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On the evolution of floral traits

Variation in complex floral phenotypes across pollination environments

Hildesheim, Laura Sophie

2025

Link to publication

Citation for published version (APA):
Hildesheim, L. S. (2025). *On the evolution of floral traits: Variation in complex floral phenotypes across pollination environments* . Lund University.

Total number of authors:
1

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On the evolution of floral traits

Variation in complex floral phenotypes across pollination environments

LAURA S. HILDESHEIM

DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

List of papers

I. Hildesheim, LS, Friberg, M, Armbruster, WS, Opedal, ØH. Scent evolution is associated with pollinator shifts in a specialized pollination system. *Unpublished manuscript*.

II. Opedal, ØH, Hildesheim, LS, Armbruster, WS (2022). Evolvability and constraint in the evolution of three-dimensional flower morphology. *American Journal of Botany*, 109(11): 1906-1917.

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Printed by Media-Tryck, Lund 2025 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Faculty of Science
Department of Biology

ISBN 978-91-8104-185-9

On the evolution of floral traits

Variation in complex floral phenotypes across pollination environments

Laura S. Hildesheim

DOCTORAL DISSERTATION

By due permission of the Faculty of Science, Lund University, Sweden.
To be defended in The Blue Hall, Ecology building, Kontaktvägen 10, Lund.
March 28, 2025.

Faculty opponent
Dr. Tia-Lynn Ashman
University of Pittsburgh, Pittsburgh, USA

Organization LUND UNIVERSITY

Document name Doctoral dissertation **Date of issue** 10-Feb-2025

Author Laura S. Hildesheim

Title and subtitle On the evolution of floral traits - Variation in complex floral phenotypes across pollination environments

Abstract

Pollinators play a central role in shaping the astounding diversity of angiosperm flowers. Flowers are complex, multidimensional, genetically and functionally integrated phenotypes, rendering it a challenge to quantify and interpret observed trait variation. Even variation in simpler trait measurements is a challenge to interpret, because of the multitude of selective agents and evolutionary processes shaping floral form. The long-term pollination environment of a plant population is reflected in its pollination- and mating system. Here, I employed a geographic comparative approach, using natural variation in floral traits across pollination environments to understand the evolution of complex floral traits along the pathway to pollination. Specifically, I studied the evolution of floral scent as a pollinator advertisement and reward trait, flower-pollinator fit traits that mediate successful pollen transfer, and pollen performance traits involved in the fertilization process. First, I associated scent evolution with pollinator shifts in a specialized pollination system. Biosynthetically novel scent compounds mediated a shift from floral scent as a pollinator advertisement to a pollinator reward and precipitated a qualitative pollinator shift. Second, I quantified genetic and functional constraints in the evolution of pollinator fit traits as part of three-dimensional flower morphology. The evolutionary potential of each trait was tightly linked to its realized evolution. Third, I quantified components of variance in pollen performance across mating systems. Patterns of pollen performance varied idiosyncratically across pollination environments due to other sources of variation. Lastly, I studied the joint evolution of pollen longevity and mating system. Pollen longevity was longest in the most unreliable pollination environment allowing for delayed pollination. This thesis contributes to a better understanding of the role of pollinators in the diversification of complex floral phenotypes. I combined an understanding of the complexity of the traits under study, profound knowledge of the ecology and natural history of the study systems, and insight into the evolutionary processes shaping trait variation, to understand the evolution of complex floral phenotypes. This work is crucial for understanding the evolution of floral diversity, as well as predicting the consequences of changing pollinator communities for plant fitness.

Key words *Arabis alpina*, biosynthetic novelty, complex phenotypes, *Dalechampia*, delayed pollination, evolvability, floral scent, flower evolution, genetic constraints, mating system, plasticity, pollen longevity, pollen performance, pollination environment, pollinator fit, pollinator reward, trait variation, specialized pollination system

Language English **Number of pages** 67

ISBN (print) 978-91-8104-185-9 **ISBN (electronic)** 978-91-8104-186-6

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On the evolution of floral traits

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Faculty of Science
Department of Biology

ISBN (print): 978-91-8104-185-9
ISBN (electronic): 978-91-8104-186-6

Printed in Sweden by Media-Tryck, Lund University
Lund 2025

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MADE IN SWEDEN

Printed matter
3041 0903

The world is full of mysteries. Life is one.
The causes of evolution (1932), p.6
J.B.S. Haldane

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Author contributions

I. **LSH**, MF, and ØHO designed the experiments. **LSH** collected the data. **LSH** lead the data analysis with suggestions from MF and ØHO. MF, WSA, and ØHO contributed to discussions. **LSH** lead the writing of the manuscript with contributions from all authors.

II. ØHO conducted field and greenhouse work, curated and analyzed data, crafted figures, and wrote the first draft of the manuscript. WSA conducted fieldwork, **LSH** conducted greenhouse work, and all authors contributed to manuscript revisions.

III. **LSH** conceived of the study; **LSH**, HET, MF and ØHO developed the study and designed the experiments; **LSH** and SB collected the data with contributions from HET; **LSH** analyzed the data with contributions from ØHO; and **LSH** lead the writing. All authors contributed to discussions and writing of the manuscript.

IV. **LSH** conceived of the study, designed the experiments with suggestions from ØHO, collected the data, and analyzed the data with advice from ØHO. **LSH** lead the writing of the manuscript with contributions from ØHO.

LSH: Laura S. Hildesheim. ØHO: Øystein H. Opedal. MF: Magne Friberg. WSA: W. Scott Armbruster. SB: Sidney Blackburn. HET: Hanna E. Thosteman.

Author description

Laura S. Hildesheim

I am an evolutionary biologist with a strong interest in pollination ecology and floral evolution. For the past decade of my life, I have been studying the evolution of floral traits in response to the pollinator environment. All the work I have been doing, all the knowledge and experience I have accumulated, and all the interests I have discovered, cumulate in this PhD thesis. The greatest lesson I have learned while completing this thesis is: Progress over perfection! Photo credit: Kamile Leed.

Acknowledgements

From the bottom of my heart, I thank **Øystein Opedal**. For the past eight years, we have witnessed each other’s careers, lives, and personalities grow. Your academic brilliance is inspiring, and knowing you, has profoundly changed my perspective on pollination ecology, evolutionary theory, and analytical methods. I am beyond grateful for your influence on me. Over the years, we have truly evolved as researchers and humans, haven´t we?

I thank **Magne Friberg**, the busiest man alive, for always making time for discussions about anything evolutionary ecology, and for your help with all the practical challenges that a PhD encompasses. You bring the fun into science, and have often been my reminder to enjoy my PhD. Your kindness (and your dad jokes) make you stand out in the best way possible.

I thank **Erik Svensson** for being my mentor. Much of my development as a researcher, academic, and evolutionary biologist have happened in conversation with you. Because you let me be an honorary member of your lab, I could not only broaden my knowledge and understanding of evolution but also make an abundance of friends. I am forever grateful for your kindness, guidance, and support, I wouldn´t be here without you!

I thank **Karin Rengefors** for being my departmental representative. You brought the structure, consistency, and continuity into my PhD that I so desperately needed. Even when you changed job responsibilities, you chose to stay a part of my PhD journey, and I genuinely appreciate you for that!

I thank the SPACE PhDs for being excellent companions since day one. **Hanna**, you have been the best office mate, friend, and PhD sister I could have wished for. You were the first friend I made in Lund and the best office mate for 5(!) years. Our discussions about science and life have kept me on track at all times, and for that I am forever thankful. **Kalle**, you have been at the core of my social life here in Lund and I fondly remember all the many times we have been out and about. I cannot put into words how much I appreciate your friendship and support. **Zach**, we started our PhDs on the same fateful day all these years ago. Since then, we have been each other’s friends, therapists, and statistics and programming advisors. I am so thankful to know you! **Alex**, you joined us a little bit later, and you have been the best addition I could have wished for. I enjoy every conversation with you! I stopped thinking about all of you as colleagues a long time ago, because you are first and foremost my friends. I am so so so grateful for all of you.

I thank the horde of flower post-docs, aspiring PhDs, students, and interns that have come through the SPACE lab, and especially the flower group, throughout the years. A special shoutout to **Aarushi**, **Anna L.**, **Cheng**, **Felipe**, **Hampus**, **Juan**, **Jule**, **Kajsa S.**, **Kate**, and **Sidney,** anyone who has you as their colleague or friend is lucky to have you. **Pam**, **Rachel**, **Sissi**, and **Yedra**, beyond being inspiring role models as researchers, academics, and women in science, I appreciate you for your genuine support during my PhD. A big thanks to everyone in the extended **SPACE lab** (present and past) for being my scientific and social support network!

I thank **Cristina**, **Rocío**, and **Scott** for being the best field buddies in French Guiana. I had so much fun and made so many unforgettable memories, because of you. Thank you for accompanying me on one of the greater adventures of my PhD (and my life)!

I thank the third-floor crowd for the genuine support, insightful chats, and fun nights out! I appreciate **Cilla** for the kindness, **Colin** for always having a smirk on your face, **Emma** for the unforgettable girls' nights out, **Erika** for being a powerhouse, **Iain** for keeping it cool, **Johan** for always having a smile on your face, **Josefin** for turning everything into fun, **Julia** for the quick wits, **Jöran** for spreading a good mood, **Lydwin** for being inspiringly ambitious, **Masahito** for being an scientific and personal inspiration, **Pedro** for always spreading a good mood, **Pet** for being a fellow foodie, **Robin** for all those very fun writing sessions, **Ruben** for immediately being a friend, **Simon** for the many hilarious or deep (or both) conversations, parties, and winter baths in the ocean, your friendship means everything, **Sofia** for supplying me with pet stories, **Sofie** for being a ray of sunshine and making my day better without fail, **Stefan** for teaching me how to be a teacher, and **Sridar** for the relaxing chats.

I thank the former and current second-floor crowd for being great companions for all these years! I appreciate **Angela** for always being kind to me, **Ann-Katrin** for always being fun to chat with, **Arne** for being a calming presence, **Elsie** for always being sweet to me, **Ernö** for always checking in on me, **Henrik** for the genuine chats about life, **Homa** for being the funniest person, **Jamie** for being a little unhinged, **Kat** for taking us all under your wing, **Linus** for keeping a handle on everything, **Qinyang** for many game nights and jokes, **Rae** for being an endless source of inappropriate jokes, **Tamara** for being the most caring person, and **Zsófia** for the genuine enthusiasm.

I thank the administrative and technical staff **Anna G.**, **Cecilia**, **Chatarina**, **Ella**, **Erling**, **Ewa**, **Helene**, **Henrik**, **Hong-Lei**, **Isabella**, **Jep**, and **Sofia**. Thank you for taking me by the hand and helping me do what needs to be done!

Zuletzt möchte ich meiner Familie und Freunden aus der Heimat aus vollem Herzen danken. Dank euch allen habe ich nie vergessen, dass es ein Leben außerhalb des Doktors gibt. Ich bin so dankbar für eure unentwegte moralische (und telefonische) Unterstützung, ohne die ich dieses Kapitel meines Lebens sicher nicht so erfolgreich abgeschlossen hätte. **Mama**, **Papa**, **Franzi**, **Jane**, und **Thomas**, dank euch habe ich immer ein zu Hause, jemanden dem ich von meinen Erfolgen erzählen kann, und jemanden der mir hilft durchzuhalten. Dafür bin ich euch unendlich dankbar. **Julia**, **Seb**, **Nick**, **Maddy**, **Esther,** und **Leon**, ich schätze mich so glücklich, dass unsere Freundschaft durch die Jahre fortbesteht. Es ist schon etwas Besonderes, so viele Meilensteine miteinander zu erleben. Danke dass ihr alle ein Teil dieses Kapitels wart. Ich hab euch alle lieb!

Last, but not least, I thank **Kitty** the cat for being my roommate, the most loyal companion, as well as my indispensable emotional support. You´re a weirdo and I love you for it. I also need to mention the very good cats and dogs **Uma**, **Bonnie**, **Hera**, **Sixten, Egon** and **Börje**. Thank you for being fun playmates and a never-ending source of conversation topics.

Life is better because of all of you. Thank you! Tack så mycket! Dankeschön!

Abstract

Pollinators play a central role in shaping the astounding diversity of angiosperm flowers. Flowers are complex, multidimensional, genetically and functionally integrated phenotypes, rendering it a challenge to quantify and interpret observed trait variation. Even variation in simpler trait measurements is a challenge to interpret, because of the multitude of selective agents and evolutionary processes shaping floral form. The long-term pollination environment of a plant population is reflected in its pollination- and mating system. Here, I employed a geographic comparative approach, using natural variation in floral traits across pollination environments to understand the evolution of complex floral traits along the pathway to pollination. Specifically, I studied the evolution of floral scent as a pollinator advertisement and reward trait, flower-pollinator fit traits that mediate successful pollen transfer, and pollen performance traits involved in the fertilization process. First, I associated scent evolution with pollinator shifts in a specialized pollination system. Biosynthetically novel scent compounds mediated a shift from floral scent as a pollinator advertisement to a pollinator reward and precipitated a qualitative pollinator shift. Second, I quantified genetic and functional constraints in the evolution of pollinator fit traits as part of three-dimensional flower morphology. The evolutionary potential of each trait was tightly linked to its realized evolution. Third, I quantified components of variance in pollen performance across mating systems. Patterns of pollen performance varied idiosyncratically across pollination environments due to other sources of variation. Lastly, I studied the joint evolution of pollen longevity and mating system. Pollen longevity was longest in the most unreliable pollination environment allowing for delayed pollination. This thesis contributes to a better understanding of the role of pollinators in the diversification of complex floral phenotypes. I combined an understanding of the complexity of the traits under study, profound knowledge of the ecology and natural history of the study systems, and insight into the evolutionary processes shaping trait variation, to understand the evolution of complex floral phenotypes. This work is crucial for understanding the evolution of floral diversity, as well as predicting the consequences of changing pollinator communities for plant fitness.

Popular science summary

Most flowering plants rely on pollinators to reproduce, and pollinators play a major role in shaping the astounding diversity of flowers. Numerous characteristics of flowers are involved in plant-insect interactions along the pathway to sexual reproduction. First, plants need to attract pollinators to the flower by advertising it, for example with floral fragrances, flashy colors, or large flowers. Once pollinators have approached the flower, many flowers reward the pollinator, for example with nutritious nectar, to ensure that the pollinators will return. Next, the pollinators need to position themselves correctly on the flower, to pick up pollen from the male parts of the flower and transport it on their bodies to the female parts of another flower. Plants ensure a good fit between pollinators and flowers by evolving, for instance, the position of the male and female parts of the flowers. Once pollen has been transferred to the female parts of the flower, the pollen grains compete in a race to achieve fertilization.

Flowers are complex multidimensional structures that only work efficiently when all the parts work together in a coordinated fashion. In addition, flowers are not only shaped by pollinators, but numerous other evolutionary processes. This makes it a challenge to understand how flowers evolve, and how plant populations will cope with pollinator declines and other human-induced changes in pollinator communities.

Pollinator communities vary in terms of the groups of pollinators that are present or absent, in pollen-transfer efficiency, and in the relative and total abundance of pollinators on a geographic scale. In the long term, this variation in pollinator communities causes evolutionary change in the flowers that they interact with. The long-term pollination environment of a plant population is reflected in its pollination system (i.e. which pollinators interact with the plant and how), and its mating system (i.e. whether they can self-pollinate or rely on pollinators to transfer pollen).

In this thesis, I used natural variation in floral traits among plant populations with different pollinator communities to understand how pollinators shape flower traits along the pathway to pollination. Specifically, I studied the evolution of floral scent that can be pollinator advertisement and/or a pollinator reward, pollinator fit traits that mediate successful pollen transfer, as well as pollen performance traits involved in the fertilization process.

In the first chapter, I connected changes in floral scent with shifts between groups of pollinators in a specialized pollination system. The plants produced new scent compounds that caused floral scent to shift from a pollinator advertisement to a pollinator reward. These new compounds also caused a shift between two groups of pollinators and therefore resulted in a new pollination system.

In the second chapter, I studied how flower traits that are involved in pollen transfer to and from the bodies of the pollinators can evolve. These pollinator fit traits are part of three-dimensional flower structures and are therefore genetically and functionally linked to each other. Therefore, they cannot evolve independently from each other, which creates a challenge in understanding their ability to evolve. The ability of a trait to evolve was tightly linked to its actual evolution.

In the third chapter, I disentangled the factors that cause variation in pollen performance traits. The pollination environment caused varying patterns of pollen performance. This was because many other factors caused variation in pollen performance and potentially obscured any pattern due to pollination environment. Some of these other factors included the age of the pollen grains at the time they had to compete with each other, and an effect of the ambient temperature on the ability of a pollen grain to compete.

In the fourth chapter, I studied the evolution of the life span of an individual pollen grain, and whether the longevity of the pollen grains varied due to the pollination environment. Pollen longevity was longest in the most unreliable pollination environments which allows the pollen grains to still be viable when pollination occurs with a delay. In general, delayed pollination can have negative consequences for plant reproduction, and therefore plant fitness.

Overall, this thesis contributes to a better understanding of the role of pollinators in shaping floral traits. I combined an understanding of the complexity of the traits under study, deep knowledge of the interaction between plants and pollinators in my study systems, both in the present and in the past with insight into the evolutionary processes that shape floral trait variation. This work is crucial for understanding the evolution of floral diversity, as well as predicting the consequences of changing pollinator communities for plant fitness.

Svensk sammanfattning

De flesta växter är beroende av pollinerare för att reproducera sig, och pollinerare spelar en stor roll i att forma den extraordinära mångfalden av blommor. Många blomkaraktärer är involverade i interaktionerna mellan växter och insekter längs vägen till pollinering. Först måste växterna locka pollinerare till blomman genom att annonsera, till exempel med blomdoft eller stora, färgglada blommor. När en pollinerare har upptäckt och undersökt blomman, belönar många blommor besökaren med exempelvis näringsrik nektar för att säkerställa att pollineraren kommer tillbaka. Nästa steg är att pollineraren måste positionera sig korrekt på blomman för att plocka upp pollen från en blomma och transportera det på sin kropp till pistillens märke i en annan blomma. Växterna säkerställer effektiv pollinering genom att optimera, till exempel, positionen av ståndare och märken så att de passar specifika typer av pollinerare. När pollen har överförts till märken, tävlar pollenkornen för att uppnå befruktning.

Blommor är komplexa, tredimensionella strukturer som endast fungerar effektivt när alla delar arbetar tillsammans på ett koordinerat sätt. Dessutom formas blommor inte bara av pollinerare, utan även av många andra evolutionära processer. Detta gör det utmanande att förstå hur blommor utvecklas och hur växtpopulationer kommer att klara av människans påverkan på pollinerar- och växtsamhällen.

Pollinerarsamhällen varierar geografiskt när det gäller vilka grupper av pollinerare som är närvarande eller frånvarande, i hur bra dessa är på att överföra pollen mellan ståndare och pistiller, och i den relativa och totala abundansen av pollinerare. På lång sikt orsakar denna variation evolutionen av de växter som de interagerar med. Den långsiktiga pollineringsmiljön för en växtpopulation återspeglas i dess pollineringssystem (dvs vilka pollinerare som interagerar med växten och hur) och dess parningssystem (dvs om de kan självpollinera eller måste förlita sig på pollinerare för att överföra pollen).

I denna avhandling använde jag naturlig variation i blomkaraktärer mellan växtpopulationer med olika pollinerarsamhällen för att förstå hur dessa formar blomkaraktärer som fyller olika funktioner när det gäller att uppnå pollinering. Specifikt studerade jag evolutionen av blomdoft som kan fungera som lockmedel och/eller en belöning för pollinerare, formkaraktärer som leder till framgångsrik pollenöverföring, samt viktiga pollenkaraktärer som är involverade i befruktningsprocessen.

I det första kapitlet kopplade jag förändringar i blomdoft till skiften mellan olika typer av pollinerare i ett specialiserat pollineringssystem. Växterna producerade nya doftämnen som gjorde att doften gick från att vara ett lockmedel till att bli en belöning för pollinerarna. Dessa nya ämnen orsakade också ett skifte mellan två olika grupper av pollinerare och resulterade därmed i ett nytt pollineringssystem.

I det andra kapitlet studerade jag hur blomkaraktärer som är involverade i pollenöverföringen till och från pollinerarnas kroppar utvecklas. Dessa tredimensionella blomstrukturer är genetiskt och funktionellt kopplade till varandra. Därför kan de inte utvecklas oberoende av varandra, vilket skapar en utmaning att förstå deras möjlighet att genomgå evolutionära förändringar. Resultaten visade att den evolutionära potentialen, dvs förmågan hos en karaktär att förändras efter att ha utsatts för naturlig selektion, var tätt kopplad till hur mycket karaktären faktiskt förändrats under evolutionen.

I det tredje kapitlet redogjorde jag för de faktorer som orsakar variation i pollenets egenskaper. Pollineringsmiljön orsakade svårförutsägbara och varierande mönster i hur väl pollentuben (som leder spermierna till växtens ägganlag) kunde växa. Detta berodde på att många andra faktorer orsakade variation i hur väl pollenet växte, vilket antagligen dolde effekterna av pollineringsmiljön. Några av dessa andra faktorer var t ex åldern på pollenkornen vid den tidpunkt de måste tävla med varandra och den omgivande temperaturen.

I det fjärde kapitlet studerade jag evolutionen av livslängden hos ett enskilt pollenkorn och huruvida pollenlivslängden varierade beroende på pollineringsmiljön. Pollenet levde som längst i de mest opålitliga pollineringsmiljöerna, vilket gör att pollenkornen fortfarande kan vara livskraftiga när pollineringen sker med en fördröjning. Generellt kan fördröjd pollinering ha negativa konsekvenser för växtens reproduktiva framgång.

Sammanfattningsvis bidrar denna avhandling till en bättre förståelse av pollinerarnas roll i att forma blommornas doft, utseende och morfologi. Jag kombinerade en förståelse för komplexiteten i de studerade egenskaperna med en djup kunskap om interaktionen mellan växter och pollinerare i mina studiesystem, både i nutid och i det förflutna, vilket ledde till nya insikter i de evolutionära processer som formar evolutionen av blommor. Studier som dessa är avgörande för att förstå evolutionen av växternas mångfald och för att förutsäga konsekvenserna av att pollinerarsamhällena just nu är stadda i snabb förändring för växtarters överlevnad och evolutionära framtid.

Background

Pollinator-mediated diversification of flowers

Pollinators play a central role in the phenotypic diversification of flowering plants. Selection by pollinator communities varies across time and space and results in the evolution of floral traits (e.g. Anderson & Johnson, 2009; Gross *et al.*, 2016; Szenteczki *et al.*, 2021; Torres-Vanegas *et al.*, 2024). Pollinator shifts may occur through quantitative variation in relative abundance and efficacy of pollinators or through qualitative shifts in functional groups of pollinators (Armbruster, 1993; Armbruster & Muchhala, 2009).

Quantitative pollinator shifts are often associated with gradual subtle change in floral trait means. For example, a shift between long-proboscid fly species between populations of *Erica junoica* (Ericaceae) in South Africa is associated with continuous quantitative variation in mean floral tube length that co-evolves with fly proboscis length, to ensure optimal pollinator fit (Anderson & Johnson, 2009; Newman *et al.*, 2014; Newman & Johnson, 2021). Meanwhile, *qualitative* pollinator shifts are often associated with functional changes of a trait, the evolution of novel traits, or discrete characters. An intriguing example are the Merianieae plants (Melastomataceae) where repeated shifts from bee pollination to passerine bird pollination are associated with changes in reward type from generalized pollen rewards to highly specialized food-body-rewards (Dellinger *et al.*, 2021). The food bodies are appendages on stamens that contain air and act like a bellow that disperses the pollen onto the birds' head (Dellinger *et al.*, 2014).

Selection by pollinators may not only cause *divergence* in floral phenotypes, but also *convergence* in floral form. Floral trait convergence emerges when suites of traits evolve a shared form for a shared function, repeatedly in independent evolutionary events. Functional groups of pollinators (or their absence) may select for a specific suite of floral traits. As a consequence of correlated selective pressures, these floral traits may be ecologically, functionally, and/or genetically correlated across independent plant taxa (Armbruster & Schwaegerle, 1996), and result in pollination syndromes (Faegri & van der Pijl, 1979; Fenster *et al.*, 2004).

Even though the concept of pollination syndromes may not be universally applicable (Ollerton *et al.*, 2015; Krakos & Austin, 2020; Castañeda-Zárate *et al.*, 2021; Hilpman & Busch, 2021), there is ample evidence for phenotypic convergence in floral traits in association with functional groups of pollinators (Dellinger, 2020; Koski, 2020). For example, hummingbird pollination is frequently associated with red, scentless flowers with deep and wide corolla tubes (Bertin, 1982; Castellanos *et al.*, 2004); noctuid moth pollination is often associated with white, strongly-scented flowers that increase night-time detectability (Kevan & Baker, 1983); and euglossine-pollinated plant species converged in their floral scent profiles within plant families (within orchids) and across plant families as unrelated as Orchidaceae (monocots) and Euphorbiaceae (dicots) (Whitten *et al.*, 1986).

This framework for analyzing floral form is most informative with regards to the principal pollinators but is less informative with regards to co-pollinators and other biotic and abiotic selective pressures. Beyond selection by the pollination environment, floral trait variation may be induced by a number of evolutionary processes and selective drivers, including mutation, polyploidization, hybridization, genome rearrangements, plasticity in response to the biotic or abiotic environment, genetic drift, and genetic constraints, or conflicting selection pressures.

The historical pollination environment (i.e. pollinator composition, abundance, and efficacy) of a plant is reflected in its mating system. The spatial and temporal variation in the efficiency and abundance of pollinators leads to distinct mating-strategies among populations, with increasing advantage of selfing with declining pollinator reliability (Lloyd, 1979, 1992; Primack, 1985; Schemske & Lande, 1985; Lande & Schemske, 1985; Fausto *et al.*, 2001; Arathi *et al.*, 2002; Kalisz & Vogler, 2003; Kalisz *et al.*, 2004; Goodwillie *et al.*, 2005; Karron *et al.*, 2012; Brys *et al.*, 2013; Opedal *et al.*, 2016). Evolutionary transitions between mating systems are a driver for the evolution of floral traits along the pathway to pollination.

Some patterns of variation in floral phenotypes emerge along a mating system gradient as part of a selfing syndrome. The selfing syndrome may result in reduced investment in advertisement traits (e.g. display size or scent) (e.g. Petrén *et al.*, 2021), reduced spatial (herkogamy) or temporal (dichogamy) separation of male and female sexual organs to facilitate autonomous selfing (e.g. Hildesheim *et al.*, 2019b), relaxed selection on traits related to sexual selection (e.g. pollen performance) (e.g. Mazer *et al.*, 2010), and the evolution of mechanisms to cope with reduced or delayed pollination (e.g. pollen or flower longevity) (Ashman & Schoen, 1994; Hildesheim *et al.*, 2019a). We may use natural floral phenotypic variation among pollination environments, e.g. among mating systems, as a natural experiment to assess the role of pollinators in floral evolution. In the following, I describe variation in floral phenotypes across pollination environments, with a focus on the set of floral traits studied in this thesis.

The pathway to pollination

Angiosperm flowers are the bridge between pollinators and plant fitness and exhibit a diversity of phenotypes (see cover illustration). In this thesis, I study the role of pollinators in shaping complex floral phenotypes along the pathway to pollination (Fig. 1). I investigate traits related to pollinator advertisement and reward, pollinator fit and pollen transfer, reproductive performance traits involved in the fertilization process itself, as well as the plant response to changes in the pollination environment.

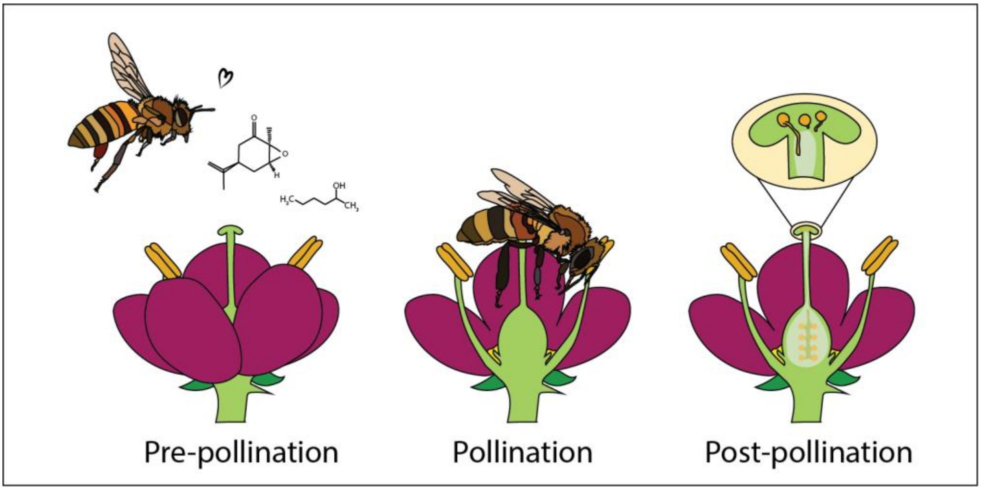


Fig. 1: Illustration of the pathway to plant sexual reproduction. First, flowers of animal-pollinated plants attract pollinators through visual or chemical signaling and incentivize visitation by providing pollinator rewards. Floral advertisement and reward traits may increase general apparency and attractiveness of the flower and may also confer pollinator specificity. Second, successful pollination depends on the fit between the body of the pollinator and floral morphology. The position of anthers, stigmas, and the reward site relative to each other, and relative to the body of the pollinator, determines successful pollen transfer. Third, once pollen has been deposited on the stigma, the pollen grains compete in a race to successfully fertilize an ovule. Floral phenotypes vary vastly across pollination environments (i.e.in terms of pollinator assemblage and relative abundance). The complexity of multidimensional floral phenotypes, the array of selective drivers, and the multitude of evolutionary processes shaping floral form, render it a challenge to predict the evolution of floral traits. Illustration: Hanna E. Thosteman.

First, the flowers of animal-pollinated plants attract pollinators through visual or chemical signaling and incentivize visitation by providing pollinator rewards (Fig. 1). Here, I focused on floral scent which can be a pollinator attractant or a specialized pollinator reward (**chapter I**) (Armbruster, 1993; Minnaar *et al.*, 2019). Floral scent varies not only quantitatively, but also qualitatively among taxa (e.g. Gfrerer *et al.*, 2021; Petrén *et al.*, 2021). Biosynthetic pathways may produce variants of chemical molecules which the pollinators may perceive as distinctly different scent bouquets (Junker *et al.*, 2017; Liu *et al.*, 2024). Even minor changes in floral scent may result in major shifts in the pollinator community.

Second, once pollinators are visiting, the flowers need to successfully transfer the pollen from the anthers to the body of the pollinator, and subsequently to a receptive stigma (Fig. 1) (Minnaar *et al.*, 2019; Opedal *et al.*, 2023b). Here, I focused on pollinator fit traits, such as the distance between the reward site and the sexual organs (**chapter II**). The relative positioning of floral pollinator fit traits may vary among plant populations in association with variation in pollinators, e.g. in terms of their reliability, size, or morphology (Armbruster, 1988). Floral morphology is rather complex due to its three-dimensionality. Estimating the evolutionary potential of pollinator fit traits in response to the pollination environment proves challenging, because some floral traits, or trait combinations, may evolve more readily than others (e.g. Hansen *et al.*, 2003).

Third, once pollen has been deposited on the stigma, the pollen grains engage in a race to successfully fertilize an ovule (Fig. 1) (Mulcahy & Mulcahy, 1975). Pollen grains need to germinate and grow the pollen tube down the style and into the ovary (e.g. Mascarenhas, 1993). Here, I studied pollen performance as an important component of male reproductive fitness (**chapter III**). Pollen performance varies dramatically among species, populations, and pollination context (Mazer *et al.*, 2010; Gutiérrez-Valencia *et al.*, 2022). Yet, it is difficult to detect patterns of pollen performance across pollination environments, as they may be obscured by physiological factors and environmental plasticity (e.g. Lankinen, 2001).

Lastly, global changes in pollinator communities may affect plant fitness due to reduced or delayed pollen transfer (Potts *et al.*, 2010; Dicks *et al.*, 2021; Rodger *et al.*, 2021). The effect of delayed pollination may affect plant fitness through pollen senescence (e.g. Proctor, 1998). Pollen longevity may vary across pollination environments (Dafni & Firmage, 2000), because of differences in how rapidly and reliably pollen may usually be transferred. Predicting the effects of delayed pollination is complicated by the interplay between ecological requirements and the physiological constraints on the pollen grain (**chapter IV**).

Floral phenotypic variation across pollination environments

Chapter I: Floral scent as an advertisement and reward trait

In pollination ecology, floral scent is primarily regarded as a pollinator advertisement trait that enhances pollinator specificity and constancy (Huber et al., 2005; Raguso, 2008; Friberg et al., 2014; Brandt et al., 2021). Floral scent may covary with the pollination environment of a population (Friberg *et al.*, 2019; Thosteman, 2024), and even small changes in the scent bouquet can lead to the attraction of an entirely different set of pollinators (Hetherington-Rauth & Ramírez, 2016; Castañeda-Zárate *et al.*, 2021). In systems where floral scent contributes minorly to pollinator attraction (e.g. in highly selfing populations), chemical advertisement may be reduced, or altered, as a consequence of relaxed pollinator-mediated selection (Petrén *et al.*, 2021), selection to reduce inadvertent attraction of antagonists (Junker & Blüthgen, 2010), or selection to reduce metabolic cost.

There is tremendous variation in floral scent at all organizational levels: among pollination and mating systems (e.g. Petrén *et al.*, 2021; Liu *et al.*, 2024), species (Huber et al., 2005; Friberg et al., 2019), populations (Friberg et al., 2019; Joffard et al., 2020), individuals (Petrén, 2020; Szenteczki et al., 2021), and floral tissues (García *et al.*, 2021). Variation in floral scent can be caused by quantitative changes of total emission rate or the relative proportion of compounds within a sample, and also through qualitative changes in bouquet composition (Friberg *et al.*, 2017, 2019; Dormont *et al.*, 2020; Petrén *et al.*, 2021). The spectacular diversity and chemical complexity of floral scent renders it not only a challenge to quantify the variation, but also to disentangle the mechanisms underlying the evolution of floral scent.

In pollination ecology, floral scent has primarily been studied as a pollinator advertisement trait, but in some systems it also functions as a pollinator reward (e.g. Armbruster, 2012). Scent rewards were first described in tropical orchids (Vogel, 1966; Dodson *et al.*, 1969), and have since been discovered in several other plant families (Whitten *et al.*, 1986; Gerlach & Schill, 1991; Armbruster, 1993; Hentrich *et al.*, 2010; Milet-Pinheiro *et al.*, 2021). Scent rewards are associated with pollination by scent-collecting male euglossine bees, and these euglossinophilous plant species may converge in their scent profiles (Whitten *et al.*, 1986; Gerlach & Schill, 1991; Liu *et al.*, 2024).

Euglossine bees (Apidae, tribe: Euglossini; common name: orchid bees) are a group of more than 200 species occurring in the Neotropics (Central- and South America) (Fig. 2) (Roubik & Hanson, 2004). Euglossine bees have evolved multiple specialized interactions with plants. Females collect floral resins to construct their nests (Fig. 2a) (Armbruster, 1984). Male euglossine bees collect floral scent that serves both as a pollinator attractant and reward (Vogel, 1966; Dodson *et al.*, 1969; Gerlach & Schill, 1991). The male bees collect the scent molecules by scratching them off of the surface of the scent source using tarsal brushes on their front tibiae, and store the compounds in pouches on their hind tibiae (Fig. 2c, d) (Vogel, 1966), for later use during mating display (Bembé, 2004; Pokorny *et al.*, 2017). Scent can be collected from a variety of resources, including floral scent (Dodson *et al.*, 1969), rotting wood (Whitten *et al.*, 1993), fungi (Cappellari & Harter-Marques, 2010), leaves, or feces (Henske *et al.*, 2025).

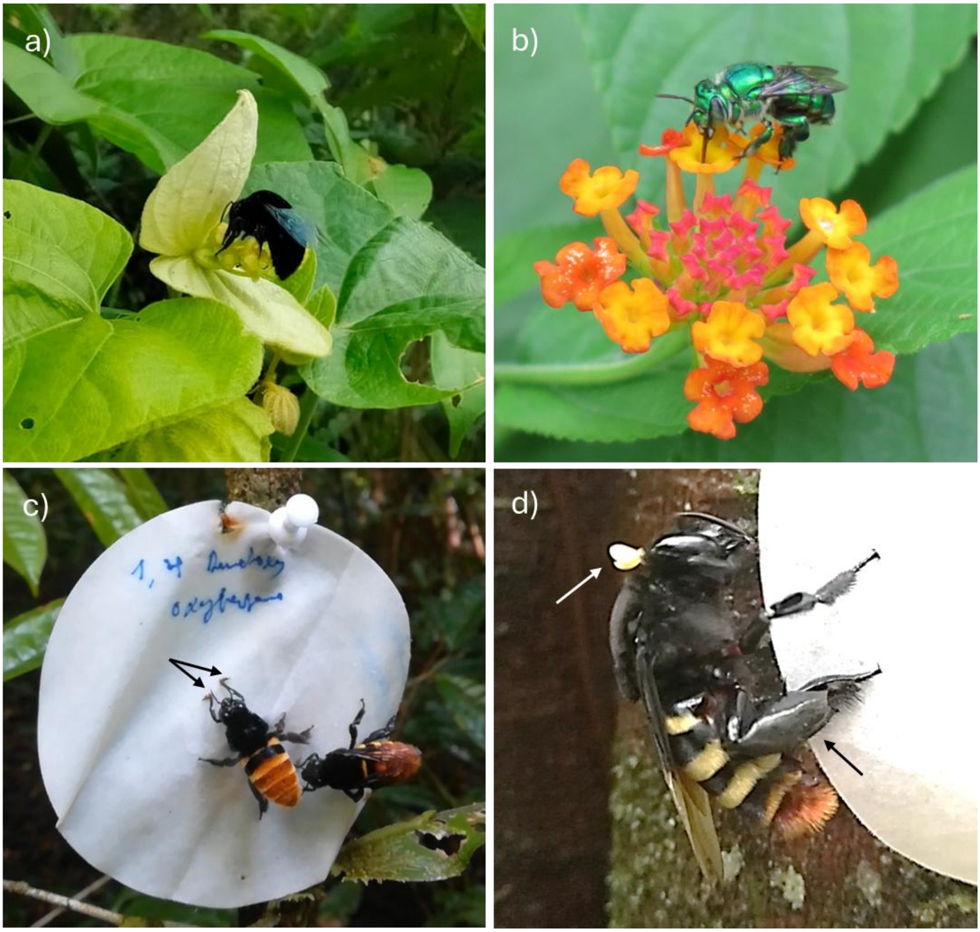


Fig. 2: The beautiful diversity of euglossine bees in French Guiana. a) Female *Eulaema nigrita* pollinating *Dalechampia tiliifolia* (Euphorbiaceae). She is collecting floral resin from the resin gland that she will use to construct her nest and pollinates the flower in the process. This is the first time the interaction between the two species has been documented! **b)** *Euglossa sp.* drinking nectar from the seemingly tasty *Lantana camara* (Verbenaceae). **c)** Two male *Eulaema sp.* collecting 1,4-dimethoxybenzene from a scent bait. They will use the compound to produce perfumes used during mating display. The arrows point to the “brushes” on their front legs with which they scratch the scent molecules off the surface of the bait (or flower). **d)** A male *Eulaema sp.* collecting scent from a scent bait. The white arrow points out an orchid pollinarium stuck to the back of the bee, hopefully on its way to be deposited on the stigma of an orchid. The black arrow points out the characteristic enlarged corbiculae on the hind legs in which the male bees store the scent molecules that are to be used for perfume production. Photo credit: Laura S. Hildesheim and Cristina Rodríguez-Otero.

Chapter II: Pollinator fit traits

Pollinator fit traits mediate pollen transfer by ensuring the fit between floral morphology and pollinator morphology. The variation in three-dimensional flower morphology, i.e. the relative positioning of the reward site and the floral sexual organs is associated with variation in pollinator assemblages, e.g. in terms of their size or morphology (Armbruster, 1988; Newman *et al.*, 2014). The distances between sexual organs and the reward site may not only evolve due to the size of the pollinators, but also as part of the selfing syndrome (e.g. Cutter, 2019).

One of the most readily measured indicators of the historical mating system of a population, may be the separation of male and female sexual functions. Outcrossing can be promoted by a greater spatial separation of male and female sexual organs (i.e. greater anther-stigma distance) (Webb & Lloyd, 1986; Opedal, 2018), or by increasing the temporal separation between male and female function (i.e. reducing overlap of the sexual functions) (Lloyd & Webb, 1986). Meanwhile, small anther-stigma distances and greater overlap of sexual functions are indicative of historically frequent autonomous selfing. In some species, variation in the anther-stigma distance (herkogamy), is associated with variation in autogamous seed set or outcrossing rates (e.g. Bodbyl Roels and Kelly 2011; Opedal *et al.* 2016). Similarly, the distance between the sexual organs and the reward site may evolve jointly with the anther-stigma distance to maintain optimal relative positioning of the floral structures to each other maintain fit to the body of the pollinator in the event of outcrossing (Armbruster *et al.*, 2009b). Evolution of any one of these traits will necessarily have consequences for the relative positioning of the other traits.

Chapter III, IV: Pollen traits

Pollen performance is the link between pollen deposition and successful ovule fertilization (Minnaar *et al.*, 2019; Opedal *et al.*, 2023b). During pollen competition, pollen performance traits are subject to both natural and sexual selection (Hormaza & Herrero, 1996; Williams *et al.*, 2010; Lankinen *et al.*, 2016; Gutiérrez-Valencia *et al.*, 2022). Despite expected strong directional selection on pollen performance, there is great observed variation in pollen traits. Variation in pollen performance has been attributed to numerous factors, including: genetic variation among pollen donors (Walsh & Charlesworth, 1992; Hormaza & Herrero, 1996; Lankinen *et al.*, 2009), pollen-pollen interactions (Ashman *et al.*, 2020), pollen-style interactions (Walsh & Charlesworth, 1992; Higashiyama & Hamamura, 2008; Lankinen *et al.*, 2016), thermal plasticity (Lankinen, 2001; Hedhly *et al.*, 2005; Peach & Mazer, 2019), pollen age (Dafni & Firmage, 2000), or variation among mating systems (Mazer *et al.*, 2010, 2018; Lankinen *et al.*, 2016; Gutiérrez-Valencia *et al.*, 2022).

In selfing plants, pollen may often compete with pollen from within the same plant individual with similar genetic make-up. This should reduce, or even remove, sexual selection and reduce the opportunity for pollen competition (Mazer *et al.*, 2010; Gutiérrez-Valencia *et al.*, 2022). Therefore, pollen performance traits may be under relaxed selection in selfing plants, while there would be stronger sexual and natural selection in more outcrossing populations. This may result in faster pollen germination, and higher pollen tube growth rates in outcrossing populations (Mazer *et al.*, 2010, 2018; Hove & Mazer, 2013). Moreover, the mating system of a plant may not only affect pollen performance traits in terms of pollen germination or pollen tube growth, but also other pollen traits. For example, pollen longevity may evolve jointly with the mating system (Dafni & Firmage, 2000). In autonomously selfing plants, pollination may often occur rapidly during early flower development. Meanwhile, outcrossing plants may evolve greater pollen longevity to account for potential delays in pollinator visitation. Hence, pollen phenotypes may vary across pollination environments.

The genetic and functional architecture of complex floral traits

Flowers are complex phenotypes with an indispensable function in plant reproduction. Flowers are three-dimensional morphological structures with multidimensional floral chemistry, designed to facilitate pollination. Despite understanding the multidimensional complexity of the trait itself, another challenge arises from the complexity of selective agents and patterns of variation, even in “simple” floral trait measurements.

During the evolution of floral traits, a multitude of selective agents and evolutionary processes are at work. This makes it challenging to quantify variation in floral phenotypes as well as disentangle the sources of variation in these traits. Furthermore, due to their functional and genetic integration, floral traits may not evolve independently, but in concert with one another (e.g. Reich *et al.*, 2020; Thosteman *et al.*, 2024). The evolution of one floral trait may have cascading effects on a suite of other floral traits and ultimately affect plant fitness. Therefore, predicting the evolution of floral traits requires knowledge of their ecological function (e.g. pollinator attractant vs. pollinator fit trait), the selective pressures acting upon those traits (e.g. variation in pollinator community vs. abiotic environment), as well as an understanding of the underlying genetic architecture and evolutionary potential of these traits.

Only with a comprehensive understanding of flowers, can we hope to make predictions about the evolution of floral traits in a changing world. In the following, I focus on the floral traits that I investigated in this thesis: floral scent as a pollinator attractant, three-dimensional flower morphology that has a function in pollinator fit and the mechanics of self-pollination, and pollen performance traits that are the link between the process of pollination and its effect on plant fitness. Any change to the pollination environment may result in the evolution of this delicate complex structure that is a flower.

Gradual evolution, exaptation, and evolutionary novelty in flowers

Multiple evolutionary processes explain the emergence of variation in floral phenotypes. Adaptive evolution of floral phenotypes allows plants to respond to changes in their environment. We may think of an adaptation as a trait that is currently under stabilizing selection, i.e. at a local fitness optimum. However, adaptations and phenotypic variation in floral traits may arise from a number of evolutionary processes. If we want to predict the evolutionary response of floral phenotypes to a changing world, we need to understand the evolutionary processes shaping the extant variation in floral form across time and space. Here, I distinguish between the gradual evolution of a continuous quantitative trait and the saltatory emergence of a functionally or developmentally discretely novel trait variant. Although it should be noted that once a trait has undergone a dramatic change in function, or emerged as a novel developmental variant, it may then continue to evolve gradually.

The literature showcases many examples of gradual evolution of morphological traits in response to variation in pollination environments. For example, pollinator advertisement- and fit traits, such as corolla tube and upper lip length of *Lavandula latifolia* (Lamiaceae) varied along a geographic mosaic of pollinator communities in the Mediterranean (Herrera *et al.*, 2006). Pollen performance traits, such as pollen tube growth rate or time until pollen germination, have been suggested to vary among populations according to their pollination environment i.e. pollen competitive environment on the stigma (Mazer *et al.*, 2010). Gradual, continuous, and *quantitative* trait variation may usually occur in floral traits with an assumed polygenic basis (i.e. the phenotype may be determined by an assumed infinite number of genes with small effect) (Fisher, 1918). Yet, even closely related species can have *qualitatively* different floral phenotypes, which cannot be explained solely by gradual evolutionary changes, but instead by exaptation or evolutionary novelty. While the term “exaptation” focuses often on the function of a trait (Gould & Vrba, 1982), the term “novelty” often refers to the developmental origin of a trait (Shubin *et al.*, 2009; Wagner & Lynch, 2010).

First, floral traits may arise via exaptation as a homology, i.e. by co-opting existing characters for novel purposes (*cf.* Gould & Vrba, 1982). For example, a floral trait may evolve as a signal to attract pollinators, but may be co-opted by a seed predator or an herbivore as a cue to find the plant for consumption (Knudsen *et al.*, 2006). Exaptations may be notoriously difficult to detect, especially when the evolutionary history of the system and the ecological function of the trait is unknown. This may explain why examples of exaptations are less represented in the literature of floral evolution and makes the documented instances even more valuable. In the example of the evolution of reward types in Merianeae plants mentioned above, the food bodies are likely an exaptation from enlarged stamens, i.e. the stamens were repurposed from their sexual function to a function as a pollinator reward (Dellinger *et al.*, 2014, 2021).

Second, floral traits may arise as an evolutionary novelty. Here, I define an evolutionary novelty as a discontinuity in the evolution of a trait, i.e. a *de novo* developmental variant of a trait (West-Eberhard, 2003), that phenotypically and/or functionally diverts from the ancestral condition (Pigliucci, 2008). Novel traits may arise from an array of mechanisms, including mutations, introgression, polyploidization, hybridization between morphs within a polymorphic system, or genomic rearrangements. We may allow for homology when defining a trait as novel (Hallgrímsson *et al.*, 2012), because even non-homologous novel traits may share genetic regulatory or developmental networks with their ancestors (Shubin *et al.*, 2009; Wagner & Lynch, 2010). For example, a new floral scent molecule may be produced due to a developmental "error" or a mutation in a biosynthetic pathway (Junker *et al.*, 2017). Because chemical compounds are discrete phenotypes, even a small difference in chemical structure may be perceived as an entirely novel signal by a flower visitor (Eltz & Lunau, 2005). Therefore, even a minute change in the floral scent profile can have profound consequences for pollination and even attract novel pollinators (Gerlach & Schill, 1991; Castañeda-Zárate *et al.*, 2021).

Disentangling exaptation from evolutionary novelty is an empirical challenge and requires good knowledge of the evolutionary history of the study system. An example is the evolution of resin rewards in the tropical plant genus *Dalechampia* (Euphorbiaceae) (Armbruster *et al.*, 1997, 2009a). Resin secretion originated as a defense mechanism to protect the male flowers from florivory. The resin-secreting bractlets rearranged into a gland-like structure that makes the resin rewards easily accessible to the pollinators. Thus, the presence of resin in the inflorescence was a pre-adaptation for resin pollinator rewards, and *vice versa*, resin pollinator rewards are an exaptation from resin defense against florivores. Note that Darwin´s term "pre-adaptation" can only be used in retrospect. For example, we can describe a trait that realized its potential to become an exaptation as a pre-adaptation (e.g. plant resin that changes function from antagonist repellent to pollinator attractant). We can also refer to a developmental machinery as a pre-adaptation for producing a developmental variant (e.g. a biosynthetic pathway that produces a new floral scent molecule).

The evolutionary potential of complex floral traits

Since Darwin's work in the 1800s, natural selection has been regarded as a major driving force behind trait evolution. However, more recent insights have raised our attention to the importance of the underlying genetic architecture of a trait in our attempts to predict its evolutionary response. Building on this insight, Lande and Arnold formulated the Lande equation $\mathbf{\Delta}z = \sigma^2\beta$ (Lande & Arnold, 1983). In its univariate form, the Lande equation predicts the expected trait change $\mathbf{\Delta}z$ given the additive genetic variance or phenotypic variance σ^2 of a trait, and the selection pressure expressed as the selection gradient β acting on the trait. Importantly, the Lande equation separates the ability to evolve (expressed as the amount of genetic variance available to selection σ^2) from natural selection (expressed as the selection gradient β). This approach can be extended to multivariate trait space as $\mathbf{\Delta}z = \mathbf{G}\beta$, where **G** represents the **G**-matrix, which is the variance-covariance matrix that models both the variance within a trait, as well as its correlations with other traits. **G**-matrices allow us to quantify evolutionary constraints that emerge from the genetic architecture of complex phenotypes. The multivariate form takes into account that floral traits do not evolve in a vacuum, but as part of genetically and functionally integrated phenotypes.

Starting in the 1990s, Houle and Hansen saw a need to further develop the approach to predicting evolution and popularized the concept of the mean-scaled evolvability, i.e. the predisposition of a trait to respond to natural selection (Houle, 1992; Hansen & Houle, 2008; Hansen *et al.*, 2011). Evolvability (i.e. the predicted trait change $\mathbf{\Delta}z$) is obtained by scaling the additive genetic variance (i.e. the proportion of the variance that is heritable and available to selection) by the trait mean (i.e. $\sigma^2/\bar{z}^2$). This results in an estimate of the expected trait change in response to a standardized strength and direction of selection (i.e. unit standardized selection of β=1), expressed as a percentage change (i.e. x100). Mean-scaled evolvability (*sensu* Hansen and Houle) is estimated as $I_A = \sigma^2/\bar{z}^2$ x 100, where I_A is the predicted trait change per generation.

The important nuance of their approach is the mean-scaling of the trait and the standardization of the assumed selection on the trait. This allows direct comparison of evolutionary potential across trait types, measurement magnitudes, taxa, as well as across timescales. In recent decades, there has been a growing interest in the *evolutionary potential* of traits (including floral traits), as it holds great predictive power for the *realized evolution* of traits, i.e. there is a strong relationship between the evolvability and the divergence of traits among taxa (Opedal *et al.*, 2023a; Holstad *et al.*, 2024; Tsuboi *et al.*, 2024).

Chapter I: The modularity of floral scent chemistry

Floral scent plays a major role in pollination ecology, as it serves to attract pollinators. Each set of pollinators may be attracted to a specific scent bouquet, but the scent compounds may not evolve independently due to their functional, as well as genetic integration (Junker *et al.*, 2017; Thosteman *et al.*, 2024). The non-independence of scent compounds due to their shared biosynthetic pathways may complicate predictions about the evolution of floral scent in response to pollinator communities. Furthermore, due to shared biosynthetic pathways, floral scent may or may not be able to evolve independently of metabolic plant chemistry or vegetative leaf chemistry (Thosteman *et al.*, 2024).

Over 1700 floral volatiles have been reported across a large number of plant taxa (Knudsen *et al.*, 2006). However, these compounds are produced by only four major biosynthetic pathways (Junker *et al.*, 2017), and can be divided into four major compound classes: monoterpenoids, sesquiterpenoids, aromatics, and fatty acid derivatives (Muhlemann *et al.*, 2014; Junker *et al.*, 2017). Each pathway can produce a large number of compounds, and we can assess biosynthetic diversity at the level of the biosynthetic pathway (e.g. monoterpenoids produced by the MEP pathway), at the level of scent modules (e.g. “cineole cassette” containing a number of commonly co-occurring monoterpenoids), or at the level of individual compounds (e.g. carvone oxide). Despite this great diversity of known scent compounds, to date, only a fraction of plant species in a small proportion of plant families has been investigated (Knudsen *et al.*, 2006), and many more compounds are to be discovered.

To draw conclusions about floral scent evolution, we need to understand the modularity of biosynthetic pathways as well as the chemical properties of scent compounds. In the following section, I introduce terpenes as an example. Terpenes are the largest compound class in flowering plants, and comprise many hundreds of compounds found in flower and leaf tissues (Pichersky & Raguso, 2018). The vast terpene diversity is the consequence of 1) multiple products being formed from a single substrate, 2) single mutations in the beginning of a pathway generating multiple new products, 3) terpene synthases being regulated by large gene families providing a large target for mutation and selection, and 4) terpene skeletons being susceptible to modification reactions (Degenhardt *et al.*, 2009; Pichersky & Raguso, 2018). Synthesis of different terpenes often only differs in a single enzyme at the final step of the pathway (Gershenzon, 1994; Junker *et al.*, 2017; Petrén *et al.*, 2023a).

Floral scent bouquets may contain compounds that are biosynthetic by-products of one another (Wise *et al.*, 1998), and due to the discrete nature of scent molecules, even a minor change may lead to a quantitative or qualitative pollinator shift (e.g. Castañeda-Zárate *et al.* 2021). This genetic, developmental, and functional integration of floral scent needs to be taken into account when aiming to understand the evolution of floral chemistry (Petrén *et al.*, 2023a; Thosteman *et al.*, 2024).

Chapter II: The functional integration of three-dimensional flower morphology

To improve predictions about the evolution of floral traits as well as understand the genetic constraints emerging in integrated multidimensional traits, recent work has developed a multivariate framework for estimating evolutionary potential (Hansen *et al.*, 2003, 2019; Blows & Hoffmann, 2005; Hansen & Houle, 2008). Estimating multivariate evolvability takes into account that traits do not exist in a vacuum, but as part of genetically and functionally integrated phenotypes. This means that traits cannot evolve independently of each other and there may be some dimensions in phenotypic space that are more evolvable than others (*cf.* Schluter's lines of least resistance; Schluter 1996).

The multivariate evolvability of a trait and its autonomy (i.e. how much it can evolve independently from other traits) are intertwined with the modularity and integration of a trait. As a special form of multivariate evolvability, conditional evolvability estimates the evolvability of a trait, while specified other traits are being held constant (as if they were under stabilizing selection) (Hansen *et al.*, 2003). However, it requires a good understanding of the study system to understand the genetic or functional correlations with other traits in order to decide which traits to condition upon.

Floral phenotypes offer a unique opportunity to study the evolutionary potential of multidimensional structures. Especially morphological traits offer an exceptional study system where we have a good understanding of the function of a trait (e.g. pollinator fit traits). Given that flowers are three-dimensional structures that are functionally integrated, usually multiple traits may evolve at the same time. Understanding the evolution of composite traits that are the result of a functional interaction between multiple traits is a special challenge. For example, herkogamy which is the distance between anthers and stigma, determines the ability of a plant to autonomously self-pollinate. Due to the three dimensionality of flower morphology, the anther-stigma distance evolves by changing the relative positioning of male and female organs. This may be accomplished by evolving either the male, female, or both sexual organs, and by changing either the protrusion, the angle, or both, between the two sexual organs.

Chapter III, IV: The complexity of "simple" floral trait measurements

In the examples above, the challenge lies in quantifying and interpreting the multidimensional complexity of the floral traits under investigation (multivariate scent or composite pollinator fit traits). However, even “simple” floral trait measurements can pose a scientific challenge, because any trait is subject to a multitude of interacting selective agents and multiple evolutionary processes causing the observed variation in phenotypes. For example, some pollen traits can be quantified in simple terms such as pollen germination as a percentage, pollen tube length in millimeters, or pollen longevity in days. Variation in these traits may be induced by the pollination environment (Dafni & Firmage, 2000; Mazer *et al.*, 2010), i.e. by differences in competitive environment, but also by genetic, developmental, and physiological processes.

First, pollen grains are haploid, and during pollen production, genetic variation is created. In the next step, during pollen competition, this variation is being selected upon. Therefore, the variation in pollen performance among pollen grains is expected to be large because it includes both fit, as well as unfit, genotypes and phenotypes. Furthermore, predictions about the evolution of pollen performance may be complicated by variation in ploidy levels among plants. Patterns of variation in pollen traits may differ among diploid plants that produce haploid pollen, and plants with higher ploidy levels, that produce pollen of higher ploidy levels, with consequences for selection of those genotypes (*cf.* Gerstein & Otto, 2009).

Second, the male gametophyte may be the most environmentally sensitive stage of the plant reproductive cycle (Rosbakh *et al.*, 2018; Heiling & Koski, 2023). Therefore, pollen performance may be greatly affected by abiotic factors and the phenotype is the result of the interaction between the genotype and its environment (Lankinen, 2001). Thus, when understanding trait variation, we have to take into account in equal parts the genetic architecture of the trait *and* trait plasticity (*cf.* Uller *et al.*, 2020).

Third, physiological processes affect floral trait variation (Mascarenhas, 1993). For example, pollen performance could vary between the autotrophic (self-sustained) and heterotrophic (sustained by the pistil) phase (Stephenson *et al.*, 2003). Furthermore, pollen performance could vary due to the age of the pollen grain and associated senescence effects (Dafni & Firmage, 2000). When measuring floral trait variation, we need to consider the effect of physiological processes on floral trait variation. Some of these processes may impose hard constraints on the evolution of pollen traits, such as a thermal maximum, while other directions in phenotypic space may evolve more freely.

Lastly, even though pollen performance is tightly linked to the male fitness component, predictions about offspring quality are not straight forward. The effect of pollen performance and pollen competition on offspring fitness may be subject to numerous other factors such as order of arrival on the stigma (Lankinen & Madjidian, 2011), competition intensity (Armbruster & Rogers, 2004; Pélabon *et al.*, 2015, 2016), parental conflict (Raunsgard *et al.*, 2018; Petrén *et al.*, 2023b), pollen load size (Hildesheim *et al.*, 2019b), or pollen age (Proctor, 1998).

Aims of the thesis

A majority of plants rely on animal pollinators for reproduction (Ollerton *et al.*, 2011). Flowers are the bridge between pollinators and plant reproductive success and the floral phenotype is at the core of successful pollination (Fig. 3). As pollinator communities continue to change, floral phenotypes are ever evolving. The complexity and diversity of floral traits and the multitude of interactions with their pollinators create a fascinating challenge for evolutionary biologists hoping to understand floral evolution. In this thesis, I focus on the evolution of floral traits in response to variation in pollination environments. Thereby, this thesis contributes to an improved understanding of the observed variation of complex floral traits along the pathway to pollination.

Flower phenotypes vary naturally in response to their pollinators in a geographic and temporal mosaic (Grant-Stebbins model; Grant & Grant, 1965; Stebbins, 1970). To study the evolution of floral traits, we may leverage the natural variation in floral phenotypes across pollination environments (i.e. in terms of pollinator assemblage, efficacy, or abundance). The historical, i.e. long-term, pollination environment of a plant population is reflected in its mating system (selfing vs. outcrossing). Here, I used floral phenotypic variation along a mating system gradient as a natural experiment to quantify the evolutionary response of floral traits to changing pollination environments. Predictions about the evolution of floral phenotypes are further complicated by the multidimensional complexity of floral structures that are functionally and genetically integrated. Furthermore, I face the challenge of disentangling the role of pollinators in shaping floral phenotypes from other evolutionary drivers, such as plasticity in response to the abiotic environment.

In this thesis, I aim to take the reader on an exciting journey through the world of pollination ecology and floral evolution along the pathway to pollination. I start with the amazing floral chemistry involved in pollinator attraction and pollinator reward (**chapter I**), follow it up with an investigation of the evolutionary potential of floral morphology involved in optimal pollinator fit (**chapter II**), take a deep dive into causes of variation in pollen performance during the relentless race for ovule fertilization (**chapter III**), and ultimately, I address the concerning consequences of changing pollinator communities for plant reproductive fitness (**chapter IV**).

Chapter I: Pollinator attraction

In **chapter I**, I aimed to understand the evolution of rather unique scent rewards and associated pollinator shifts in the genus *Dalechampia* (Fig. 3). In this plant genus, some species potentially use floral scent as a pollinator advertisement to female euglossine bees. Meanwhile, other species of this genus provide the scent molecules as a pollinator reward to male euglossine bees who collect scent molecules to use in their mating display. I considered two hypotheses for the evolution of scent rewards and the associated qualitative pollinator shift between female and male bees: evolution as a biosynthetic novelty (indicated by distinct scent profiles in scent-and resin-rewarding species) or evolution via exaptation by repurposing an attractive compound as a reward compound (indicated by an overlap in scent compounds in scent-and resin-rewarding species).

Chapter II: Pollinator fit and pollen deposition

In **chapter II**, I aimed to understand the evolutionary potential of three-dimensional floral morphology in the bisexual *Dalechampia* blossoms. The positioning of the reward site relative to anthers and stigmas, is crucial for pollinator positioning during visitation (pollinator fit) and determines pollination success. Meanwhile, the relative positioning of anthers and stigmas (herkogamy) determines the ability for autonomous self-pollination. Therefore, floral reproductive structures are composed of functionally interacting traits that are likely genetically correlated. These genetic constraints complicate predictions of the evolutionary potential of these traits in response to variation in the pollination environment.

Chapter III: Pollen performance

In **chapter III**, I aimed to understand the evolution of pollen performance traits. Once pollen is deposited on the stigma, the pollen grains must germinate and grow down the style in the competition for ovule fertilization. Despite an expectation of strong directional selection for ever-faster pollen tube growth, the literature suggests enormous variation in pollen performance. In this study, I focused on variation in pollen performance along a mating-system gradient (selfing vs. outcrossing) in *Arabis alpina*. I expected faster pollen tube growth rates in outcrossing populations due to a more competitive environment, however, previous studies have yielded mixed results. Patterns in pollen performance across mating systems, could be obscured by genetic variation among- and within populations, environmental plasticity, or physiological effects.

Chapter IV: Consequences of changing pollinator communities

In **chapter IV**, I aimed to understand the consequences of delayed pollination on seed production. When pollination is delayed, it may occur more frequently with older pollen with potential negative effects on seed production. Consequently, pollen longevity may evolve jointly with the mating system of a population. I first formulated general predictions about the joint evolution of pollen longevity and the mating system. Predictions of pollen longevity across mating systems may be complicated by mechanisms such as sequential pollen presentation that ensures that fresh pollen is being presented throughout anthesis. Then, I empirically tested the predictions in populations of *D. scandens* that fall along a gradient from highly selfing to more outcrossing.

Fig: 3: Plant-pollinator interactions play a major role in shaping floral phenotypes. In this photo, a female euglossine bee approaches a blossom inflorescence of *Dalechampia scandens* (Euphorbiaceae). She is aiming to collect resin from the resin gland within the inflorescence and will likely pollinate the blossom in the process. Visible on her hind legs are resin droplets that she may have collected from previously visited blossoms. She will use the resin to construct her nest. This busy little bee deserves the reward for her hard work. Photo credit: Elena Albertsen.

Methods

Study systems

On the genus *Dalechampia*

The plant genus *Dalechampia* L. (Euphorbiaceae) comprises about 130 described species that occur in tropical regions across the world (Fig. 4). *Dalechampia* are perennial, mixed-mating vines and shrubs. Here, I focus on the neotropical species of *Dalechampia* that offer specialized resin- or scent-rewards and are pollinated by, among others, euglossine bees. In the resin-rewarding species, resin is secreted from a resin “gland” located above the male flowers and is collected by *female* euglossine and megachilid bees that use it to construct their nests (Fig. 3) (Armbruster, 1984). In the scent-rewarding species, scent rewards are either secreted from the stigmatic surface (Armbruster *et al.*, 1992), or from papillate bractlets that form a “scent gland” that is homologous to the resin gland. Scent rewards are collected by *male* euglossines (Whitten *et al.*, 1986; Armbruster *et al.*, 1989; Armbruster, 1993).

In *Dalechampia*, resin rewards have evolved once and have subsequently been lost multiple times (Armbruster, 1993, 2012). Within the genus, shifts from pollination by resin-collecting female euglossine bees to pollination by scent-collecting male euglossine bees has occurred at least three to four times independently. Owing to 50 years of research on the interaction between *Dalechampia* and their pollinators, we have extensive knowledge of the natural history and pollination systems within the genus. Hence, the genus *Dalechampia* is a suitable system to study the evolution of floral phenotypes in response to the pollination environment.

Fig. 4: Illustration of the variation in floral phenotypes in the genus *Dalechampia*. For decades, researchers have been fascinated by the floral diversity and pollination ecology of the genus *Dalechampia* L. (Euphorbiaceae). The variation in floral form among species, but also within species, still holds many mysteries. Top row (left to right): *D. scandens, D. scandens, D. dioscoreifolia, D. schottii.* Bottom row: *D. aristolochiifolia, D. pernambuscensis, D. bella, D. heteromorpha*. Blossom sizes not to scale.

Dalechampia blossoms are functionally bisexual and protogynous. Each blossom comprises three female flowers with three ovules each (Fig. 5). Depending on the species, between four and 16 male flowers are located in a cluster above the female flowers. The male flowers are fused together with the resin- or scent-gland forming the male cymule (Fig. 5). Most species of *Dalechampia* have involucral bracts that open and close daily around the sexual organs, and may have a function in protecting the sexual organs and seeds from predators and the abiotic environment (Song *et al.*, 2024).

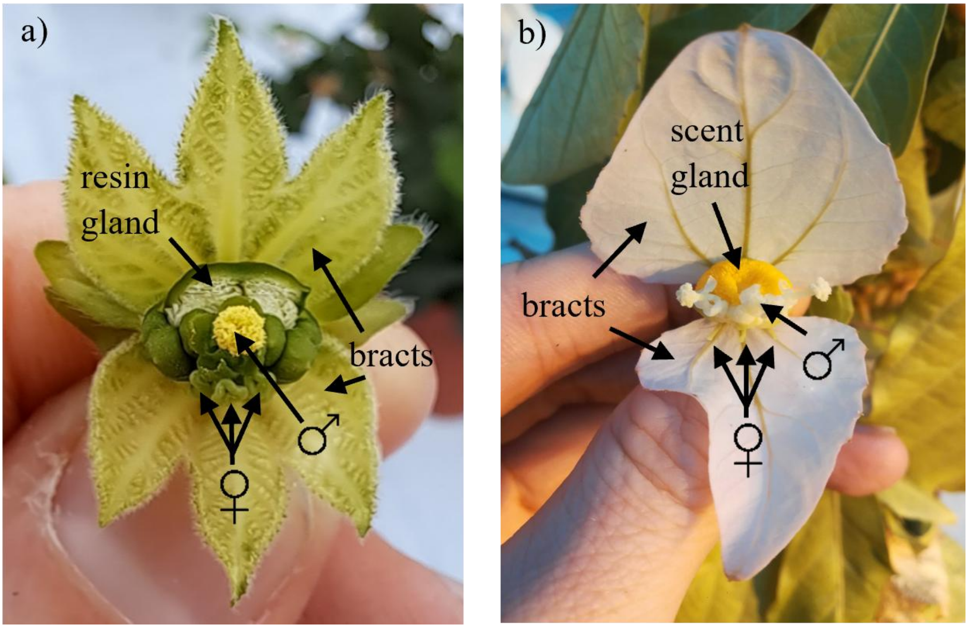


Fig. 5: Blossom inflorescences of *Dalechampia* in a greenhouse common garden at Lund University. a) A blossom of the resin-rewarding *D. scandens* on the first day of the bisexual phase with one open male flower presenting yellow pollen. **b)** A blossom of the scent-rewarding *D. spathulata* during the bisexual phase with multiple open male flowers. The arrows indicate the three female flowers within the blossom, the male flowers within the blossom, the resin or scent glands that secret the pollinator rewards, as well as the involucral bracts.

Floral ontogeny begins with a female phase during which the three stigmas are receptive (Fig. 6b). After a few days, the first male flower within the blossom dehisces (usually the central male flower), and the blossom enters the bisexual phase (Fig. 6c). The remaining male flowers in the male cluster open sequentially over the course of the next days (Fig. 6d), resulting in presentation of fresh pollen all throughout anthesis. Meanwhile, the older male flowers within a blossom start senescing and are ultimately shed by cleanly breaking off the pedicel stalk (Fig. 6e). When all male flowers have opened, the entire male cluster including the reward gland is shed, by cleanly breaking off the pedicel stalk (Fig. 6f). Subsequently, the blossoms mature the seeds, which are dispersed by explosively dehiscing the seed capsules.

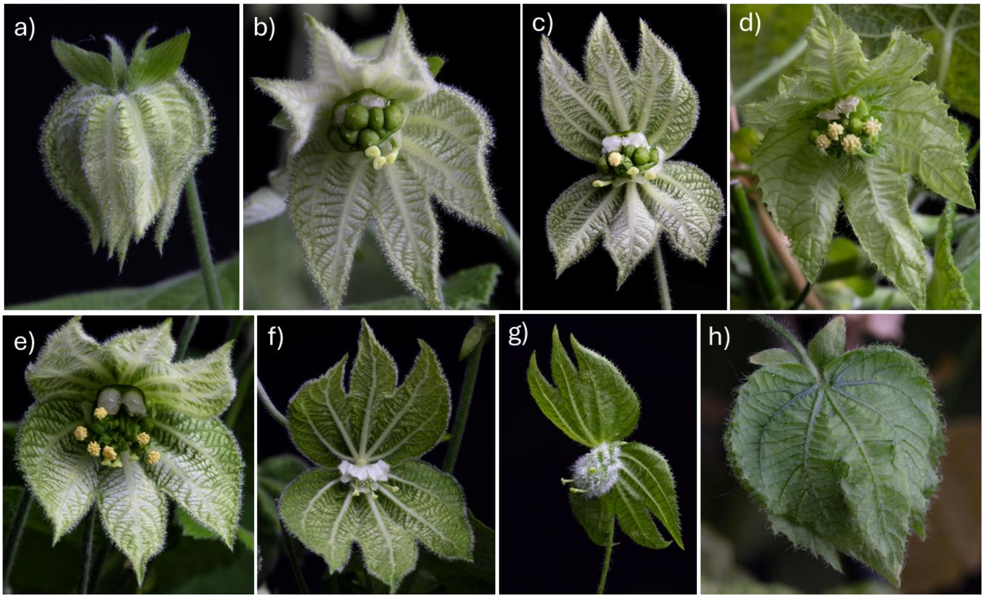


Fig 6: Blossom ontogeny of the protogynous *Dalechampia*. a) Blossom bud. **b)** Female-phase blossom. The stigmas are receptive while the male flower buds have not yet opened. The arrows indicate the three female flowers, the cluster of male flowers (cymule) with up to ten male flower buds, the resin gland, and the upper and lower bracts. **c)** First day of bisexual phase. The first male flower (usually the central flower) presents the yellow pollen while the stigmas continue to be receptive. **d)** Advanced bisexual phase. Multiple male flowers are presenting pollen simultaneously, but some male flower buds are still closed. **e)** Late bisexual phase. Multiple male flowers present pollen simultaneously, and some male flowers have already wilted and were shed. The arrow indicates the remaining pedicel stalk of a shed male flower. **f)** Old blossom. The entire male cymule has been shed, while the stigmas still remain. The arrow indicates the pedicel stalk, where the male cymule, including the resin gland, broke off. **g)** Blossom during early seed maturation. The maturing fruit is protected by hairy spines that will get stuck in the skin of careless greenhouse workers. In this case, the bracts of the blossom inflorescence remained open during seed development, while in most populations, the bracts close around the maturing fruit to protect it from seed predators. **h)** Closed blossom inflorescence. The bracts open and close daily throughout the flowering period. They may have a function in protecting the male and female flowers within the inflorescence, the pollen grains, as well as the maturing seeds. The delicate structures are shielded from predators and weather, and the bracts may also perform photosynthesis to provision the flowers or seeds within. The figure is assembled from photos of multiple taxa within the *D. scandens* species complex. Photo credit: Per Harald Olsen.

Dalechampia are self-compatible and may self-pollinate autonomously during the bisexual phase. Taxa vary in their outcrossing rates from highly selfing to mostly outcrossing. The ability to autonomously self-pollinate depends greatly on the distance between anthers and stigmas during the bisexual phase. Both, among- and within species, outcrossing rates are positively correlated with anther-stigma distances (see Armbruster, 1988; Opedal *et al.*, 2016). Therefore, I used anther-stigma distances to infer the mating system of a population, where smaller distances correlate with high selfing rates and larger distances indicate frequent outcrossing.

On *Arabis alpina*

The generalist herb *Arabis alpina* L. (Brassicaceae) occurs in arctic-alpine mountain regions of the northern hemisphere (Fig. 7a) (Koch *et al.*, 2006; Wötzel *et al.*, 2022). The multiple flowers borne in each inflorescence are bisexual and protogynous. The stigma at the center of the bisexual flower is surrounded by four long and two short stamens (Fig. 7b). Stigmas are receptive throughout the first few days of the flowering period, while anthers open during later ontogeny, with temporal overlap of male and female function (Toräng *et al.*, 2017). In self-compatible populations, autonomous selfing may occur during the bisexual phase.

Here, I focus on six European populations that differ in their pollinator communities and span a mating system gradient from highly selfing and mixed-mating (i.e. self-compatible) to entirely outcrossing (i.e. self-incompatible) (Ansell *et al.*, 2008; Toräng *et al.*, 2017; Petrén *et al.*, 2023b). The wide mating system gradient and the variation in self-compatibility, makes this system suitable to study patterns of floral phenotypes across pollination environments.

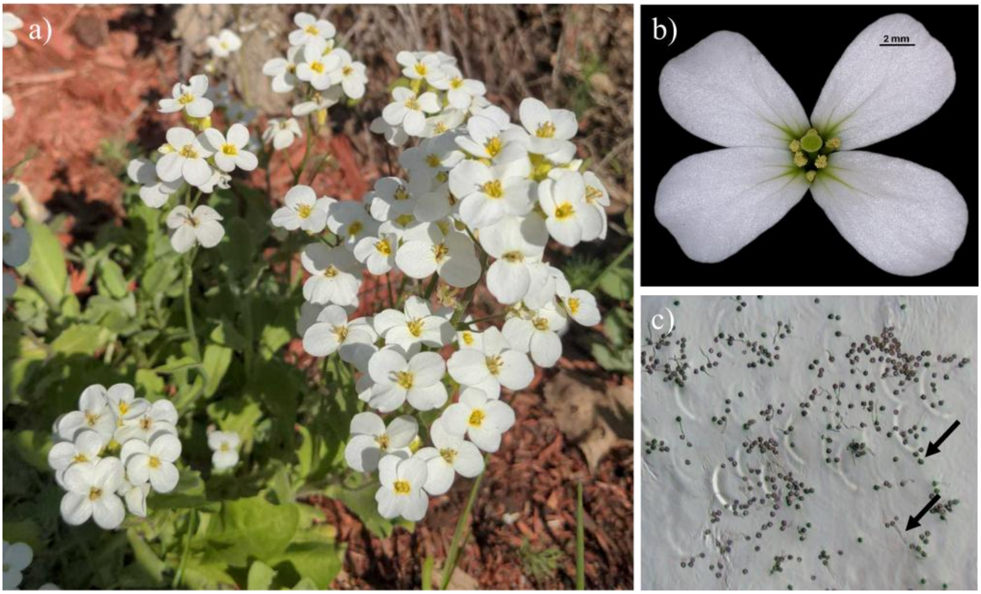


Fig. 7: *Arabis alpina* (Brassicaceae). a) Adult *A. alpina* flowering in front of the ecology building. The brilliant white petals draw the attention of any onlooker. **b)** A perfect *A. alpina* flower during the bisexual phase. The stigma at the center of the perfect flower is surrounded by four long and two short stamens. **c)** Microscopic photo of *A. alpina* pollen in vitro. The upper arrow indicates a pollen grain that failed to germinate. The lower arrow indicates a pollen grain that has grown a pollen tube. Photo credit: a and c) Laura S. Hildesheim, b) Hanna E. Thosteman.

Study design, experimental setup, and data analysis

Using natural trait variation to understand floral evolution

The historical long-term pollination environment of plant populations is reflected in their evolved pollination system (e.g. in terms of functional groups of pollinators) and evolved mating system (selfing vs. outcrossing). To estimate the effect of variation in pollination environments on floral evolution, I utilized natural variation in floral phenotypes among pollination systems and mating systems. The geographic comparative approach was coupled with a deep understanding of the function of floral traits. This approach allowed me to isolate pollinator-mediated variation in floral phenotypes and to test predictions about the evolution of flowers and pollination interactions. Using natural variation in pollination environments among plant populations, i.e. variation in mating systems, allowed me to empirically test predictions of the role of pollinators in shaping floral phenotypes in greenhouse experiments. In this thesis, I combined empirical data obtained from plants raised in greenhouse common gardens with data collected by colleagues in the field over the course of many field seasons.

Table 1: *Dalechampia* species included in this thesis, in alphabetical order. All taxa are self-compatible.

Species	Chapter	ASD (mm)	SE	GSD (mm)	SE
scent-rewarding species					
D. spathulata	I	4.61	0.66	4.18	0.22
resin-rewarding species					
D. aristolochiifolia	I, II	5.27	0.53	5.05	0.19
D. *sp. nov.* "bella"	I, II	2.26	0.25	6.91	0.31
D. dioscoreifolia *var.* pubescens	I, II	7.54	0.36	3.95	0.51
D. heteromorpha	I, II	0.03	0.01	4.3	0.22
D. *sp. nov.* "leucocarpa"	II	4.73	0.50	8.11	0.37
D. osana	II	3.11	0.72	7.27	0.71
D. magnistipulata	I, II	7.98	1.12	12.09	1.13
D. pernambucensis	I	1.66	0.09	3.95	0.14
D. scandens	I, II, IV	0	0	4.5	0.18
D. *aff.* scandens complex	I, II, IV	1.17 - 3.08		5.36 - 6.87	
D. schottii	I	1.67	0.47	3.82	0.55
D. tiliifolia	II	2.8	0.34	13.44	0.39
D. websteri	II	3.25	0.34	8.95	0.29

Chapter refers to the thesis chapters in which the species was included.
ASD = anther-stigma distance
GSD = gland-stigma distance

All experimental plants were grown in a greenhouse common garden at Lund University, Sweden (Table 1, 2). Plants were raised from seeds collected in the field, or from seed material obtained from within-population crosses or self-pollination in the greenhouse. For scent analysis, I grew one scent-rewarding species as well as 11 resin-rewarding species and populations of *Dalechampia* sampled from across the phylogeny. For analysis of the evolutionary potential of three-dimensional flower morphology, I grew plants from one Costa-Rican population of *D. scandens* in a diallel crossing design, which allowed us to estimate a **G**-matrix. For analysis of pollen tube growth rates across mating systems, I grew six European populations of *Arabis alpina*, reflecting a mating-system gradient from self-compatible to self-incompatible. For analysis of the effects of pollen age on plant fitness, I grew five populations of *D. scandens* reflecting a mating-system gradient from highly selfing to more outcrossing.

Table 2: *Arabis alpina* populations included in this thesis, ordered from most selfing to most outcrossing population.

Population	Self-compatible	Outcrossing rate
Sweden (S1)	yes	0.0169*
France2 (FR2)	yes	0.139*
France1 (FR1)	yes	0.266*
Italy (IT10)	no	>0.75°
Greece3 (GR3)	no	>0.90°
Greece4 (GR4)	no	>0.90°

*Toräng et al., 2015

°Estimated based on comparable populations and statistics in Laenen et al., 2018 and Gutiérrez-Valencia et al., 2023

Chapter I: Floral scent variation across pollination systems

To understand the evolution of floral scent rewards in the genus *Dalechampia*, I quantified floral scent variation among 12 taxa of *Dalechampia*, including 11 resin-rewarding and one scent-rewarding species. To compare scent composition and scent emission rates between resin-and scent-rewarding species, I collected scent from whole blossoms. To identify the emission site of the scent molecules, I dissected blossoms into three parts, 1) “male tissues + gland” including the cluster of male flowers that is fused together with the resin- or scent-gland, 2) “female tissues”, including the pistils of the three female flowers within a blossom, and 3) the involucral “bracts” which are modified leaves. Samples were taken during the bisexual phase.

I estimated relative floral scent composition using solid-phase microextractions (SPME) (Fig. 8a). I obtained scent samples by enclosing the plant material in an airtight container, letting the scent accumulate in the headspace within the airtight container, and exposing the SPME fiber to headspace air following a standardized protocol (*cf.* Friberg *et al.*, 2013, 2019). The samples were stored in the fridge until further analysis.

To obtain scent chromatograms, I analyzed the SPME and dynamic headspace samples with gas chromatography/mass spectrometry (GC/MS). The gas chromatograph was equipped with a polar DB-wax column. I used pure helium as a carrier gas at a constant flow and ran the samples at standardized temperature programs. I manually analyzed the chromatograms for floral volatiles using the software Xcalibur Qual Browser. I identified floral volatile compounds using at least one of three methods: 1) comparison with authentic reference standards 2) comparison of Kovats retention index values with those in the literature obtained from polar wax columns equivalent to the column used in the present study, 3) library suggestions from a reference library of chemical compounds. To be able to standardize scent emission rates across plant taxa and different tissue types of vastly different sizes, I weighed the dry mass of the plant materials after drying them in an oven.

I used the statistical software R for all analyses (R Core Team, 2020). I analyzed scent variation among species and tissues using Bray-Curtis dissimilarities among samples, based on the relative proportions of a scent compounds within each sample (`R` package `vegan;` Oksanen *et al*., 2018) and visualized the variation in scent composition with a non-metric multidimensional scaling (NMDS) plot. I compared mean total scent emission across species and tissues based on SPME samples. To quantify components of variance in scent, I analyzed the scent data with a hierarchical joint model (`R` package `HMSC`; Tikhonov *et al.*, 2020). The model included square-root-transformed and scaled scent abundance data as a response matrix. I included reward type (2 levels: scent vs. resin), tissue (3 levels: male tissue incl. gland, female tissue, and bract tissue), species-mean anther-stigma distance, and species-mean gland-stigma distance as fixed effects, and sample ID, SPME sampling unit, sample date, taxon, individual, and blossom as random effects.

Chapter II: Multivariate evolvability and evolutionary divergence of pollinator fit traits

To understand the multivariate evolvability and constraints in the evolution of three-dimensional blossom structures, I focused on three blossom traits involved in pollinator fit (gland-anther distance and gland-stigma distance) and the ability to self-pollinate (anther-stigma distance) in one Costa Rican population of *Dalechampia*. The evolvability estimates for the three blossom traits were derived from a genetic variance-covariance matrix (**G**-matrix), which in turn was yielded from a diallel crossing design in a greenhouse common garden.

To obtain data for the **G**-matrix, I performed crosses among the parental individuals in a diallel block design (Fig. 8b), where each of ten blocks consisted of four parental individuals. I performed all possible cross-combinations, aiming for 16 crosses per block and a total of 40 half-sibling families. Second, I raised the offspring from each cross, and measured gland-anther distance, gland-stigma distance, and anther-stigma distance on two blossoms from two individuals.

The **G**-matrix was estimated from a multivariate animal model (`R` package `MCMCglmm`; Hadfield, 2010). Subsequently, mean evolvability, autonomy, and conditional evolvability of gland-anther distance, gland-stigma distance, and anther-stigma were calculated (`R` package `evolvability`; Bolstad *et al.*, 2014). To yield a distribution of multivariate evolvability, selection was simulated in 1000 random directions of phenotypic space, allowing to explore selection on random combinations of the three traits. Some directions in phenotypic space may be more evolvable than others, but the mean of a large number of evolvability estimates should approximate the mean evolvability across the G-matrix.

Subsequently, to estimate the divergence-evolvability relationship (Bolstad *et al.*, 2014; Houle *et al.*, 2017; Holstad *et al.*, 2024), the simulated evolvability estimates (i.e. the evolutionary potential) were related to divergence of phenotypic trait means among species and populations (i.e. realized evolution). Measurements of trait means across taxa were collected during prior greenhouse work (Opedal *et al.*, 2016) and multiple field seasons.

Chapter III: Pollen performance across pollination environments

I quantified variation in pollen performance across mating systems in six populations of *Arabis alpina*, varying in mating system from highly selfing to entirely outcrossing. Because patterns of pollen performance across mating systems may be obscured by other factors, we also quantified pollen age effects and thermal plasticity in pollen performance. Each pollen donor served as a block, so that pollen of each plant individual was germinated in each temperature treatment (Fig. 8c). I germinated the pollen grains *in vitro* in a Hoekstra medium (Hoekstra & Bruinsma, 1975). After 4.5h in incubators, I counted the percentage of germinated pollen grains and measured the pollen tube length by taking photos of the samples under a microscope and analyzing the images with ImageJ.

To test for a relationship between mating system and pollen performance, I fitted a series of mixed-effect models (`R` package `glmmTMB;` Brooks *et al.*, 2017), including mating system and temperature treatments as fixed effects and pollen donor as a random effect. To estimate variance components in pollen performance, I fitted a series of linear mixed-effect models with gaussian error distribution (`R` package `MCMCglmm;` Hadfield, 2010). In separate models of pollen performance for each mating system (self-compatible vs. self-incompatible), I partitioned variance into components related to population (genetic variation), temperature (plastic component), pollen age (physiological component) and pollen donor (opportunity for sexual selection).

Chapter IV: Pollen longevity and consequences of delayed pollination

I quantified the joint evolution of pollen longevity with the pollination environment, as well as the fitness consequences of delayed pollination in the male function across pollination environments in five populations of the *Dalechampia scandens* species complex ranging from highly selfing to more outcrossing. First, I quantified the physiological longevity of the pollen, as well as the consequences of delayed pollination (in the male function) on seed production, by hand-pollinating fresh emasculated blossoms (i.e. pollen receivers or "females") with 0-8 days old pollen.

Second, sequential pollen presentation within a blossom results in continuous presentation of fresh pollen throughout anthesis (see explanation of *Dalechampia* blossom ontogeny above). I estimated the effect of the pollen presentation schedule on seed production by pollinating with pollen-donating ("male") blossoms from early to late anthesis. Because older male flowers wilt and are shed naturally, the number of currently pollen-presenting male flowers within a blossom is usually smaller than the total number of male flowers that have already opened throughout the blossom lifespan. Therefore, this experiment varied the overall age of the pollen-donating blossom, and the number of male flowers used for pollination.

If a pollinated blossom failed to produce seeds, I recorded the cross as unsuccessful. If the blossom set seed, I counted the number of seeds per blossom (max. 9) and weighed each seed individually (Fig. 8d). In separate models for each population, I analyzed the effect of pollen age, overall blossom age, or the number of male flowers used for pollinations on seed number with generalized linear mixed-effect models following the hurdle approach (`R` package `lme4`; Bates *et al.*, 2024). I included mother ID as a random effect to account for the non-independence of fruits within a plant. To analyze the effects of the pollination treatments on seed mass (mg), I fitted linear mixed-effect models (`R` package `lme4`) with peduncle diameter ("blossom stem") as a covariate to account for effects of blossom size, seed number to account for trade-off effects between seed number and seed mass, and cross ID nested within mother ID as random effects to account for maternal effects on seed mass, the non-independence of fruits within a plant, and the non-independence of seeds within a blossom.

Fig. 8: Illustration of the data collection process. a) Scent collection for chapter I. The photo shows dissected Dalechampia blossom tissues enclosed in glass vials that are sealed airtight. The scent of the floral tissues diffuses into the glass vial where it gets trapped. Then, I insert the scent-collection device (SPME sampler) into the vial to collect scent. **b)** Diallel crosses for chapter II. The photo shows me inspecting the plants used for the diallel cross design that was used to create a **G**-matrix for *D. scandens*. The white bags will catch the seeds upon the explosive dehiscence of the seed capsules. **c)** Pollen performance trials for chapter III. The photo shows a tray of *Arabis alpina* plants in the background. In the foreground are boxes that are prepared with petri dishes for the in vitro pollen performance trials at five different temperatures. I am about to sprinkle pollen onto a pollen tube growth medium that is on the microscope slides within the petri dishes. **d)** Seed weighing for chapter IV. The photo illustrates *Dalechampia* seeds and seed capsules that are ready to be weighed on a precision scale. Seeds are released by explosive dehiscence of the seed capsules.

Results and discussion

Chapter I: Pollinator advertisement and reward

In animal-pollinated plants, the first step towards pollination, is pollinator attraction to the plant via floral advertisement and oftentimes reward. In **chapter I**, I aimed to understand the evolution of scent rewards and associated pollinator shifts in the genus *Dalechampia*. I considered two mechanisms for the evolution of scent rewards. Scent reward compounds may have evolved as a biosynthetic novelty, indicated by distinctly different scent bouquets between scent-and resin-rewarding species. Alternatively, the reward scents may have evolved as an exaptation, by repurposing advertisement compounds as reward compounds, indicated by an overlap in scent compounds between scent-and resin-rewarding species.

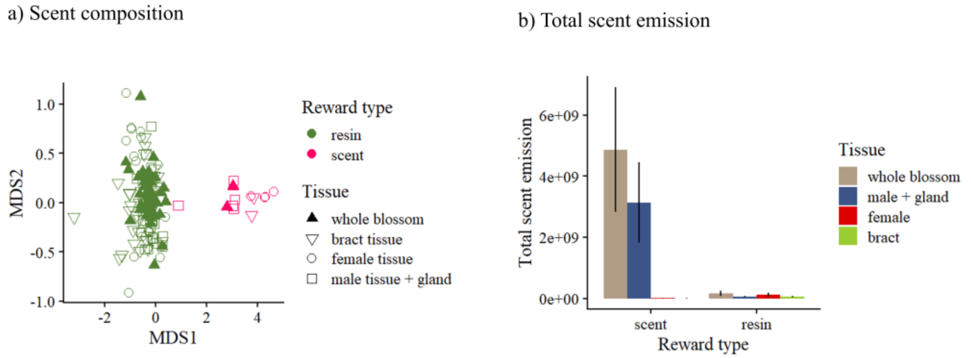


Fig. 9: Floral scent variation in the euglossine bee pollinated genus *Dalechampia*. a) NMDS plot illustrating that floral scent of the scent-rewarding *D. spathulata* is distinctly different from the scent of the resin-rewarding taxa. **b)** Total floral scent emission is drastically higher in the scent-rewarding *D. spathulata* than in resin-rewarding taxa. The scent in the scent-rewarding *D. spathulata* is primarily emitted from the scent gland, while the resin-rewarding taxa emit scent diffusely from all blossom tissues.

The scent of the scent-rewarding *D. spathulata* was distinctly different from the scent of 11 resin-rewarding *Dalechampia* taxa (Fig. 9a). The scent-rewarding species produced a set of cyclic monoterpenoid compounds (e.g. carvone and carvone oxide), while the resin-rewarding taxa produced linear monoterpenoids (primarily linalool and ocimene). Carvone oxide, a well-known male euglossine attractant (Whitten *et al.*, 1986), was the most abundant compound in *D. spathulata*. Producing this compound may have precipitated a qualitative pollinator shift from pollination by female resin-collecting bees to male scent-collecting euglossine bees in this specialized pollination system.

The main difference in the chemical structure of linear and cyclic monoterpenoids is the closure of a carbon ring. I propose that the scent reward compounds may have evolved as a biosynthetic novelty via a modification to the biosynthetic pathway that produces the linear monoterpenoids (i.e. closure of the carbon ring). Recently, Liu *et al.* (2024) proposed the same mechanism to have mediated the evolution of scent rewards in a large set of male-euglossine pollinated species.

Floral scent also varied moderately among resin-rewarding taxa, and some of this variation may be attributed to quantitative variation in pollinator communities. The scent-rewarding species emitted dramatically more scent (primarily from the scent gland) than the resin-rewarding taxa (diffuse scent emission across tissues) (Fig. 9b). This pattern emphasizes the importance of floral scent for attracting male euglossine bees. While I cannot exclude a role of floral scent as an advertisement trait in the resin-rewarding species, scent appears to play a smaller role in the interaction with female euglossine and megachilid pollinator communities.

Here, I associated quantitative and qualitative variation in floral scent with quantitative and qualitative pollinator shifts. I was able to propose the evolutionary mechanism that may have led to the evolution of floral scent rewards, owing to over five decades of extensive research into the pollination system of the genus *Dalechampia*, as well as an understanding of the biosynthetic modularity of floral scent compounds. By sharing biosynthetic pathways in the resin- and scent-rewarding *Dalechampia*, this genus was pre-adapted to evolve cyclic monoterpenes as a biosynthetic novelty.

This intriguing example of scent evolution in a specialized pollination system contributes to a better understanding of the mechanisms underlying the evolution of floral scent, specialized reward types, and specialized pollination systems. The insights gained in this chapter further our understanding of the evolution of complex pollinator advertisement and reward traits, which are the first in a series of traits mediating the interaction between flowers and pollinators.

In **chapter I**, I used the extensive knowledge on the natural history of the genus *Dalechampia* to conclude that qualitative variation in scent between scent-and resin-rewarding species emerged as a biosynthetic novelty at the compound level. Thereby, I was able to pinpoint the evolutionary mechanism explaining the association between floral scent as pollinator reward and pollinator assemblages in this specialized pollination system. These results highlight the value of a comprehensive understanding of our model systems when aiming to understand the evolutionary mechanisms that shape floral phenotypic variation.

Chapter II: Pollinator fit and pollen deposition

Once a pollinator has been attracted to a flower, successful pollen transfer depends on the fit between pollinator, floral reward site, and floral sexual organs (i.e. pollinator fit). In **chapter II**, I investigated the evolutionary potential of three-dimensional floral morphology in the bisexual *Dalechampia* blossoms (Fig. 10a). The individual floral structures that make up the composite pollinator fit trait (e.g. anther-stigma distance) are functionally, and likely, also genetically correlated. These genetic and functional constraints complicate predictions about the ability of these traits to respond to selection by pollinator communities.

Some directions in phenotypic space had higher evolvabilities, while others appeared to be more genetically constrained (Fig. 10b). On average, multivariate evolvability of three-dimensional blossom morphology was very high (mean multivariate evolvability = 2.6%), i.e. composite morphological traits may change by 2.6% per generation in response to unit-strength selection. Pollinator fit traits (i.e. positioning of the reward site in relation to anthers and stigmas) and anther-stigma distances (i.e. proxy for the ability to autonomously self-pollinate) exhibited fairly high independent evolutionary potential despite being functionally and genetically correlated. Autonomy was 53%, meaning that about half of the genetic variation was available for independent evolution, assuming a random combination of the original traits.

Ultimately, morphological traits with highest evolvability (i.e. highest evolutionary potential) diverged most among taxa (i.e. highest realized evolution), as indicated by a strong divergence-evolvability relationship. This pattern held true at the population level (Fig. 10c) and the species level (Fig. 10d). These results contributed to establishing a broad-scale pattern that indicates a tight link between evolutionary potential (evolvability) and realized evolution (trait divergence) across timescales, taxa, trait types, and trait measurements (Holstad *et al.*, 2024).

This complex example of the relationship between evolutionary potential and realized evolution of floral morphology showcases the strength of a quantitative genetic approach for understanding floral trait evolution. My results underscore the recently emerging appreciation of the predictive power of evolvabilities in an attempt to understand floral trait evolution in a changing world (e.g. Opedal *et al.*, 2023; Holstad *et al.*, 2024; Tsuboi *et al.*, 2024). This work also furthers our understanding of the evolution of pollinator fit traits and mating-system variation, which are crucial traits in successful pollination.

In **chapter II**, I quantified the genetic and functional constraints underlying the evolution of floral morphology in response to variation in pollinator assemblages. While selection undoubtedly plays a major role in floral evolution, this work focused on a traits ability to in fact *respond* to selection. Ultimately, this work established a strong relationship between the evolutionary potential of a trait and its realized evolution. This research makes an important contribution to our ability to make predictions about evolutionary changes in plant populations in response to anthropogenic changes to the environment.

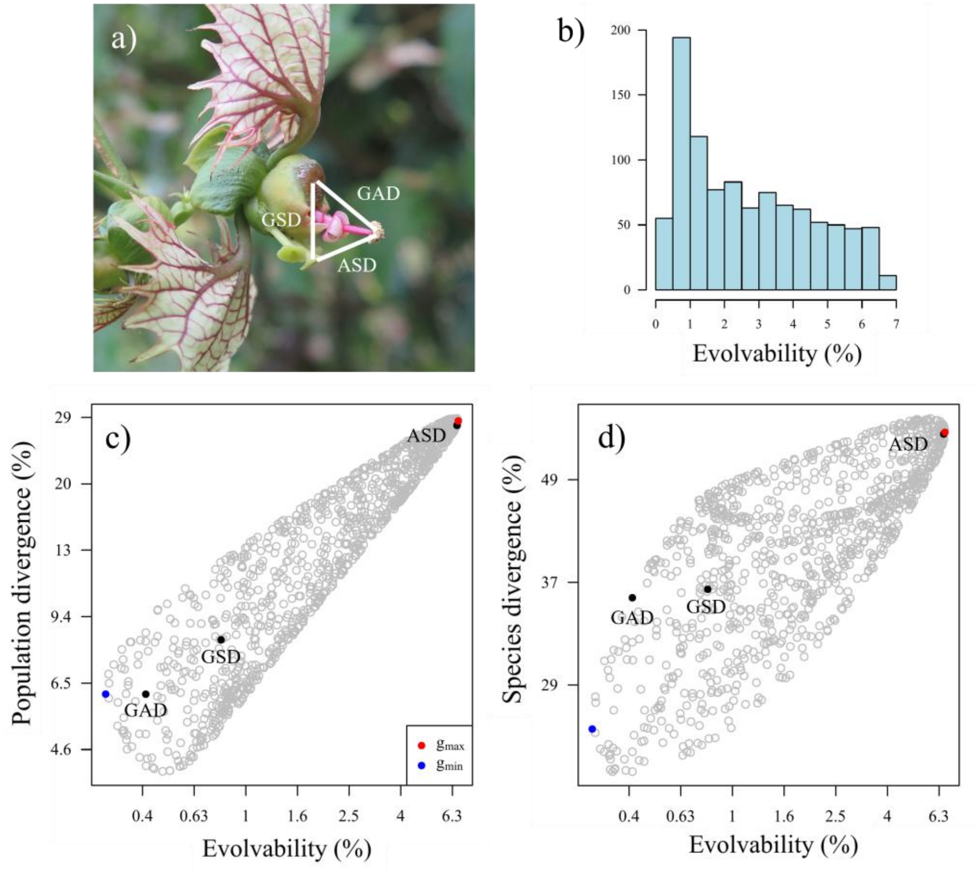
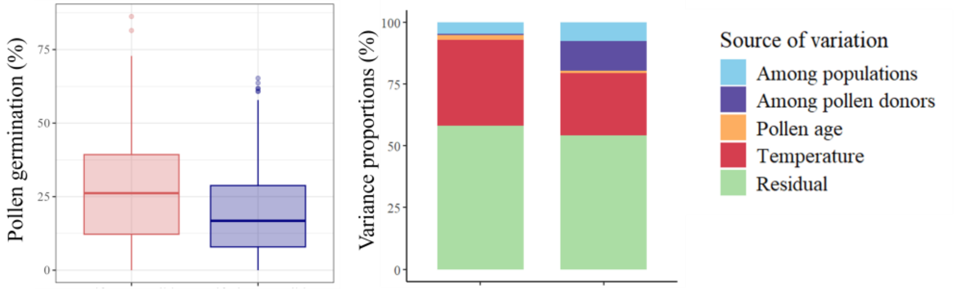


Fig. 10: Genetic and functional constraints in the evolution of pollinator fit traits in three-dimensional *Dalechampia* blossoms. a) Illustration of pollinator fit traits in a *D. dioscoreifolia* blossom; ASD: anther-stigma distance, GAD: gland-anther distance, GSD: gland-stigma distance. **b)** Distribution of multivariate evolvabilities (mean = 2.6%) along 1000 simulated random unit-length selection gradients. Divergence-evolvability relationship among **c)** populations and **d)** species. The evolutionary potential (evolvability) of a trait predicts its realized evolutionary change (divergence) across timescales. G_{min} indicates the the least evolvable traits and g_{max} indicates the most evolvable traits of the **G**-matrix, i.e. the traits with the lowest and highest evolutionary potential, respectively. Figure material modified from Opedal *et al.*, 2022: © 2022 The Authors.

Chapter III: Pollen performance

Once pollen is deposited on the stigma, the pollen grains must germinate and grow down the style in competition for ovule fertilization. Despite an expectation of strong directional selection for ever-faster pollen tube growth, the literature suggests tremendous variation in pollen performance. In **chapter III**, I focused on variation in pollen performance across pollination environments (as indicated by the mating system: selfing vs outcrossing).

a) Pollen germination



b) Pollen tube length

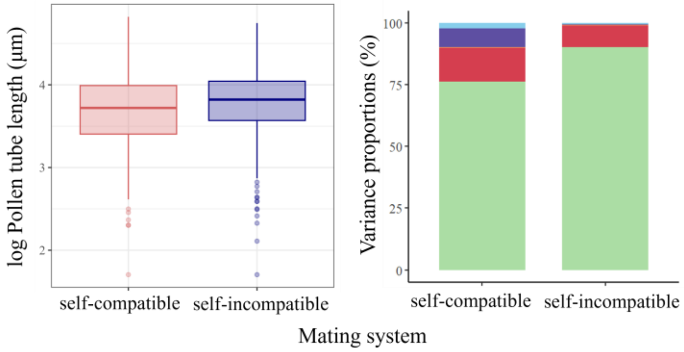


Fig. 11: Variation in pollen performance across pollination environments. Pollen performance was estimated as **a)** pollen germination per sample (%), and **b)** log pollen tube length (µm). The boxplots illustrate the average pollen performance across mating systems. The barplots illustrate the components of variance contributing to variation in pollen performance

I expected greater pollen performance in outcrossing populations of *A. alpina* due to stronger sexual selection (Walsh & Charlesworth, 1992; Mazer *et al.*, 2010; Gutiérrez-Valencia *et al.*, 2022), however, previous studies have yielded mixed results (Hove & Mazer, 2013; Mazer *et al.*, 2018). Any patterns in pollen performance across mating systems could be obscured by genetic variation among populations (e.g. due to local adaptation or genetic drift) and within populations (among-pollen donor variance), environmental plasticity (exemplified by thermal plasticity), as well as physiological effects (exemplified by pollen-age effects).

While pollen germination was greater in self-compatible *A. alpina* (Fig. 11a), pollen tube length was greater in self-incompatible plants (Fig. 11b), most strongly reflected in the longest pollen tubes within a sample being 34% longer in self-incompatible populations. Variance in pollen germination was greater in self-compatible than self-incompatible plants. This pattern was mainly driven by thermal plasticity (Fig. 11a), while other factors under study contributed comparatively less to variation in pollen performance. The idiosyncratic patterns of pollen performance across pollination environments may be explained by the complex interplay between numerous factors contributing to variation in pollen performance. These factors may ultimately obscure mating-system variation in pollen performance.

My results showcase the challenge of quantifying broader patterns of trait variation in response to the pollinator environment, when these patterns are obscured by a multitude of genetic, abiotic, and physiological factors. The context-dependency of pollen performance may maintain the observed variation in pollen performance traits. This work also furthers our understanding of the evolution of pollen performance traits, which represent one of the last steps in the long journey to successful plant reproduction.

In **chapter III**, I discovered idiosyncratic patterns of variation in pollen performance across pollination environments. These patterns may be explained by thermal plasticity in pollen performance which may have obscured any pattern of variation across pollination environments. These results imply that any pattern in floral trait variation across pollination environments may be obscured by numerous other factors contributing to floral diversity. Studies that combine multiple selective agents on floral trait variation at once may be the path forward to improve our understanding of floral evolution.

Chapter IV: Fitness consequences of changing pollinator communities

Fourth, changing pollinator communities can affect plant fitness, not only through reduced, but also through delayed pollination. When pollination is delayed, it may occur more frequently with older pollen with potential negative effects on seed production. In **chapter IV**, I aimed to understand variation in pollen longevity across pollination environments, as well as the consequences of delayed pollination for seed production. I expected pollen longevity to evolve jointly with the mating system in response to the pollination environment.

Confirming the predictions, pollen longevity was shortest in highly autonomously selfing *D. scandens* populations (Fig. 12a). Meanwhile, more outcrossing populations that may regularly experience delayed pollination, had greater pollen longevity to account for delayed pollinator visits. Pollination with older pollen negatively affected seed quantity (Fig. 12a), but not seed quality. Negative effects of delayed pollination were most strongly expressed in highly selfing populations that may not usually experience delayed pollination. Pollen longevity appears to evolve jointly with the mating system in response to variation in the pollination environment.

Plants may evolve strategies to ensure stable seed production when pollination is delayed. Sequential pollen presentation and pollen competition within inflorescences mitigated the negative effects of pollination with old pollen and led to continuously stable and high seed quantity and quality (Fig. 12b). My results suggest that sequential pollen presentation schedules may have an underappreciated function in alleviating the cost of delayed pollination.

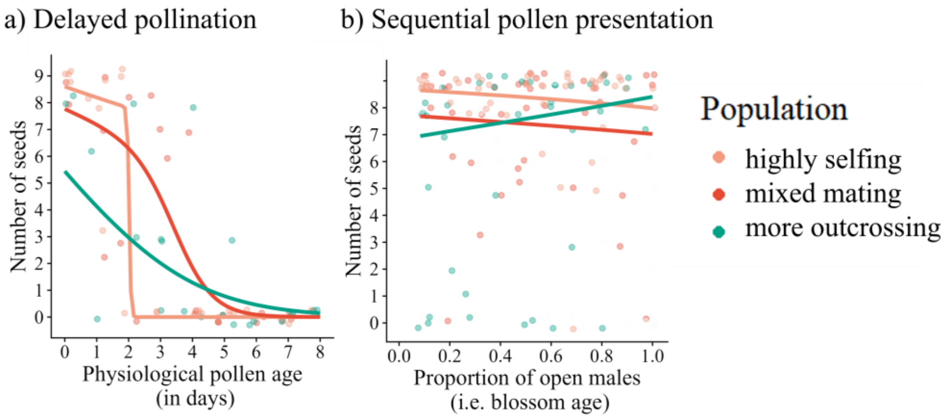


Fig. 12: Effects of delayed pollination and sequential pollen presentation on seed set in a subset of the populations used in chapter IV. **a)** The effect of physiological pollen age on the number of seeds per blossom (max. 9 seeds). Pollination with old pollen results in reduced seed set. **b)** Effect of the proportion of opened male flowers per blossom (proxy for overall blossom age) on seed set. **c)** Effect of the number of male flowers within a blossom used for hand-pollination (proxy for the potential variance in pollen age) on seed set. **b)** Sequential pollen presentation (i.e. sequential opening of male flowers) within a blossom results in stable seed set, despite an older age of the overall blossom, or older pollen being present within the pollen load.

Global changes in pollinator communities have negative consequences for plant reproduction (Potts *et al*., 2010; Rodger *et al*., 2021). Reduced and delayed pollination may result in the evolution of plant mating systems (Kalisz & Vogler, 2003; Eckert *et al.*, 2006), with cascading effects on numerous floral traits. While the consequences of reduced pollination have been studied in some detail (e.g. Hildesheim *et al.*, 2019), the consequences of delayed pollination have received less attention. Prior research on the effects of delayed pollination has focused primarily on fitness consequences expressed in the female function (e.g. Castro *et al.*, 2008; Hildesheim *et al.*, 2019b), or on male ecological longevity in terms of pollen removal rates (e.g. Ashman & Schoen, 1994).

In **chapter IV**, I added a layer of complexity to our understanding of the consequences of global changes in pollinator communities for plant populations. While focusing on the evolution of pollen longevity, I provided evidence that numerous floral traits evolve jointly with the plant mating system due to a common selection pressure by the pollination environment. I demonstrated that pollen age effects are an important consideration in the study of pollinator declines, because they mediate the evolution of pollen longevity and hence the ability to cope with delayed pollination. These results highlight the importance of considering the cascading effects of changes in pollinator communities for the evolution of floral diversity.

Conclusions and outlook

Flowers exhibit an astounding diversity of phenotypes. Much of this variation is associated with variation in pollinator communities (Grant & Grant, 1965; Stebbins, 1970). The multidimensional complexity of flower structures, quantitative and qualitative variation in floral traits, and the multitude of selective agents shaping floral form, render it a challenge to understand the tremendous observed variation in floral phenotypes. In this thesis, I studied a series of floral traits along the pathway to pollination: floral scent as a pollinator advertisement and reward, three-dimensional flower morphology involved in pollinator fit and pollen deposition, and post-pollination processes such as pollen performance traits.

Floral complexity, for example in terms of multidimensional scent chemistry or three-dimensional flower morphology, holds challenges during data analysis and interpretation. The complex floral trait types studied in this thesis are fundamentally different from each other. Pollinator fit traits vary quantitatively and follow a gaussian distribution, i.e. a minor change in genotype may have a minor effect on phenotype. This implies that minor deviations from optimum pollinator fit may still result in successful pollen transfer. Meanwhile, floral scent compounds are qualitatively different from each other. A novel scent compound may arise from a minor modification to a biosynthetic pathway and be structurally very similar to the other scent compounds in the scent bouquet. Yet, the resulting novel scent compound may qualitatively alter the scent bouquet so drastically that it attracts an entirely novel set of pollinators, i.e. a minor change in genotype may have major effects on phenotype.

Even when considering “simple” trait measurements such as pollen tube length in millimeters or pollen longevity in days, the complexity of underlying mechanisms that cause variation in floral phenotypes, introduces challenges to interpreting the observed phenotypic variation. This leaves open opportunities for continued improvement of quantitative analytical methods beyond a descriptive approach of floral phenotypic variation. In combination with observations of the ecology of flowers and the natural history of our study species, are we then able to understand the evolutionary processes shaping floral diversity. This trait complexity complicates predictions about the evolution of floral traits in changing environments.

Throughout this thesis, I used a geographic comparative approach for studying floral phenotypic variation. The long-term pollination environment (i.e. pollinator assemblage, abundance, and efficacy) is reflected in the evolved plant mating system (selfing vs. outcrossing). Note that the evolved plant mating system is the result of the long-term pollination environment, i.e. populations that have chronically suffered from inadequate pollinator-mediated pollination may have evolved the ability for autonomous selfing. However, this does not exclude the possibility that the plants presently receive sufficient pollinator visits, i.e. it is important to distinguish between the evolved mating system and the present ecology of a plant population.

In this thesis, I employed natural variation in pollination environments (i.e. evolved mating systems) to understand the role of pollinators in shaping the evolution of complex floral traits. This approach resembles a natural experiment where the pollinator assemblages and pollinator reliability are varied across plant populations. By comparing floral traits in selfing and outcrossing populations, i.e. populations that have historically received insufficient pollinator visits versus populations that have received sufficient pollinator visits, we may draw conclusions about the evolution of floral traits given human-induced pollinator declines. Thus, the geographic comparative approach is a powerful tool that can help us disentangle pollinator-mediated floral trait variation from other sources of variation on floral form.

My work highlights the challenges in disentangling the contribution of multifarious changes in the environment to floral variation. The complex interplay of genetic variation among and within populations, physiological effects within the flower, and the adaptive and plastic response to the abiotic environment, contribute to maintaining the tremendous observed variation in floral traits. These results imply that the context-dependency of floral trait evolution may maintain the observed variation in floral phenotypes. Disentangling sources of variation in floral phenotypes is invaluable to understand consequences of anthropogenic changes to the environment, e.g. in terms of pollinator declines or climate change. By considering effects of delayed pollination (reflected in pollen age effects) and changes in the abiotic environment (reflected in the temperature response), I was able to assess the relative importance of some of the major sources of variation that may be subject to ongoing changes worldwide.

In this thesis, I contributed to a better understanding of the role of pollinators in shaping the beautiful diversity of complex floral phenotypes. Profound knowledge of the evolutionary processes underlying floral evolution is crucial for predicting the consequences of anthropogenic changes in the environment for plant fitness. Still, the co-evolution between flowers and pollinators still holds many mysteries, and I am excited to see what research on the ecology and evolution of flowers has yet to bring.

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