the most recently visited flowers will be better represented on the female stigmas than pollen from flowers visited earlier in the sequence (a negative relationship between positions in the sequence and the number of grains exported). Our control allowed us to verify the effect of time once the pollinator visit the flower, if it is a time effect, the control will be smaller than the others. A negative or positive relationship between the sequence and the amount of pollen will demonstrate the evidence of pollen layering. We expect that in many cases, no pollen will be transferred from some donors. As a control for time effect, we presented flowers without reproductive structure on second male place, which allow us to have a reference in case the presence of pollen was actually related to the amount of time pollen is on pollinator body.
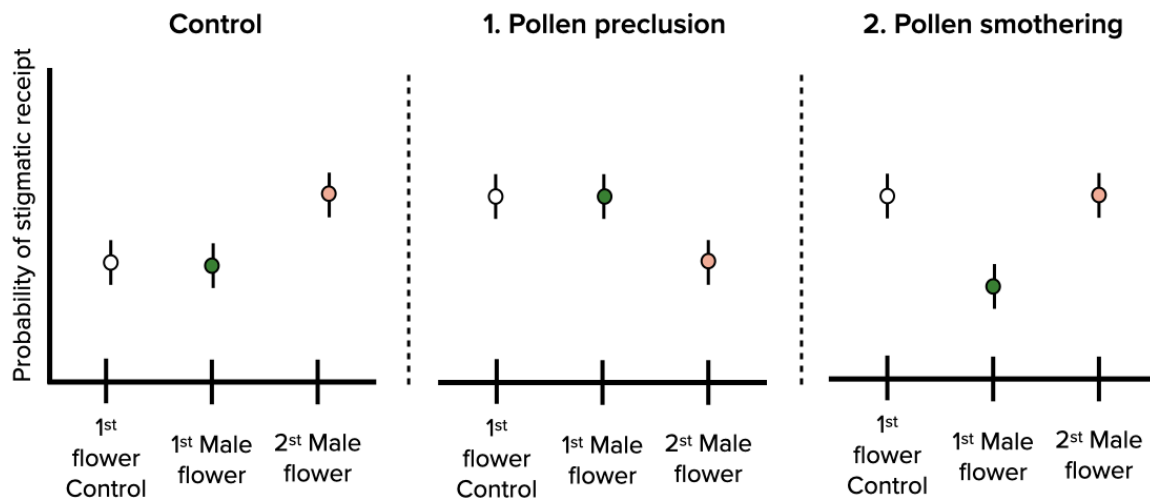
Figure 3: In our **control**, we expect that the second male flower is better represented because its pollen has spent less time on pollinator body than either the control flower or the first male flower. In addition, we tested two alternative predictions: 1) **Pollen preclusion** - the first flower is better represented than the second flower because it prevents the second flower from placing pollen on the pollinator 2) **Pollen smothering -** the second flower is better represented than the first flower but the control flower is also well represented because it is not smothered by the pollen from a second flower.

To test these hypotheses, we counted the number of pollen grains deposited by each of the male flowers onto the stigma of the female flower. Twelve of the 43 replications (~27%) did not place pollen on the stigma. Therefore, to analyze this data we first looked at the probability of achieving the stigma by analyzing the data from presence x absence of pollen grains (a binomial perspective). We used generalized linear mixed-effects binomial models (with a logit link function and Laplace maximum likelihood approximation of theta) in which the success of achieving the stigma by pollen grains was the response variable in function of the visit sequence number and treatment. The identity of the bird was added to the models as a random factor. Pollen transfer was highly variable (varying from 0 to 150 pollen grains). We compared negative binomial, hurdle (poisson and negative binomial) and zero-inflated models (poisson and negative binomial). The last two types of models split the response variable into two latent variables and account for more than one process

generating the failure of achieving the stigma. For instance, pollen was not deposited onto the pollinator body, alternatively, pollen may be deposited on the pollinator body, but was not deposited onto the stigma surface. It means that there is a zero-response probability and a count portion probability (the magnitude of the variable - number of pollen grains that achieved the stigma). We used model selection based on Akaike Information Criterion (AIC) to infer the models that best fit our data and we used the criteria of equality plausible models with an AIC lower than 2. We compared the pairwise factor combinations of our selected model using Marginal means (or least-squared means) through *emmeans* (v1.5.5-1 Lenth, R.V. 2021) and *phia* (Rosario-Martínez, H. 2015 v0.2.1) packages, by computing contrasts of EMMs between the levels of fixed factors. The confidence level adjustment was conducted with Tukey method for comparing a family of 3 estimates and we back-transformed from the log scale to obtain the estimates. All analyses were done in R (R Core Team, 2022, version 4.0.2), using the packages *lme4* for linear models (Bates et al., 2015), *MASS* (Venables and Ripley, 2015), *pscl* (Jackman, 2015), *AER* (Kleiber and Zeileis, 2022).

Results

Pollen transfer was often ineffective and when we found pollen on stigmas, the mean number of pollen grains was low (Treatment mean: 0,70 - Control mean: 3,7 grains), while the variance was high (Treatment variance: 3,31 - Control variance: 61,04 grains). Our results indicated a significant treatment effect on the probability of depositing pollen onto the stigma of the last flower (Figure 4). In particular, the control (95% confidence interval: 0,28-0,68) and the second male (95% confidence interval: 0,06-0,34) had double the probability of depositing pollen, compared to the first male (95% confidence interval: 0,33-0,73). The higher pollen deposition by the

second male is suggestive of a smothering effect, equal deposition of the control and second male suggests that there is no time effect.
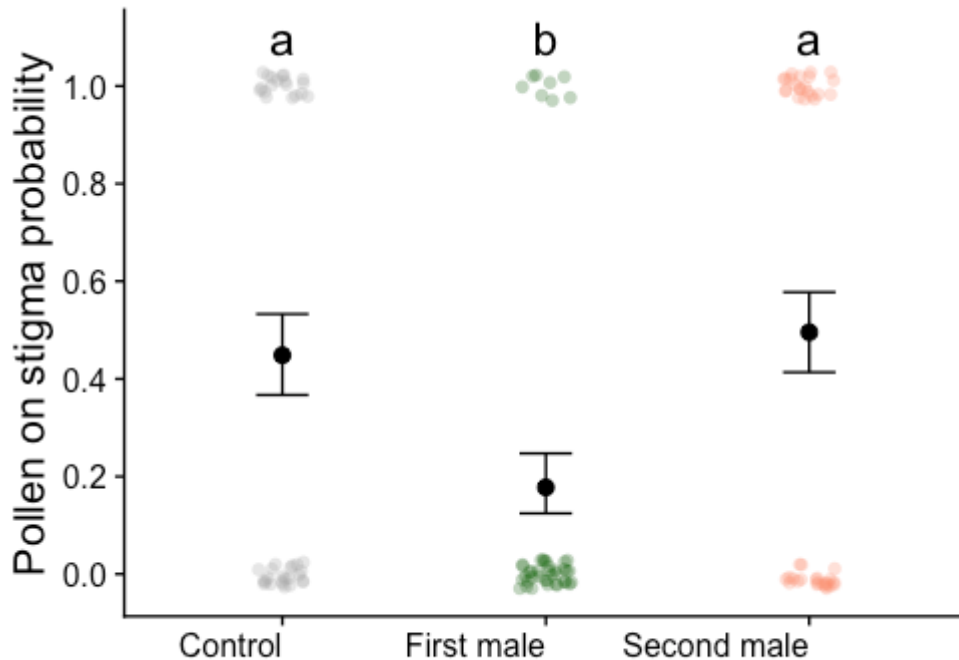

Figure 4: Probability of pollen achieving the stigma according to the sequence of visits. Estimates from the model (mean; inferior value of standard error - SE inf; superior value of standard error - SE sup): Control (0,3; 0,45; 0,53); Treatments - First male (0,12;0,18;0,25); Second male (0,41; 0,49;0,57). Letters show statistically significant differences among treatments according to Tukey method for pairwise comparisons of interval confidence.

The pattern was similar when we looked at the number of pollen grains on the stigma. The control deposition (95% confidence interval: 0,675-3,009) was not different from the deposition by the second male (95% confidence interval: 0,958-4,517), but it was different from the first male (95% confidence interval: 0,154-0,983). However, the second male deposited five times more pollen than the first male (Figure 5) onto the stigmas of subsequently visited flowers.
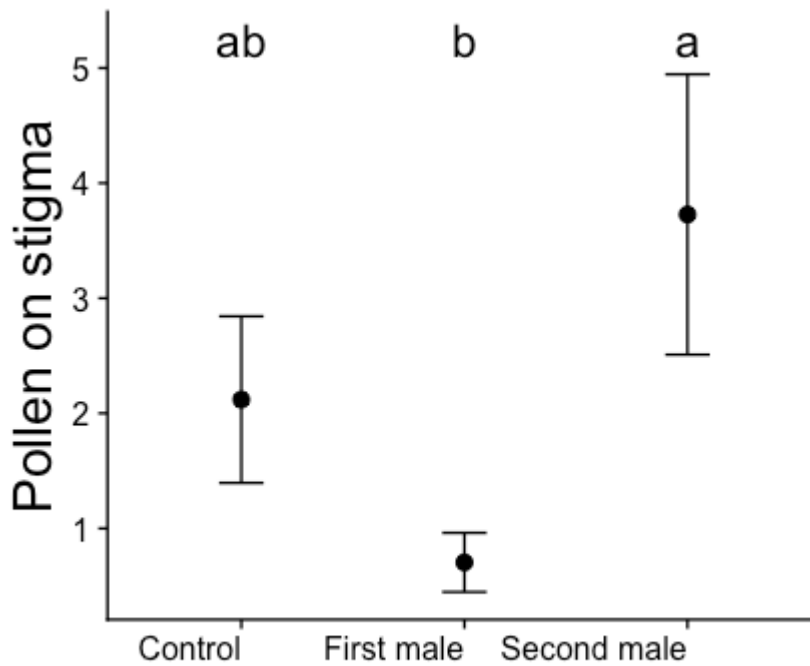
Figure 5: Number of pollen grains deposited, depending on the sequence of visiting. Estimates from the model (mean; SE inf; SE sup): Control (2,12; 1,4; 2,84); Treatments - First male (0,70; 0,96; 0,44); Second male (3,73; 2,51; 4,95). Letters show statistically significant differences among treatments according to pairwise comparisons.

Discussion

We show that the second flower has a greater probability of depositing pollen onto the stigma of a female flower and also deposits more pollen than the first flower visited. We also show that this pattern cannot be attributed to a time effect as the second flower and the first control flower were equally successful. Overall, these results suggest that smothering may be taking place in this experimental setup, where pollen from the most recently visited flowers is better positioned for pollen removal than pollen from previously visited flowers. This is the first study to demonstrate such a smothering effect in plants with granular pollen. Below, we discuss how our findings contribute to the understanding of the pollen movement, particularly about how

smothering strategy may be a result of male-male competition, and thus, affect flower fitness via male pathway.

The evidence of layering highlight traits that may affect plant reproductive ecology and sexual selection in plants (Minnaar et al., 2019). Several aspects are involved in the efficiency of pollen transfer from the anthers to stigma of another individual, like the mechanical fit with pollinators (Campbell et al., 2014). Flower morphology may have a central role in generating pollen smothering. In the case of *T. antholyza,* upper petal may act pressing the anther on pollinator head and allowing the smothering. The evolution of the pollen smothering x pollen preclusion is probably a consequence of the interplay between the microscale of pollen structure and chemistries and pollinator surface in an evolutionary trajectory of optimizing the efficiency of pollen transfer. Lin et al (2013) showed that a combination of surface morphology of the pollen (size and shape of echinate or reticulate features) with the pollenkitt volume provides pollens with a remarkably adhesion to surfaces. They found that the adherence capacity was higher for plants that depend on insect-pollination related to wind-pollinated flowers (Lin et al., 2013). The exact mechanism of pollen adhesion is still unknown, however this is probably an important trait under selection to generate either pollen preclusion or pollen smothering. Other floral traits may also play a role, like: 1) pollen viability; 2) mode of pollen dispensing; 3) anthers structure; 4) flower longevity; 5) flower display size, besides others which are connected to the pollinator traits, as 1) pollinators' body texture and 2) foraging patterns. All these traits may affect the success of these two strategies. For instance, pollinators that groom or eat pollen, like bees, which use pollen to feed their young, have a few protected positions on their bodies where they are not able to reach what decreases the surface that pollen can be attached and have success (Tong and Huang, 2018). The decrease in surface to transport of pollen can increase competition but also shake pollen layers. Smothering may work for pollinators that do not groom, once

pollen will be able to be in the pollinator's body for a period of time, as is the case of sunbirds. In addition, pollen smothering may be linked to feathers, fur or hairs, which take longer to saturate (Muchhala and Thomson, 2010). On the other side, pollen preclusion can be linked to firm substrates, as it is easier to attach. The existence of layering may be the explanation of why some flowers, such as Lobelias, present structures that may brush and displace the previous pollen grains on pollinator's body before depositing their own, which can be a counter-strategy (Minnaar et al., 2019).

Pollen smothering promoted by *T. antholyza* flowers may have an even more important role to male fitness pathway when we consider the differences that flowers in the male and female phases present in the order of maturation and in the position that they occur in the plant. *Tritoniopsis antholyza* female-phase flowers develop first, in the bottom of plant and are typically visited by sunbirds before the male-phase flowers, which develop after and on top of the plant. Harder et al. (2021) already registered similar behavior for bee pollinators, which usually visit flowers from the bottom upwards, visiting first the female flowers and then the male flowers. While this is known to reduce geitonogamy (Harder et al., 2000), it may also increase pollen export if the female - phase flowers clean pre-existing pollen from rival individuals, allowing the male - phase flowers to place pollen onto relatively pollen-free pollinators. In addition, combined with our results, visiting more than one male-phase flower can increase pollen export for one individual (Figure 5). A similar process is sperm precedence in animals that have outside fertilization (*e.g.* Dubey et al., 2018, , see (Wade and Arnold, 1980). However, different from animals, in which sperm precedence means different donors of sperm, for plants it can be the same donor. Therefore, pollen smothering may compose the total pollen in the pollinator and increase the chance of dispersion to the next individual from the youngest flowers and pollen grains, which can be more viable (Souza et al., 2022). In this way, pollen smothering would promote gamete inundation on the stigmatic surface, by a higher

deposition of pollen, as we show in our results. By depositing a higher amount of pollen grains, it increases the chance of the gamete pools to be represented on the stigmatic surfaces and to have, at least, one gamete able to succeed in ovule fertilization (Ganeshaiah and Shaanker;, 2001; Stanton, 1994). This process is probably more prominent in plants with few ovules, since the more limited are female gametes, the higher the competition the male should face. An alternative interpretation to our results is that the second male may have an advantage because previous pollen grains increase the adherence of new pollen grains.

Moore & Pannell (2011) proposed that coevolution plays a role in floral trait evolution specially through male fitness, because male gametes are more abundant and have the potential to increase the total fitness of a plant, therefore intensifying sexual selection. We have evidence that sexual selection may act on exine traits that facilitate male mating success by influencing the transfer of pollen from the anther to the body of the pollinator and that these traits vary among pollinators (Lynn et al., 2020). Therefore, through male - male competition plants may have evolved the ability of covering previous pollen grains. An important caveat is that in our study, it is possible that pollinators never got fully saturated with pollen, because we allowed only few visits. Therefore, further studies are important to better-comprehend pollen movement and the role that male fitness pathway may play in floral evolution and ecology.

Conclusions

Our study shows that the sequence of visiting flowers affected the probability of reaching the next stigma. Pollen from the last male-phase flower visited can smother the pollen from previously visited flowers. This can increase the reproductive success of the most recently visited flowers and male x male competition may promote the evolution of smothering as an evolutionary strategy. However, it is still unclear

how the relative roles of smothering versus preclusion will play out under different ecological contexts. There is knowledge gap concerning the role of pollination transport and pollinators on male-male competition and floral evolution.

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Conclusão geral

Essa tese investigou as consequências de assimetrias que ocorrem em interações mutualistas e como elas influenciam a dinâmica coevolutiva gerando padrões em características que medeiam as interações. Para tal, nós unimos conhecimento da literatura de história natural de interações com modelos matemáticos e experimentos a fim de contribuir com o entendimento mecanístico de processos coevolutivos. Por meio deste estudo, foi possível verificar fontes de variação capazes de gerar assimetrias nas forças evolutivas e que estão espalhadas pela natureza. Contudo, estas assimetrias não estão distribuídas de forma aleatória, mas estão estruturadas em diferentes aspectos. Primeiro, a assimetria de força seletiva ocorrerá de maneira distinta de acordo com quanto uma determinada característica contribui para a eficiência da interação. Para casos de polinização, em especial, propomos verificar os processos coevolutivos em dois passos: *i)* a atração do polinizador e a identificação da flor pelo polinizador; *ii)* o encaixe morfológico entre as estruturas da flor e a morfologia e fisiologia do polinizador. Potencialmente, processos coevolutivos simétricos ocorrem primariamente no segundo passo, dado que são características que não são utilizadas para outras funções essenciais do polinizador. Desta maneira, espera-se uma forte pressão de seleção simétrica entre essas características e potencialmente coevolução. Segundo, nós exploramos como assimetrias de seleção estruturadas nas guildas podem mudar a propagação de efeitos (co)evolutivos em redes mutualistas. Nós encontramos que as assimetrias entre guildas alteram o efeito que a estrutura da rede possui para a propagação de efeitos evolutivos em cada guilda. Adicionalmente, encontramos que os efeitos indiretos se tornam concentrados em uma única guilda. Por fim, podem gerar padrões de distribuição de características com maior convergência em uma guilda do que em outra. Estes resultados indicam que padrões de convergência em características que medeiam interações são um resultado não só de pressões diretas e assimétricas, mas também de como os efeitos evolutivos indiretos se propagam pela rede. Esta consequência está diretamente ligada à estrutura bipartida da rede. Terceiro, a

assimetria também está estruturada nas redes de interação, por meio do papel que a espécie ocupa na rede. Por fim, como a maioria das plantas são hermafroditas, nós investigamos como o comportamento de visita do polinizador às flores pode afetar as chances de competição entre machos. Nossos resultados indicam que a estratégia evolutiva de cobrir grãos de pólen de potenciais competidores pode ter evoluído em plantas que apresentam o grão de pólen de forma concentrada e polinizadores que possuem penas. Conjuntamente, nossos resultados contribuem para a compreensão de como assimetrias no papel funcional das características entre espécies que interagem, diferentes benefícios provenientes da interação e a estrutura da rede podem alterar padrões fenotípicos em comunidades.

Para estudos futuros, vejo cinco passos importantes a serem dados: *i)* identificar quais são os genes sob seleção na interação. A identificação de tais genes pode ser feita por meio do uso de mapeamento genético (*manhattan plots*) em estudos de genomas completos (*genome-wide association studies* - GWAS); *ii)* estimar a força da pressão de seleção para ambas as espécies que estão interagindo, de maneira que seja possível quantificar as assimetrias. A maioria dos estudos se concentram em impacto da interação no valor adaptativo das plantas, que são de mais fácil manipulação. A definição do valor adaptativo da interação para os animais é mais difícil ser estimada já que manter animais por toda a sua vida em condições controladas é desafiador. Contudo, só saberemos como a simetria varia na natureza se pudermos estimar a força de seleção em ambas as espécies e em mais de uma característica envolvida na seleção; *iii)* unir a estimativa de valor adaptativo para o componente feminino e masculino, pois a maioria dos estudos estimam o valor adaptativo da interação para a planta somente com o componente feminino. Porém, o componente masculino pode ser ainda mais importante em dinâmicas coevolutivas, como discutido no último capítulo desta tese. Para isso, será necessário unir as rotas de dispersão de pólen com análises de paternidade; *iv)* verificar qual a contribuição de diferentes espécies ao valor adaptativo final de uma espécie focal, ampliando a escala da dinâmica coevolutiva para o contexto da comunidade. É importante compreender se tais pressões são

conflitantes; *v)* estimar empiricamente a contribuição de efeitos indiretos para a dinâmica coevolutiva em redes de interação, pois tal contribuição é conhecida somente de forma teórica. Para tanto, estudos de seleção natural e evolução precisam ser feitos em distintos ambientes e contextos de múltiplas espécies; *vi*) conectar padrões fenotípicos em mais de uma característica envolvida na interação nas comunidades ecológicas, através de medidas quantitativas das características em espécies que co-ocorrem. Tais medidas nos permitirão testar as previsões teóricas dos modelos matemáticos de coevolução em comunidades e através de módulos funcionais.