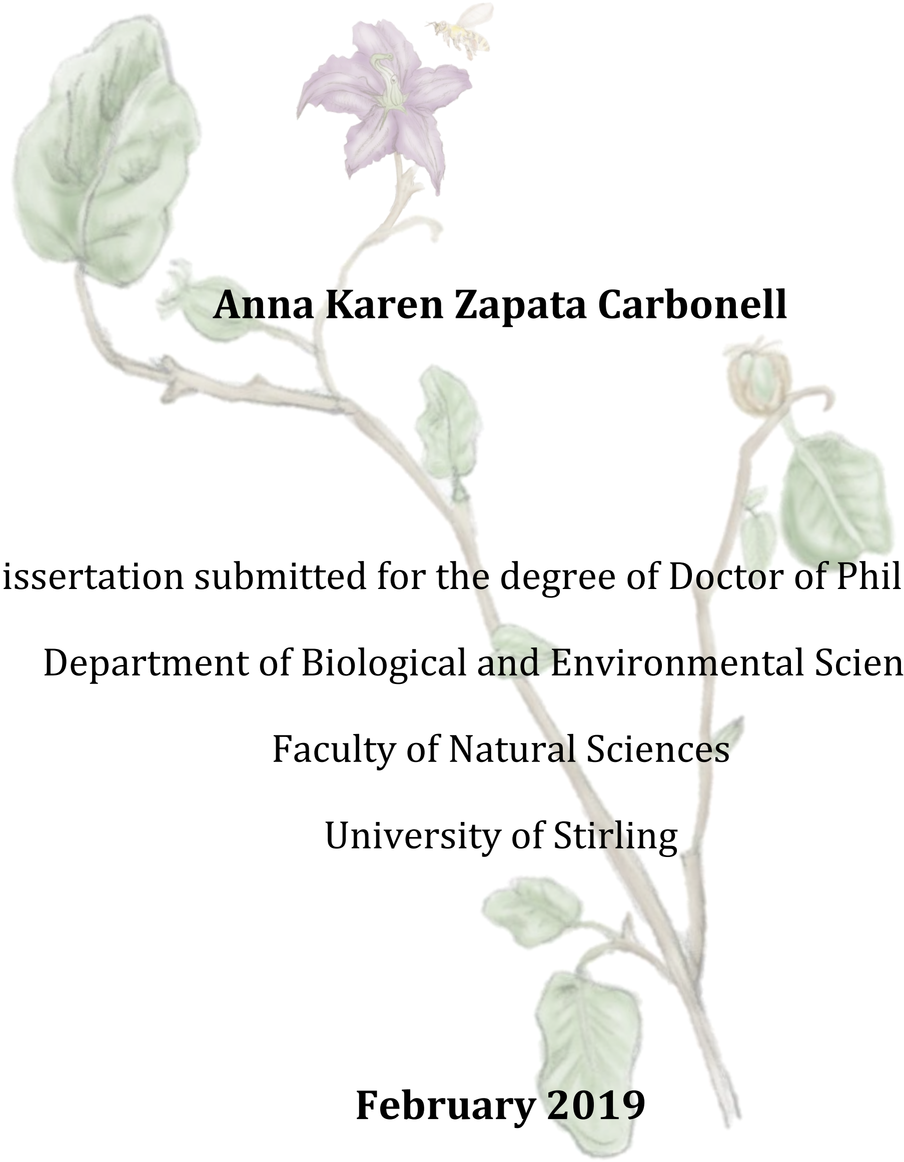
Expression and functional significance of andromonoecy in *Solanum houstonii* Martyn



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Dissertation submitted for the degree of Doctor of Philosophy
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February 2019

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Declaration

I hereby declare that this dissertation is an original piece of work that embodies the results of my own research. All work contained herein has not been submitted for any other degree. Where appropriate, I have acknowledged the nature and extent of work carried out in collaboration with others.

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Acknowledgements

First, I would like to thank my sponsors CONACYT, COQCYT and the University of Stirling, in particular the Biological and Environmental Sciences Department for funding my studies through scholarships to pay for my living costs and tuition fees.

Thanks to my supervisors Mario and Luc, for encouraging me since the beginning of my PhD until the end. Mario thank you very much for the opportunity to develop this PhD project with you. For sharing your enthusiasm for science and teaching me how to become a better researcher. Thank you for your support and for encouraging me to do my best. Luc thank you very much for always being there for meetings and cheering me up. For all your support while writing up and simple explanations that helped me to understand statistical models. Thank you for your empathy, patience and kind disposition.

Thank you to the Vallejo-Marin lab, past and present members, for all the great chats, drinks and words of encouragement.

Thanks to everyone who helped me during my field, lab and glasshouse work. Particularly, I am grateful to Leticia Mimeza, Joel Puc, Tere Aguilar, Indira Angélica, Kevin Baaz, Alejandra Euan, Saturnino Durán, Paula Sosenski, Jonás Aguirre, Isabel Castillo, Mario Durán, Flor Carbonell and Gonzalo Zapata for their help during my fieldwork in Mexico. Thank you George MacLeod, Kat Raines, Blanca Arroyo, David Real, Sofie Meeus, Pauline Pantoja, Lucy Nevard, John Patterson and Pakkapol Thaowetsuan for their assistance during my glasshouse and laboratory experiments.

I want to thank the institutes and research groups in where I did short stays during my PhD research. In particular, thanks to the Tropical Ecology Department at Universidad Autónoma de Yucatán that received me during my fieldwork season in Mexico. Thank you Victor Parra-Tabla and Paula Sosenski, for your logistic support and encouragement during my field trips. Thank you to the Royal Botanic Gardens of Edinburgh for providing the facilities to conduct microscopy and SEM analyses and Dr. Louis Ronse de Crane for accepting me in his lab and teaching me how to dissect and prepare my samples. I also will like to thank the staff from the Estates and Campus Services team of University of Stirling. Thanks to Eileen, Jacqueline and all the people that works in the grounds and garden team for their help and advice to take a better care of my plants.

I am very grateful to all the support my family provided. Thank you Gonzalo Zapata, Flor Carbonell and Jose Carbonell for your unconditional help, love and all the words of encouragement during this journey. Thank you Saturnino, my partner and best friend, for helping me to get through the most difficult days, to improve my fitness and health, for the endless chats, jokes and for being the light at the end of road.

A very special thank you goes to Mario Duran for his amazing art that I had the fortune to show in this book.

Lastly, thank you to all the people I share this journey with. My office and university mates Anisha Kewsani, Pauline Pantoja, Chris Pollard, Emma Sheard, Emma Bush Becca Lawrence, Maria Aullo, Izzy Jones, Erin Stoll, Kat Raines and Jess Goodman for the uncountable chats, the help with codes and laughs. Thank you to my Edinburgh and Mexican friends, Nora and Tom, Jonti, Nathan, Miroslawa, Lenka, Anna, Keila, Angeles, Karime. Thank you very much for being there when I needed it the most.

Lastly, thank you to my examiners, Rocio Perez-Barrales, Matt Tinsley and David Copplestone, who brought upon themselves with this long document and a long viva.

Abstract

Andromonoecy, the production of both hermaphrodite and female-sterile (staminate) flowers in the same plant, is a sexual system that has evolved independently numerous times and is found in 33 families and ~4000 species of flowering plants. Over the last three decades, andromonoecy has been used as a model to study resource allocation in plants, and to investigate the evolution of unisexual flowers. However, large gaps remain in our knowledge of the mechanisms that promote the production of staminate flowers. In this thesis, I investigated the expression and functional significance of andromonoecy in *Solanum houstonii*, a Mexican endemic perennial shrub, by 1) assessing sex determination of staminate flowers, 2) examining the functional role of staminate flowers and 3) evaluating the reproductive consequences of andromonoecy in natural populations. In this thesis, first, I characterised the andromonoecy of *S. houstonii*. I achieved this by determining the main morphological differences among flower types (hermaphrodite and staminate) and establishing how these differences arise through floral development (Chapter 2). Second, I assessed the lability in the production of hermaphrodite and staminate flowers within an individual in order to determine whether sex expression is a plastic response to environmental changes in resource availability (Chapter 3). Third, I investigated the functional role of staminate flowers on their efficiency at pollen donation, pollinator attraction and in siring seeds in a laboratory experiment using commercial bumblebees as pollinators (Chapter 4). Finally, I conducted field surveys in natural populations to evaluate the reproductive success of *S. houstonii* and the ecological factors that may maintain andromonoecy in this species (Chapter 5). Overall, my results demonstrated that in *S. houstonii* the suppression of female organs on staminate flowers occurs at early stages of development and is influenced by inflorescence architecture. Staminate flowers do not promote pollen donation or pollinator attraction in laboratory experiments more than hermaphrodite flowers. However, in natural populations, staminate flowers may increase pollen export and deposition as I found a relatively high incidence of pollinators and that fruit and seed set were not limited by pollen receipt. These findings provide new insights into the mechanisms involved in the production of staminate flowers, the functional significance of staminate flowers, and the reproductive success of an, andromonoecious species.

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Chapter 1
General introduction

Chapter 1. General introduction

Angiosperms have a striking variety of floral forms and remarkable variation in their reproductive strategies (Barrett, 2002a). The origin and maintenance of plant sexual structures have been a major focus of study to improve our understanding of the selective forces responsible for the evolution and functional significance of sexual reproduction in angiosperms (Barrett, 2002a, 2010). The study of plant reproductive systems (also known as breeding systems) encompasses all the aspects of sexual and asexual reproduction, from embryo development to pollination (Cardoso *et al.*, 2018). Sexual systems, the arrangements of different sexual organs on different flowers and/or individuals (Cardoso *et al.*, 2018), have received considerable attention over the past decades as they can clarify the origin and maintenance of angiosperm reproductive systems (Sakai & Weller, 1999; Webb, 1999; Barrett, 2002a; Miller & Venable, 2002; Mitchell & Diggle, 2005; Barrett & Hough, 2013).

Most angiosperms produce exclusively bisexual or hermaphrodite flowers, which bear both female and male function in the same flower (Yampolsky & Yampolsky, 1922; Barrett, 2002a; Cardoso *et al.*, 2018). Hermaphroditism is widespread, occuring in approximately 72-90% of the species (Yampolsky & Yampolsky, 1922; Torices *et al.*, 2011) and is the ancestral sexual system of angiosperms (Sauquet *et al.*, 2017). However, a small proportion of species possess unisexual flowers either female (pistillate) or male (staminate) flowers, either in the same or in different individuals (Yampolsky & Yampolsky, 1922; Torices *et al.*, 2011; Renner, 2014; Christenhusz & Byng, 2016). The systems with unisexual flowers within the same individual are classified as monoecious if they bear pistillate and staminate flowers, andromonoecious if they bear hermaphrodite and staminate flowers and gynomonoecious if they produced hermaphrodite and pistillate flowers. When unisexual flowers are produced by different individuals, the sexual system is dioecious if individuals produce either pistillate or staminate flowers, androdioecious if they produced hermaphrodite or staminate flowers and gynodioecious if individuals bear either hermaphrodite or pistillate flowers (Sakai & Weller, 1999; Cardoso *et al.*, 2018). Unisexuality is rare; occurring in only approximately 6% of angiosperms species (Yampolsky & Yampolsky, 1922; Torices *et al.*, 2011; Renner, 2014). Although evidence suggests that bisexuality is the ancestral condition of angiosperms, it is still unclear whether all these sexual systems have evolved directly from this condition or if some have arisen via other non-bisexual systems.

Two main pathways have been proposed to explain the transition from bisexuality to unisexuality. In the first one, gynodioecy is an intermediate stage before full separation of sexes, while in the second monoecy is the intermediate. In the gynodioecy pathway, hermaphrodite populations that experience reduced outcrossing, inbreeding depression and reduced fitness experience a rapid spread of male-sterility mutations, causing populations to transition from hermaphrodite to gynodioecious. Selection then favours male function in hermaphrodites by the presence genetic modifiers on female fertility (Charlesworth, 1999, 2006; Barrett, 2002a; Dufay *et al.*, 2014). Alternatively, in the monoecy pathway, sexes are separated into different flowers due to developmental changes caused as a strategy to improve sex allocation (Charlesworth & Charlesworth, 1979; Bertin, 1982; de Jong *et al.*, 2008). Disruptive selection on variation in the proportion of pistillate and staminate flowers (sex expression) then increases the gender specialisation culminating in the origin of female and male plants. The evolution of dioecy on these two main pathways has received a lot of attention and support through research on some model species and families (Thomson & Barrett, 1981; Pannell, 1997, 2002; Sarkissian *et al.*, 2001; Ashman, 2006; Dorken & Pannell, 2009; Torices *et al.*, 2011; Dufay *et al.*, 2014; Anderson *et al.*, 2015). Less attention, however, has been paid to the relationships among other sexual systems that do not directly involve the evolution of dioecy, such as andromonoecy and gynomonoecy. Some of the studies investigating evolutionary transitions between sexual systems are focused on the species level (Webb, 1999; Huang, 2003; Vallejo-Marín & Rausher, 2007a; Boualem *et al.*, 2008) or at subgenus, clade or family level (Bertin & Kerwin, 1998; Sakai & Weller, 1999; Miller & Diggle, 2003; Martine *et al.*, 2006, 2009; Torices *et al.*, 2011). This research has provided valuable data on pathways towards the evolution of separate sexes. However, the evidence collected through the last three decades is still a small fraction in the broad research necessary to understand the evolution of sexual systems.

Andromonoecy

Andromonoecy is the sexual system of plants in which hermaphrodite and staminate flowers (female sterile) are produced in the same individual (Cardoso *et al.*, 2018). This is a rare system occurring in approximately 4000 species, which is only 1.7% of flowering plants (Miller & Diggle, 2003; Torices *et al.*, 2011). Despite this infrequent occurrence, an understanding of the fitness consequences of andromonoecy is important as it can elucidate the transition pathways for the evolution of dioecy from hermaphroditism (Barrett, 2002a; Charlesworth, 2006; Torices *et al.*, 2011; Dai & Galloway, 2012). Several studies have focused on understanding the evolution of this sexual system and at least four main hypothesis are commonly used to explain the production of staminate flowers (Primack & Lloyd, 1980; Bertin, 1982; Charnov, 1982; Solomon, 1985; Diggle, 1991a, 1993; Elle & Meagher, 2000; Vallejo-Marín & Rausher, 2007b; Zhang & Tan, 2009).

One major hypothesis proposed for the evolution of andromonoecy is the *resource reallocation hypothesis*. This hypothesis suggests andromonoecy evolves as a reproductive energy-saving strategy, as it assumes staminate flowers are less costly than hermaphrodite flowers, and resources saved by producing staminate flowers can be reallocated towards fitness-enhancing traits of both male and female function. For instance, resources could be allocated towards the male function by the production of more staminate flowers and/or floral adaptations that increase pollen export or towards the female function by production of bigger ovaries in hermaphrodite flowers and large fruits (Primack & Lloyd, 1980; Bertin, 1982; Charnov, 1982; Vallejo-Marín & Rausher, 2007b). This hypothesis makes two assumptions: 1) staminate flowers are cheaper to produce than hermaphrodite flowers, 2) the production of staminate flowers does not reduce female fitness (i.e. fruit set) but rather staminate flower production increases male fitness and compensates for the resources invested in their production (Bertin, 1982). Some studies that support this hypothesis have demonstrated staminate flowers are smaller or lighter than hermaphrodite flowers (Primack & Lloyd, 1980; Anderson & Symon, 1989; Diggle, 1991a; Elle & Meagher, 2000; Cuevas & Polito, 2004; Diggle & Miller, 2004; Zhang & Tan, 2009; Dai & Galloway, 2012). However, other studies have shown the opposite or shown staminate and hermaphrodite flowers do not differ in size (Huang, 2003; Narbona *et al.*, 2008; Anderson *et al.*, 2014), suggesting staminate flowers are not always cheaper to produce. Regardless of those results, andromonoecy provides a strategy to optimally allocate resources to male and female function, either as a plastic response or as a fixed mechanism, and because of this, it is predicted to occur in species where the cost of maturing fruits is substantial (Primack & Lloyd, 1980; Bertin, 1982; Diggle, 1993). For instance, in changing environments where resource availability for plant growth and fruit production is reduced, andromonoecious species exhibit variation in their sex expression (relative production of staminate flowers) as a strategy to maximise resource reallocation towards fruit production (female fitness) (Solomon, 1985; Diggle, 1991b; Korpelainen, 1998). This variation in sex expression reveals that in some species, the production of staminate flowers can be phenotypically plastic (Solomon, 1985; Diggle, 1993; Miller & Diggle, 2003). Nevertheless, in other species sex expression is fixed regardless of differences in resource availability, as producing staminate flowers ensures there are enough resources for fruit production (Miller & Diggle, 2003, 2007). How often variation in the sex expression of occurs among andromonoecious species, and which are the processes that determine whether a species is plastic or non-plastic phenotypes are important questions to understand the role of the resource allocation hypothesis in the evolution of andromonoecy.

A second hypothesis suggests that staminate flowers are more efficient at pollen donation than hermaphrodite flowers (Bertin, 1982; Whalen & Costich, 1986; Podolsky, 1993). This is known as the *pollen donation hypothesis* and it could occur if one or more of the following occur: 1) staminate flowers are more attractive for pollinators; 2) staminate flowers are more successful at pollen export or 3) staminate flowers produce more or better quality pollen (Podolsky, 1992, 1993; Harder & Barrett, 1996; Elle & Meagher, 2000; Barrett, 2002b; Huang, 2003; Cuevas & Polito, 2004; Vallejo-Marín & Rausher, 2007b). The few studies that have attempted to test this hypothesis have yielded mixed results. Some studies have found staminate flowers are not always better at siring seeds than hermaphrodite flowers (Cuevas & Polito, 2004; Sunnichan *et al.*, 2004; Luo *et al.*, 2009), while others have found that staminate flowers produce less or equal amounts of pollen as hermaphrodite flowers and sometimes that pollen is a lower quality (Solomon, 1985; Cuevas & Polito, 2004; Vallejo-Marín & Rausher, 2007b) or even that staminate flowers are less effective in dispersing pollen grains (Podolsky, 1993).

A third hypothesis to explain the evolution of andromonoecy, known as the *increased pollen receipt hypothesis*, suggests that a relatively high production of staminate flowers causes an increased ratio of pollen to ovules per plant, promoting pollen accumulation on pollinators bodies, which as a result increases the probability of pollen deposition on stigmas (Bertin, 1982; Podolsky, 1993). It has been suggested this hypothesis might be experimentally tested by determining whether flowers produce high amounts of pollen or develop a morphology that enhances both pollen dispersion and pollinator efficiency at pollen extraction and deposition (Bertin, 1982;

Whalen & Costich, 1986; Podolsky, 1993). Finally, a fourth hypothesis to explain the evolution of andromonoecy is the *sexual interference hypothesis*, which suggests that segregating male and female function in different flowers reduces the interference between sexual organs during pollen removal and deposition (Solomon, 1986; Diggle & Miller, 2004; Quesada-Aguilar *et al.*, 2008). Previous work has found that pollen removal and deposition on hermaphrodite flowers can be less efficient due to the presence of the pistil, which interferes with the placement of pollinators in the flower during pollen extraction (Quesada-Aguilar *et al.*, 2008).

These four hypotheses are not mutually exclusive as staminate flowers could enhance pollen donation more efficiently than perfect flowers, either by redirecting resources from pistil development to pollen production or by developing an enhanced morphology better suited for pollen dispersal (i.e. heteranthery) (Bertin, 1982; Whalen & Costich, 1986; Podolsky, 1993). A specialised morphology not only would increase the probability of pollen reaching the stigma but also reduce interference during pollen extraction and deposition.

Solanum: floral traits and sexual systems

Solanum is a large genus of approximately 1400-1700 species that belongs to the Solanaceae family, one of the most diverse families in the Neotropics (Knapp, 2010; Echeverría-Londoño *et al.*, 2018). *Solanum* includes crops such as tomato (*S. lycopersicum*), potato (*S. tuberosum*) and eggplant (*S. melongena*) that are of economic importance. Species in this genus show a remarkable morphological and ecological diversity and a wide distribution, including temperate and tropical climates accross all continents (Levin *et al.*, 2006; Stern *et al.*, 2011; Vorontsova & Stern, 2013; Knapp *et al.*, 2017).

Solanum is traditionally divided into two major groups, the spiny and non-spiny solanums. The spiny solanums, known as the Leptostemonum clade (or subgenus *Leptostemonum* Bitter), comprise approximately 420 species and can be identified by the presence of prickles, stellate trichomes, and long anthers with small poricidal pores (Levin *et al.*, 2006; Martine *et al.*, 2006; Vorontsova & Stern, 2013; Echeverría-Londoño *et al.*, 2018). Spiny solanums are most diverse in the Neotropics (ca. 150 species), Australia (ca. 130), and Africa (ca. 79) and comprise a great variety of morphologies and breeding systems. This high diversity of spiny solanums out of the

Americas contrasts with the distribution of the diversity of the non-spiny solanums, which has more than 90% of its diversity distributed in the Neotropics (Echeverría-Londoño *et al.*, 2018).

Subgenus *Leptostemonum* comprises a great diversity of floral morphologies and sexual systems (Levin *et al.*, 2006). Flowers of *Solanum* are characterised for possessing five petals, a radially symmetric corolla and anthers that dehisce by terminal pores (Symon, 1979; Bohs *et al.*, 2007). Anthers bear pollen that acts as the main reward for pollinators, but in order to released, anthers need to be vibrated by bees capable of producing high frequency vibrations (Symon, 1979; Buchmann, 1983). However, some species also exhibit unusual floral traits such as four-merous and/or zygomorphic corollas, unequal stamens, and style deflection to one side of the flower in a left- or right-handed arrangement (i.e. enantiostyly) (Bohs *et al.*, 2007). *Solanum* species are characterised for being hermaphrodite, andromonoecious or dioecious (Symon, 1979; Whalen, 1984). In the subgenus *Leptostemonum,* the majority of species are either hermaphrodite or andromonoecious, and less than 1% are dioecious (Whalen & Costich, 1986; Levin *et al.*, 2006; Martine *et al.*, 2006). More precisely only 18 species are dioecious and are concentrated in four subgenera of the thirteen that conform the genus (Anderson *et al.*, 2015).

Andromonoecious plants in *Solanum* produce both hermaphrodite and female sterile flowers (staminate flowers). Usually, staminate flowers are identified by their reduced, non-functional gynoecia, large poricidal anthers and their occurrence in distal positions in the inflorescences. Hermaphrodite flowers often are the first flowers to develop in the inflorescences, they have prickly calyxes and a style longer than the anthers (Symon, 1979; Whalen & Costich, 1986; Anderson & Symon, 1989). In many species of *Solanum*, the proportion of hermaphrodite and staminate flowers present in the inflorescences (sex expression) varies (Diggle, 1991a, 1993). This variation causes many species to differ in their strength of andromonoecy. For instance species with many hermaphrodite flowers are considered weakly andromonoecious, while species with one hermaphrodite flower at the base of the inflorescence are considered strongly andromonoecious (Diggle, 1993; Diggle & Miller, 2013). Evidence suggests differences among species in their sex expression can be caused by environmental changes that induce developmental phenotypic plasticity or by genetic variation in plasticity (Diggle, 1991a, 1993; Diggle & Miller, 2013).

The dioecious species of *Solanum* are morphologically androdioecious but functionally dioecious (Anderson & Symon, 1989). In this system, plants bear either staminate flowers or morphological hermaphrodite flowers. Despite producing anthers and pollen, hermaphrodite flowers are pistillate and bear inaperturate, non-germinable pollen (Anderson & Symon, 1989; Zavada & Anderson, 1997; Knapp *et al.*, 1998). This is known as cryptic dioecy and it is the only form of dioecy in *Solanum* (Anderson & Symon, 1989; Knapp *et al.*, 1998; Martine *et al.*, 2009).

Solanum is an ideal group in which to study the evolutionary transitions from hermaphroditism to unisexuality, because of the presence of a considerable variation in breeding systems. Morphological and phylogenetic studies have hypothesised that andromonoecy is the ancestor of dioecy (Symon, 1981; Anderson & Stebbins, 1984; Anderson & Symon, 1989; Knapp *et al.*, 1998; Martine *et al.*, 2006, 2009). However, it is not clear yet how transitions from bisexuality to unisexuality occurred. One theory for the evolution of dioecy proposed by Knapp *et al*. (1998) suggests that in populations with inbreeding depression and reduced fitness, the appearance of a male sterility mutation is likely to spread rapidly. In an andromonoecious taxon, with a high variability in its sex expression, dioecy is more likely to develop. Moreover, selection for the retention of at least some pollen function or presence is favoured in *Solanum*, as pollen is the only reward for floral visitors. In order to assess the relative importance of these theories we need to investigate: 1) the level of female and male sterility, 2) the lability of sex expression of individuals and populations, 3) the developmental transitions among flowers of different sex, and 4) the success of natural pollination (Anderson & Symon, 1989; Knapp *et al.*, 1998; Martine *et al.*, 2009).

Study species

Solanum houstonii Martyn is a perennial shrub native to Mexico, widespread in in South central Mexico on both coasts, from the Yucatán Peninsula and Veracruz to Sinaloa and Sonora (Nee, 1993; Knapp *et al.*, 2017). *Solanum houstonii* plants naturally grow in a wide variety of dry and semi-deciduous forests, from thorn scrub in Sinaloa to humid semi-deciduous forest and coastal dunes in the Caribbean (Knapp *et al.*, 2017). *Solanum houstonii* individuals flower all year, and especially during the rainy season between June and September (Nee, 1993; Herbario-CICY, 2010).

Solanum houstonii belongs to the subgenus *Leptostemonum*, and the section *Elaeagnifolium* (Knapp *et al.*, 2017; Echeverría-Londoño *et al.*, 2018). It is characterised as strongly andromonoecious, as plants possess one basal hermaphrodite flower and several staminate flowers in each inflorescence. As for other *Solanum* species, hermaphrodite flowers have five lilac or purple petals and five similarly sized, yellow or purple poricidal anthers that require sonication in order to release pollen. However, unlike other andromonoecious species, staminate flowers are markedly dimorphic: they are short styled and possess two morphologically and functionally distinct sets of anthers of the same colour, a condition known as heteranthery (Knapp *et al.*, 2017). The first set are two short adaxial anthers that provide pollen for visiting insects and function as feeding anthers. The second set are three longer, curved abaxial anthers known as pollinating anthers, that presumably contribute to pollen transfer to the stigma of hermaphrodite flowers (Knapp *et al.*, 2017; Papaj *et al.*, 2017).

Pollinators of this species were unknown before the study carried out in this thesis, but were presumed to include bees capable of producing vibration similar in size to bumblebees such as *Bombus impatiens* (Papaj *et al.*, 2017). Experiments using captive *B. impatiens* have shown that bees prefer to vibrate feeding anthers and while doing so they hold these anthers and position their body in such way that pollen from the feeding anther pores will release pollen directly onto the underside of the abdomen or thorax (Papaj *et al.*, 2017). However, it remains to be investigated whether native bees from Mexico have a similar behaviour or whether the morphological adaptations in the anthers promote pollen transference among natural populations.

Solanum houstonii provides a good opportunity to investigate the functional and evolutionary significance of andromonoecy, as this species produces a relatively high proportion of staminate flowers and possess morphological adaptations, such as heteranthery and herkogamy that presumably enhance pollen export and deposition. Moreover, this species is closely related to other species with different strengths of andromonoecy and dioecy, as the section *Elaeagnifolium* is the sister of the clade of the Old World solanums which includes the majority of dioecious species (Knapp *et al.*, 2017; Echeverría-Londoño *et al.*, 2018).

Aims and approaches

In this thesis, I investigated the ecological and evolutionary consequences of andromonoecy in *Solanum houstonii*, a Mexican endemic perennial shrub, by assessing sex determination of staminate flowers, examining the functional role of staminate flowers and evaluating the reproductive consequences of andromonoecy in natural populations. Below I described the rationale and specific questions I set to address in each chapter.

Chapter 2: Development and morphology

Most angiosperms are hermaphrodite, and are characterised by producing bisexual flowers. However, unisexuality has evolved independently in many different plant families (Diggle *et al.*, 2011), suggesting there are different developmental and genetic pathways for its determination (Kater *et al.*, 2001; Boualem *et al.*, 2008; Li & Liu, 2017). Elucidating the developmental mechanisms for sex determination can shed light on the evolutionary pathways from hermaphroditism to unisexuality. Model species that possess unisexual and bisexual flowers, such as melon, maize and cucumber, have been used to determine the developmental mechanisms involved in the transition of bisexuality to unisexuality. However, we still lack a comprehensive explanation of how sex determination is achieved; therefore, more research on non-model species is needed. In species such as *S. houstonii* that produce hermaphrodite and staminate (male) flowers, it has been hypothesised that unisexuality is achieved due to organ arrest in later stages of development. Therefore, in Chapter 2, I characterised the morphological differences of hermaphrodite and staminate flowers at different stages of development, in order to determine when the unisexuality is achieved. To address that, I specifically asked the following questions:

1) Does sex determination in staminate flowers occur by gynoecium arrest at later stages of development?
2) When does the main differences in anther size between hermaphrodite and staminate flowers arise?
3) What are the main morphological differences associated with hermaphrodite and staminate flowers?

Chapter 3: Patterns of sex expression

Andromonoecy is often considered a strategy to maximise fitness by optimal allocation of reproductive resources to male and female functions. In *Solanum* many species exhibit patterns of sex expression that are plastic in response to changes in resource availability for plant growth. However, a reduced number of species possess phenotypes that are non-plastic and are constrained by architectural effects within the inflorescence. Very often, the species that possess a plastic phenotype vary in their production of hermaphrodite and staminate flowers, but non-plastic species phenotypes maintain a relatively high proportion of staminate flowers that does not vary. *Solanum houstonii* is characterised for possessing one hermaphrodite flower and several staminate flowers per inflorescence, which suggests it is non-plastic with respect to sex expression. In this chapter, I aimed to determine whether the production of hermaphrodite and staminate flowers is a plastic response to resource availability. To achieve this, first, I characterised sex expression in natural populations and in plants grown under controlled conditions and, second, I experimentally increased and decreased the resources available for plant growth. Specifically, I asked the following questions:

1) How does sex expression vary in natural populations?
2) Does flower position in the inflorescence have an effect on sex expression?
3) Does increased and decreased resource availability affect sex expression at the inflorescence level?
4) Does the removal of the basal flower, often characterised as hermaphrodite, affect sex expression in the inflorescence?

Chapter 4: Functional significance of staminate flowers

One of the principal hypotheses for the evolution of andromonoecy suggests staminate flowers promote pollen donation and pollinator attraction. However, the support for this hypothesis is very inconsistent, as some studies suggest different roles for staminate flowers in different environments. Therefore, in Chapter 4, I aimed to determine whether the main role of staminate flowers is to promote pollinator attraction and pollen donation. I specifically asked:

1) Is pollen from staminate flowers better at siring fruits than pollen from hermaphrodite flowers?
2) Do pollinators prefer visiting staminate flowers over hermaphrodite flowers?
3) Are staminate flowers more efficient at transferring pollen to the stigma than anthers of hermaphrodite flowers?

Chapter 5: Reproductive success and pollination ecology

Plants that are buzz-pollinated that rely on pollinators for reproduction or possess separate sexes, often experience high levels of pollen theft, which can potentially have negative effects on seed production (Ashman *et al.*, 2004; Hargreaves *et al.*, 2009; Koski *et al.*, 2018a). *Solanum houstonii* is an andromonoecious, buzz-pollinated plant distributed across the tropical dry forest and disturbed areas of Mexico, which like other Mexican *Solanum* species, is expected to be exploited by bees due to its great pollen availability. However, little is known about the pollination ecology of this species and whether its reproductive success is limited by pollen or pollinators. In Chapter 5, I conducted experiments in natural populations in Yucatan to assess the extent to which reproduction in this species depends on pollinators and to determine the effect of pollen theft on the reproductive success of *S. houstonii.* To achieve this, I addressed the following questions:

1) Does *S. houstonii* require pollinators to produce seed?
2) Are natural populations of *S. houstonii* pollen limited?
3) Which are the main floral visitors? What is their behaviour on flowers?
4) Does the presence of pollen thieves negatively impact the reproductive success of *S. houstonii*?

Chapter 2

Development and morphology

Chapter 2. Development and morphology of hermaphrodite and staminate flowers of *Solanum houstonii*

2.1 Abstract

Andromonoecious plant species are characterised by the presence of hermaphrodite and female-sterile (staminate) flowers in the same individual. In *Solanum* (Solanaceae) andromonoecy is very common and has evolved repeatedly. Some *Solanum* species exhibit a plastic response to resource availability and vary in the proportion of hermaphrodite and staminate flowers they produced. Sex determination in these species is suggested to be labile and to occur in later stages of development, a few days before flower anthesis. Other species exhibit a more fixed expression of the proportion of hermaphrodite and staminate flowers, however, in these species it remains unknown whether sex determination occurs similarly in later stages of development or from inception. Here, I investigated the developmental patterns of sex determination in *Solanum houstonii*, a species that apparently possess a fixed pattern of expression. To determine the developmental origin of unisexuality, I measured a range of floral traits on floral buds at different stages of development. Additionally, I characterised the morphological traits in mature flowers to determine the main morphological differences associated with each flower sex. My results indicated that early arrest of gynoecium growth resulted in female-sterility on buds at distal positions of the inflorescence. Gynoecial arrest occurred when buds reached 3-4 mm in length, indicating that buds became unisexual at this stage. At this length, anther dimorphism, also arose, but only in buds where gynoecium arrest occurred. In mature flowers, I found strong differences in size and shape between hermaphrodite and staminate flowers, such as long styles and short anthers in hermaphrodite flowers and anther dimorphism and zygomorphy in staminate flowers. These findings indicate that at inception all flowers of *S. houstonii* were bisexual, but after they reached a size of 3-4mm flowers from distal positions of the inflorescence became unisexual due to early arrest of gynoecium. These results contrast with those for other species of *Solanum* that exhibit phenotypic plasticity in sex expression, suggesting developmental patterns of sex determination may generally differ between species that exhibit plastic phenotypes and species that exhibit fixed sex expression.

2.2 Introduction

There are approximately 369,000 species of flowering plants in the world (Willis, 2017), and the majority are hermaphrodite, producing female and male organs within the same flower. However, a small percentage of plants have unisexual flowers, both male and female sexes present on the same plant (monoecy) or with flower sexes separated on different individuals (dioecy) (Torices *et al.*, 2011; Renner, 2014; Christenhusz & Byng, 2016). Sex determination is a developmental process that occurs by the selective abortion or arrest of either male or female reproductive organs at any point during flower development, from initiation to maturation (Mitchell & Diggle, 2005; Diggle *et al.*, 2011; Mao *et al.*, 2017). In female and male flowers of the same species, sex determination tends to happen at similar stages, sometimes due to antagonistic role of hormones that control alternative male or female developmental process (Diggle *et al.*, 2011; Pannell, 2017). Unisexual flowers have evolved independently in many different plant families (Diggle *et al.*, 2011) and previous studies on model plant species, such as melon, maize and cucumber, suggests there are different developmental and genetic pathways for its determination (Kater *et al.*, 2001; Boualem *et al.*, 2008; Li & Liu, 2017). However, because of the multiple origins of unisexuality, we still lack of a comprehensive explanation of how sex determination is achieved; therefore, more research on non-model species with atypical morphologies are need to clarify the possible pathways that have led to this impressive diversity.

Andromonoecy is a sexual system where individual plants possess both hermaphrodite and female-sterile (hereafter, staminate) flowers. It has evolved independently several times and is found in approximately 4000 species of at least 33 families (Miller & Diggle, 2003; Torices *et al.*, 2011). In andromonoecious species, sex determination of staminate flowers often occurs by selective arrest of the female carpel (Diggle, 1991b; Kater *et al.*, 2001). For instance, in some species of Cucurbitaceae, male sex determination of andromonoecious and monoecious plants occurs due to the loss an active enzyme (encoded by the *ACS7* gene) that inhibits the development of male organs, while female and hermaphrodite sex determination is mediated by the activity of the same enzyme in the ethylene pathway (Boualem *et al.*, 2008; Rodriguez-Granados *et al.*, 2017).

Andromonoecy is widespread among more than 1500 species of the large genus *Solanum* (Solanaceae), particularly in the subgenus *Leptostemonum* (the "spiny Solanum"), which besides andromonoecy includes hermaphrodite and dioecious species and a diverse floral morphology (Levin *et al.*, 2006; Weese & Bohs, 2007). In many species of *Solanum*, andromonoecy sex expression can be labile, which means the proportion of hermaphrodite and staminate flowers varies among inflorescences, or sex expression can be fixed at certain position of the inflorescences, which means the relative proportion of hermaphrodite and staminate flowers is maintained (Diggle & Miller, 2004). Sex expression varies from weak andromonoecy, in which plants express a higher proportion of hermaphrodite flowers than staminate flowers per inflorescence, to strong andromonoecy, in which plants only possess one hermaphrodite flower per inflorescence (Whalen & Costich, 1986; Miller & Diggle, 2003; Diggle & Miller, 2004).

Flowers of andromonoecious species in *Solanum* commonly possess five poricidal anthers of approximately the same size and shape, arranged in a compact to loose cluster at the centre of a five-merous radially symmetric corolla (Bohs *et al.*, 2007). Usually, staminate flowers have infertile pistilloids that are shorter than stamens, while hermaphrodite flowers usually possess long pistils that can vary in their size (Whalen & Costich, 1986; Knapp *et al.*, 1998). The arrangement of hermaphrodite and staminate flowers in inflorescences varies depending on the degree of sex expression. For instance, in weakly andromonoecious species, hermaphrodite flowers are commonly distributed at different positions across the inflorescence, while in strongly andromonoecious plants, hermaphrodite flowers tend to be located at more basal positions (Miller & Diggle, 2003; Diggle & Miller, 2004). Although many species are characterised by this morphology, a small subset of species exhibit floral morphological adaptations (i.e. enantiostyly, heteranthery, zygomorphy) that promote pollen donation and enhance differentiation among sexes (Knapp, 2002; Bohs *et al.*, 2007; Knapp *et al.*, 2017). This subset provides a unique opportunity to clarify the conditions that generate variable sexual systems in plants.

One of the unusual species in this group is *Solanum houstonii*, a strongly andromonoecious species that often possesses one hermaphrodite flower and several staminate flowers per inflorescence. Unlike other andromonoecious *Solanum* species, flowers of *S. houstonii* not only differ in the style length, but also in the anther length. Staminate flowers display two markedly distinct sets of anthers that differ in both size and shape (heteranthery) while in hermaphrodite flowers this dimorphism between anthers does not exist (Knapp *et al.*, 2017). It has been suggested that sex determination of staminate flowers in labile andromonoecious species in *Solanum* occurs by gynoecium arrest at later stages of development. For instance in *S. hirtum*, developmental analyses indicated that sex determination of unisexual flowers happens 6-7 days before anthesis to allow this species to adjust its sex expression in response to resource availability and fruit production (Diggle, 1991a,b, 1994). However, in species that produce only one hermaphrodite flower per inflorescence, like *S. houstonii*, it remains to be tested whether sex determination also occurs by abortion of gynoecium at later or earlier stages of development, determining these patterns could provide insights in the evolutionary pathway to achieve unisexuality in *Solanum*.

In this study, I examined the developmental patterns among the flowers of *Solanum houstonii* with the aim to determine when unisexuality is achieved in staminate flowers. Moreover, because of the marked morphological differentiation in anther size between hermaphrodite and staminate flowers, I examined the main morphological differences among flowers and determined whether they were associated with sex determination. I specifically asked the following questions: 1) does sex determination in staminate flowers occur by gynoecium arrest at later stages of development? 2) When does the main differences in anther size between hermaphrodite and staminate flowers arise? 3) What are the main morphological differences between to hermaphrodite and staminate flowers?

2.3 Methods

2.3.1 Study species

Solanum houstonii Martyn is a perennial shrub endemic of Mexico (Knapp *et al.*, 2017). This species is characterised by strong andromonoecy for possessing in each inflorescence one basal hermaphrodite flower and up to 35 staminate (female sterile) flowers (Knapp *et al.*, 2017). Inflorescences are scorpioid-cymes that bear up to five open flowers at a time that last two days in natural populations and up to five days under controlled conditions (AKZC personal observation). Flowers have five lilac or purple petals and five yellow or purple poricidal anthers, are heterandrous, nectarless and markedly dimorphic (Knapp *et al.*, 2017). Hermaphrodite flowers are characterised for their five straight anthers of relatively similar size, a long style (three times larger than anthers) and their prickly calyx. Staminate flowers, on the contrary, are short styled, and characterised for possessing two morphologically and functionally distinct set of anthers (Knapp *et al.*, 2017; Papaj *et al.*, 2017) (Figure 2.1, 2.2). Two short adaxial anthers provide pollen for visiting insects and function as feeding anthers and three longer, curved abaxial anthers (similar in size to the style of in hermaphrodite flowers, Knapp *et al*., 2017), known as pollinating anthers, presumably contribute to pollen reaching the stigmas of hermaphrodite flowers (Papaj *et al.*, 2017). *Solanum houstonii* individuals bloom all year, especially during the rainy season between June and September (Nee, 1993; Herbario-CICY, 2010), and are distributed in dry and humid semi-deciduous forest and disturbed areas across Mexico (Knapp *et al.*, 2017).

To study flower development and morphology in *S. houstonii* I used plants grown under environmentally controlled conditions in the glasshouse at the University of Stirling, UK and plants from natural populations from Yucatan. Plants from the glasshouse were grown from seeds collected in Mexico between 2007 and 2015. Seeds were germinated during April-May in 2015. To induce germination, seeds were pre-treated for 24h with 2000ppm aqueous solution of gibberellic acid (GA_3, Sigma-Aldrich, Dorset, UK). I sowed seeds in plastic trays containing a mix of All Purpose Growing Medium (William Sinclair Horticulture PLC, Lincoln, UK) and Perlite Standard (Sinclair) in 3:1 proportion and kept them in a glasshouse at 16-20°C with a natural daylight cycle of 16h light and 8h dark. Daylight was supplemented using compact-fluorescent lamps. Plants from natural populations were surveyed during two fieldwork seasons in January 2016 and in September-October 2017 at different locations in the tropical dry forest of Yucatan, Mexico (supplementary material, Table s2.1).

2.3.2 Floral development

To investigate the developmental patterns of sex determination and changes in floral morphology in *S. houstonii*, we collected floral buds at different stages of development from ten plants belonging to one population (Cerro-Colorado, see Table s2.1). Buds were fixed in formalin-acid acetic-alcohol (FAA) and kept in 70% ethanol until dissection. To determine when sex determination between hermaphrodite and staminate buds occurs and to describe changes in size of anthers and other organs during development, I randomly selected inflorescences of different stages of development that included both hermaphrodite and staminate floral buds. In each inflorescence, I sampled floral buds at different positions of the inflorescences, from the most basal bud, which is often described as hermaphrodite, to buds at distal positions that usually develop into staminate flowers (Knapp *et al.*, 2017).

To detect whether unisexuality occurred by organ abortion, in the inflorescences that were starting to develop (primordia), I observed the organography of floral buds. I dissected buds under a dissecting microscope (Wild MZ8), critical-point-dried with CO2 and sputter-coated with platinum, before performing observations with a scanning electron microscope (Cambridge Leo Supra) at the laboratories of the Royal Botanic Gardens of Edinburgh. During these observations, I was able to confirm all floral buds in *S. houstonii* were bisexual at inception and that unisexuality occurred later in the development. Additionally, I determined that the first bud to develop in each inflorescence was always hermaphrodite and later buds were unisexual (staminate buds). Therefore, to detect the differences among sexes I decided to divide buds into two categories: 1) **basal buds** that develop in the first position in the inflorescence and are seemingly hermaphrodite and 2) **distal buds** that develop from the second position onwards and often are characterised as staminate (female-infertile). In these buds, I measured the following floral traits (Figure 2.1): **bud length**, from the calyx base to the corolla apex, **corolla length** from the insertion of the petals to their apices, **calyx length**, from the base of the receptacle to the tip of the sepal, **anther height** in two positions: anthers inserted in the adaxial (same position as feeding anthers in staminate flowers) and abaxial (same position as pollinating anthers in staminate flowers) surfaces of the flower, **style height**, from the ovary to the tip of the stigma, and **ovary width**, from the base of the receptacle to the beginning of style.

Herkogamy is the separation between anthers and stigma that occurs in hermaphrodite flowers, and heteranthery is the morphological and functional differentiation of anthers that occurs in staminate flowers of *S. houstonii* (Knapp *et al.*, 2017; Cardoso *et al.*, 2018). In addition to the measurements for each floral bud, I measured the distance between the adaxial an abaxial anthers and the distance between the adaxial and stigma between basal and distal buds (Figure 2.1), in order to determine when those morphological adaptations of the flowers arose.

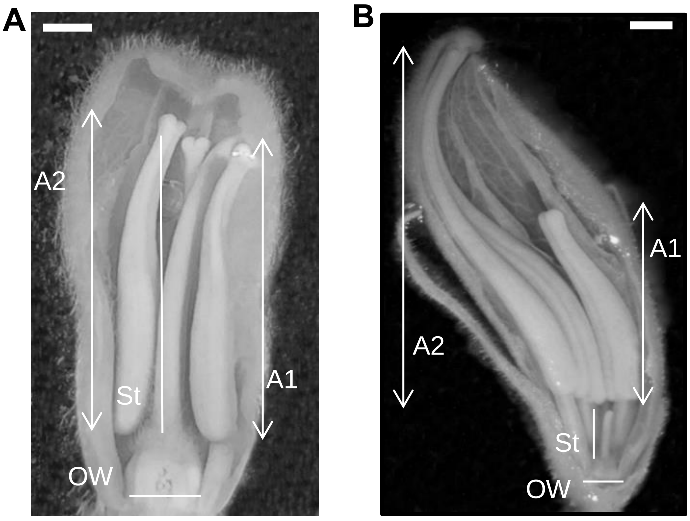


Figure 2.1. Hermaphrodite (A) and staminate (B) buds of *S. houstonii*. A1-adaxial anther height, A2- abaxial anther height, St-style height, OW-ovary width. Scale bars on top margins of 2 mm for reference.

2.3.3 Floral morphology of mature flowers

To characterise the morphological differences associated with each flower sex, I measured mature flowers of *S. houstonii* individuals from plants grown in the glasshouse and from natural populations. I randomly selected 55 hermaphrodite flowers from 33 individuals grown in the glasshouse (mean ± SE: 6±3 flowers per individual belonging to 9 populations) and 49 hermaphrodite flowers from 51 individuals of 25 natural populations (1.3±0.1 flowers per individual). Additionally I selected 56 staminate flowers grown in the glasshouse (5±2 flowers per individual (n=27) belonging to 9 populations) and 70 staminate flowers belonging to 64 individuals from 32 natural populations (1±0.1 flowers per individual). In each flower, I took the following measurements (Figure 2.2): corolla length and width, calyx length, height of feeding and pollinating anthers of staminate flowers, height of feeding anthers of hermaphrodite flowers at two positions: abaxial and adaxial (see Figure 2.1 for reference), style height, distance between feeding and pollinating anthers and distance between feeding anthers and stigma. All measurements were performed using digital callipers to the nearest 0.01 mm (Absolute Digimatic Calliper, Mitutoyo Ltd., Hampshire, UK).

2.3.4 Pollen quantity and size

In plants with heteranthery, differentiation in pollen grains size and viability has been suggested to be a reflection of the *division of labour* hypothesis for pollination (Müller, 1981; Luo *et al.*, 2009; Paulino *et al.*, 2016). Thus in this study, as part of the morphological characterisation and to detect differences among pollen grains, I quantified the number of pollen grains per anther and per flower and measured the size of pollen grains of hermaphrodite and staminate flowers. Pollen counts and measurements were performed in the anthers of hermaphrodite flowers and in anthers of staminate flowers. In hermaphrodite flowers, I collected all the five anthers of one flower and stored in 1 ml of 70% ethanol, while in staminate flowers, I stored the two short anthers and the three long anthers in separate containers with 1 ml of 70% ethanol in each. This decision was made based on the assumption that short anthers in *S. houstonii* are considered feeding anthers and long anthers as pollinating anthers (Papaj *et al.*, 2017). I collected the anthers of 17 hermaphrodite and 24 staminate flowers. I extracted pollen from poricidal anthers by vortexing them for ten seconds, three consecutive times, obtained four subsamples of 10μl and I quantified the total number pollen grains in each sample using a haemocytometer under a compound light microscope (Olympus CX31). In each subsample, I quantified the total number of pollen grains in five out of the nine cells. Then, I calculated the total number of pollen grains per subsample using the following formula:

$$\text{pollen grains per ml} = \left(\frac{number\ of\ grains\ counted}{(proportion\ of\ chamber\ counted) \times (volume\ of\ squares\ counted)}\right)(volume\ of\ orginal\ sample)$$

The average of the four subsamples was used to calculate the total number of pollen grains per anther and flower. In addition, I measured the diameter of four grains per sample using an eyepiece micrometre and took the average to estimate pollen grain size.

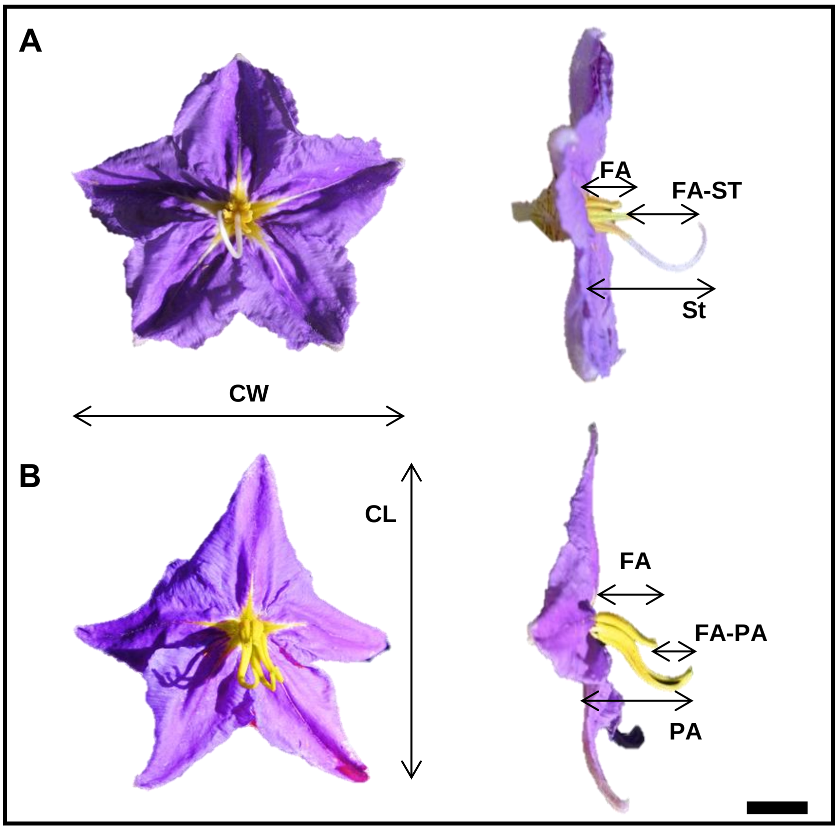


Figure 2.2. Measurements taken from hermaphrodite (A) and staminate (B) flowers of *S. houstonii*. CL-corolla length, CW-corolla width, FA-feeding anther height (adaxial), PA-pollinating anther height (abaxial), St-style height, FA-PA-distance between feeding and pollinating anther, FA-ST-distance between feeding anther and stigma. Scale bar at bottom right of 10 mm for reference.

2.3.5 Statistical analyses

To examine the growth pattern of each floral bud sex, I fitted the best model of growth rate for each floral organs measured and log transformed variables when needed. For this, I used linear mixed effect models (LMM) with Gaussian considering floral organ length/height as response variables. I used bud length, sex (hermaphrodite or staminate buds) and their interaction as fixed variables and plant ID as a random variable. Models were run with the *lmer* function of *lme4* package (Bates *et al.*, 2015) in *R* version 3.4.0 (R Development Core Team, 2013). Additionally, due to significant deviation in the intercepts of style height and ovary width, which could suggest sex determination arose in early stages of development, I took a subsample of the data for style height and ovary width that included young buds of 0.1-3mm of length and ran LMMs. These models were similar as the Gaussian models described above and indicated that slopes and intercepts were not statistically different (supplementary material, Table s2.2).

Additionally, to investigate the differences in overall floral morphology between hermaphrodite and staminate flowers I performed a linear discriminant analysis (LDA) using the *lda* function of the *MASS* package (Venables & Ripley, 2002). LDA is a multivariate technique that uses multiple traits to calculate a new set of variables that maximise the differentiation among predefined groups, in my case, different flower sex and plant collection. For the LDA I included the following variables: corolla length and width, calyx length, feeding and pollinating anthers, distance between feeding and pollinating anthers and style length, and used as grouping variables flower sex (hermaphrodite and staminate), and plant collection (glasshouse and natural populations).

To understand further the relationship among floral organs' growth, I performed allometric analyses in flowers from natural populations and grown in the glasshouse. Using a LMM, I fitted the same floral organs measured during development as a function of corolla width (used as a measured of overall flower size) of hermaphrodite and staminate flowers. I performed separate models with Gaussian distribution for each of the plant collections, using the *lmer* function. The length of each floral trait was used as response variable in the models, with corolla width, sex and plant collection as fixed variables, and plant ID as a random variable.

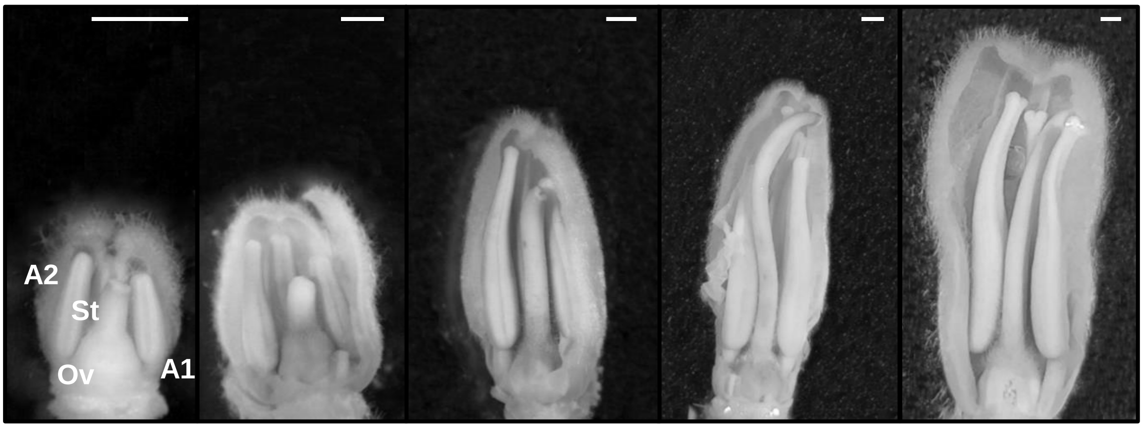
To evaluate differences in pollen size and quantity between feeding and pollinating anthers, I modelled either pollen grain size or quantity per anther or per flower as response variable, anther-type as fixed effect and plant ID as a random factor.

2.4 Results

2.4.1 Organography and growth of floral organs in developing buds

During initial flower development and early floral bud growth, buds from basal and distal positions on the inflorescence were indistinguishable. The two types of floral buds initiated organ development of calyx lobes in a spiral pattern, followed by corolla lobes, which also initiated in a spiral pattern but opposite to the sepals. Sepal lobes curved over the corolla lobes very early in the development but did not enclose the primordium. A similar pattern occurred with corolla lobes that curve over the developing stamens, but in this case covering them. As the floral buds continued developing, the tips of the corolla lobes were joined tightly by trichomes and curled around the tips of stamens. Stamens initiation occurred just after the corolla curved. In floral buds from all positions, stamens size was equal until floral buds reached 1mm long; the distance between adaxial and abaxial anthers differentiated in buds from the most basal position of the inflorescence by ~0.05 mm and in buds from the distal positions by ~0.1mm. Gynoecium development and growth occurred similarly in all buds after they were 0.4-0.5 mm long. At this stage, the sizes of the other floral organs remained similar in the two type of floral buds (Figure 2.3).

After development and early growth of floral organs, buds at the most basal and distal positions differed in their patterns of growth (Figure 2.4, Table 2.1). For instance, the corolla of distal buds had a more rapid linear growth pattern than buds at the most basal position (Figure 2.4A). This pattern was similar for adaxial and abaxial anthers in the two type of buds, whereas calyx and ovary length showed a rapid increase in growth until it stabilised when buds reached anthesis (Figure 2.4). In the case of style height and ovary width, growth in basal buds continued until anthesis, while in buds at distal positions, style and ovary growth stopped after buds reached approximately 3-4 mm of length (Figure 2.4C,F). These differences in growth indicated that buds at basal positions developed as hermaphrodite flowers and buds at distal positions as staminate flowers.



Hermaphrodite floral buds

Staminate floral buds

Figure 2.3. Comparable growth of hermaphrodite and staminate floral buds at different bud lengths. Pictures were taken under stereoscopic microscope light; two petals, one feeding and one pollinating anthers were removed. Scale bars on each picture are 1 mm for reference. FA-feeding anthers, FA1-feeding anthers at hermaphrodite buds in same position as pollinating anthers in staminate buds, PA-pollinating anthers, St-style.

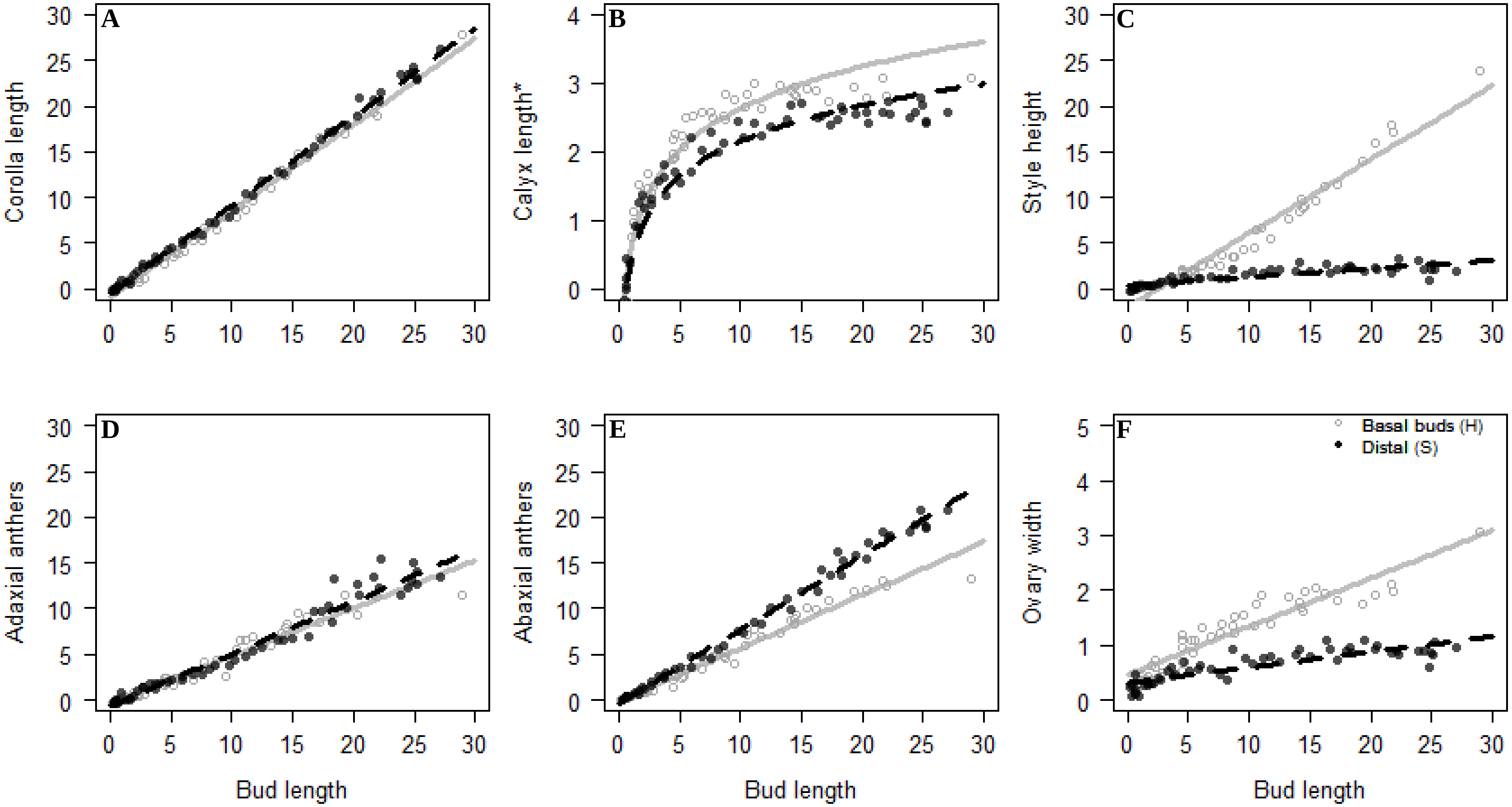


Figure 2.4. Floral organs (in mm) at different growth stages of basal (open symbols) and distal (closed symbols) buds. Each panel shows the length in mm of different floral organs and a fitted line (linear or exponential). Note1: buds at basal positions are developed as hermaphroditebuds and buds at distal positions are developed as staminate buds. Note2: adaxial anthers correspond to feeding anthers in stamiante buds and abaxial anthers correspond to pollinating anthers in stamiante buds, while for hermaphrodite flowers both adaxial and abaxial are feeding anthers. Note 3: calyx length is show in logaritimic scale. Model coefficientes in Table 2.1.

Table 2.1. Models coefficients describing the growth of floral organs in relation to bud length of *S. houstonii*. LMM: Linear mixed effects models.

Response variable	**Fixed and random variables**	**Estimate/ Variance***	**SE/ SD***	***t***	***P***	***N***	**Figures in text**
Corolla length	Intercept	-0.92	0.22	-4.2	0.009	111	2.4A
	Bud length	0.94	0.01	82.4	<0.001		
	Sex (Staminate)	0.40	0.16	2.5	0.014		
	Bud length:Sex(Staminate)	0.02	0.01	1.7	0.091		
	Plant ID (random)	0.13	0.36			4	
Calyx length#	Intercept	0.58	0.09	6.2	<0.001	114	2.4B
	Bud length	0.89	0.03	29.9	<0.001		
	Sex (Staminate)	-0.18	0.07	-2.4	0.018		
	Bud length:Sex(Staminate)	-0.13	0.04	-3.7	<0.001		
	Plant ID (random)	0.02	0.14			4	
Style height	Intercept	-2.03	0.24	-8.4	<0.001	103	2.4C
	Bud length	0.08	0.02	41.9	<0.001		
	Sex (Staminate)	2.4	0.28	8.6	<0.001		
	Bud length:Sex(Staminate)	-0.72	0.02	-30.8	<0.001		
	Plant ID (random)	0.06	0.24			4	
Adaxial anthers length	Intercept	-0.39	0.31	-1.2	0.252	106	2.4D
	Bud length	0.52	0.02	24.9	<0.001		
	Sex (Staminate)	-0.28	0.31	-0.9	0.365		
	Bud length:Sex(Staminate)	0.05	0.03	1.9	0.050		
	Plant ID (random)	0.20	0.44			4	
Abaxial anthers length	Intercept	-0.14	0.16	-0.9	0.402	106	2.4E
	Bud length	0.58	0.02	39.8	<0.001		
	Sex (Staminate)	-0.28	0.22	-1.3	0.208		
	Bud length:Sex(Staminate)	0.23	0.02	12.5	<0.001		
	Plant ID (random)	<0.01	0.03			4	
Ovary width	Intercept	0.46	0.07	6.4	0.001	103	2.4F
	Bud length	0.09	<0.01	19.8	<0.001		
	Sex (Staminate)	-0.15	0.07	-2.3	0.026		
	Bud length:Sex(Staminate)	-0.06	0.01	-11.3	<0.001		
	Plant ID (random)	0.01	0.11			4	
Distance adaxial-abaxial anthers	Intercept	0.21	0.24	0.9	0.406	106	2.5A
	Bud length	0.06	0.02	3.7	<0.001		
	Sex (Staminate)	0.04	0.23	0.2	0.857		
	Bud length:Sex(Staminate)	0.18	0.02	9.3	<0.001		
	Plant ID (random)	0.11	0.33			4	
Distance adaxial anthers- stigma	Intercept	-1.69	0.39	-4.4	<0.001	103	2.5B
	Bud length	0.29	0.03	9.2	<0.001		
	Sex (Staminate)	-2.78	0.46	6.1	<0.001		
	Bud length:Sex(Staminate)	-0.77	0.04	-20.4	<0.001		
	Plant ID (random)	0.14	0.37			4	

*SE and estimate for fixed variables, SD and variance for random variables.

#Calyx length is log transformed.

2.4.2 Development of heteranthery and style dimorphism

Anther growth differed between basal buds (hereafter, hermaphrodite buds) and distal buds (hereafter staminate buds), specifically in abaxial anthers (Figure 2.3A, Table 2.1). Abaxial anthers in staminate buds grew more than adaxial anthers (feeding anthers) of both hermaphrodite and staminate buds. The distance between the two types of anthers in hermaphrodite buds barely increased with bud length, while the distance in staminate buds increased as bud reached anthesis (Figure 2.5A). This separation among anthers became different between hermaphrodite and staminate buds after they reached 3-4mm in length, indicating the development of heteranthery in staminate buds. The distance in staminate buds one day before anthesis was approximately three times higher than in hermaphrodite buds (Figure 2.5A).

The distance between anthers and stigma in hermaphrodite buds arose late in development when buds were 15mm in length and styles surpass the size of anthers (Figure 2.5B). For staminate buds, there was a strong difference in size after style growth stopped that continued with bud and anther growth (Figure 2.5B)

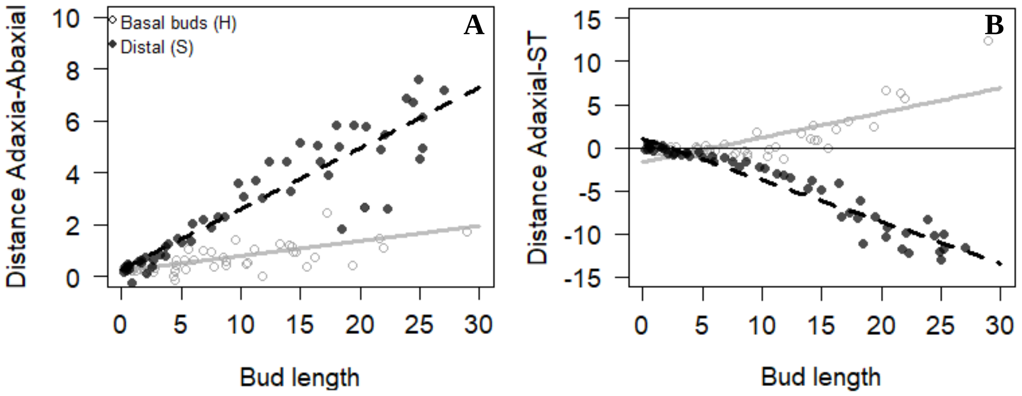


Figure 2.5. Patterns of development of heteranthery (A) and style dimorphism (B) of hermaphrodite and staminate floral buds. Differences between adaxial and abaxial refers to heteranthery and difference between adaxial anthers and stigma (ST) refers to style dimorphism. Line at zero in B represents equal size of anthers and stigma. Adaxial anthers correspond to feeding anthers in staminate flowers, while abaxial anthers correspond to pollinating anthers in staminate flowers.

2.4.3 Floral morphology and allometric relationships in mature flowers

The linear discriminant analysis showed that hermaphrodite and staminate flowers of *S. houstonii* differed in most of the floral traits measured (Figure 2.6). The first linear axis explained 97% of the trace and was able to differentiate flowers into hermaphrodite and staminate, while the second linear axis explained 1.8% and showed slight differences in plants from different collections (supplementary material, Table s2.3). Overall, staminate flowers tended to have larger corollas and anthers, while hermaphrodite flowers tended to have larger styles, ovaries and calyxes (Table 2.2). Hermaphrodite flowers were characterised by a large separation between anthers and stigma of 10 mm approximately (Table 2.2). This separation was also present in staminate flowers, but it was not of the same size as in hermaphrodite flowers and was mainly due to style growth arrest early during bud growth (see Figure 2.5B). I observed a separation between the two adaxial and the three abaxial anthers of both hermaphrodite and staminate flower; however, this separation was approximately three times larger in staminate flowers than in hermaphrodite flowers (Table 2.2). Separation of anthers (heteranthery), thus, was characteristic of staminate flowers.

I found flowers grown in the glasshouse were larger than flowers from natural populations. Few traits such as style height of hermaphrodite flowers and pollinating anthers height were of the same size in plants from both glasshouse and natural population collections (Table 2.2)

Allometric relationships between floral traits of mature flowers showed the differences among floral traits (Figure 2.7, Table 2.3). For both hermaphrodite and staminate flowers, corolla length, calyx length, style length, anther lengths and distance between anthers were significantly associated with corolla width and were significantly different among flower sexes (Table 2.3). Only ovary size and distance between anthers and style were not associated with corolla length, but their intercepts were significantly different between hermaphrodite and staminate flowers (Table 2.3).

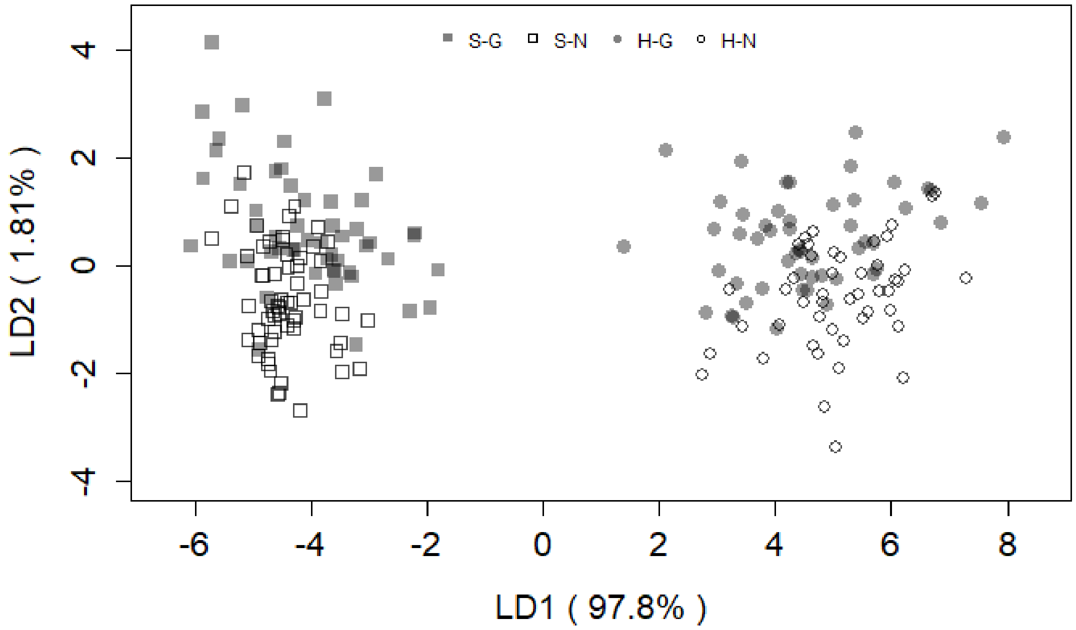


Figure 2.6. The first two axes of the linear discriminant analysis of the floral traits in hermaphrodite (H) and staminate (S) flowers of S. *houstonii* grown under control conditions (G) and from natural populations (N). Circles denote hermaphrodite flowers and squares staminate flowers, open symbols represent plants grown under controlled conditions at the greenhouse and closed symbols represent flowers collected in natural populations in Yucatan (see coefficients in Table s2.3 supplementary material).

Table 2.2. Summary statistics of floral traits measured (in mm) in hermaphrodite (H) and staminate (S) flowers of *S. houstonii* individuals from natural populations in Yucatan and plants grown in the glasshouse. Mean and standard error are shown for each floral sex and each plant collection.

Floral trait	**Glasshouse**		**Natural populations**	
	H	**S**	**H**	**S**
Corolla length	41.3 ± 1.1	44.1 ± 1.3	34.6 ± 0.9	38.4 ± 0.8
Corolla width	41.7 ± 1.2	44.6 ± 1.1	35.9 ± 1.0	39.6 ± 0.8
Style height	18.1 ± 0.5	3.4 ± 0.4	18.2 ± 1.0	1.8 ± 0.1
Feeding anthers height	8.4± 0.3	8.9 ± 0.3	6.6 ± 0.1	8.2 ± 0.1
Pollinating anthers height	10.7 ± 0.3	14.7 + 0.4	8.5 ± 0.2	14.8 ± 0.2
Distance feeding and pollinating anthers	2.3 ± 0.1	5.4± 0.3	1.9 ± 0.2	6.5 ± 0.2
Distance feeding anthers and stigma	9.6 ± 0.3	-5.9 ± 0.5	11.3 ± 0.3	-6.5 ± 0.2
Ovary width	2.5± 0.1	1.2± 0.1	1.7± 0.1	0.6± 0.02
Calyx length	15.3 ± 0.7	11.1 ± 0.3	14.0 ± 0.4	10.9 ± 0.2

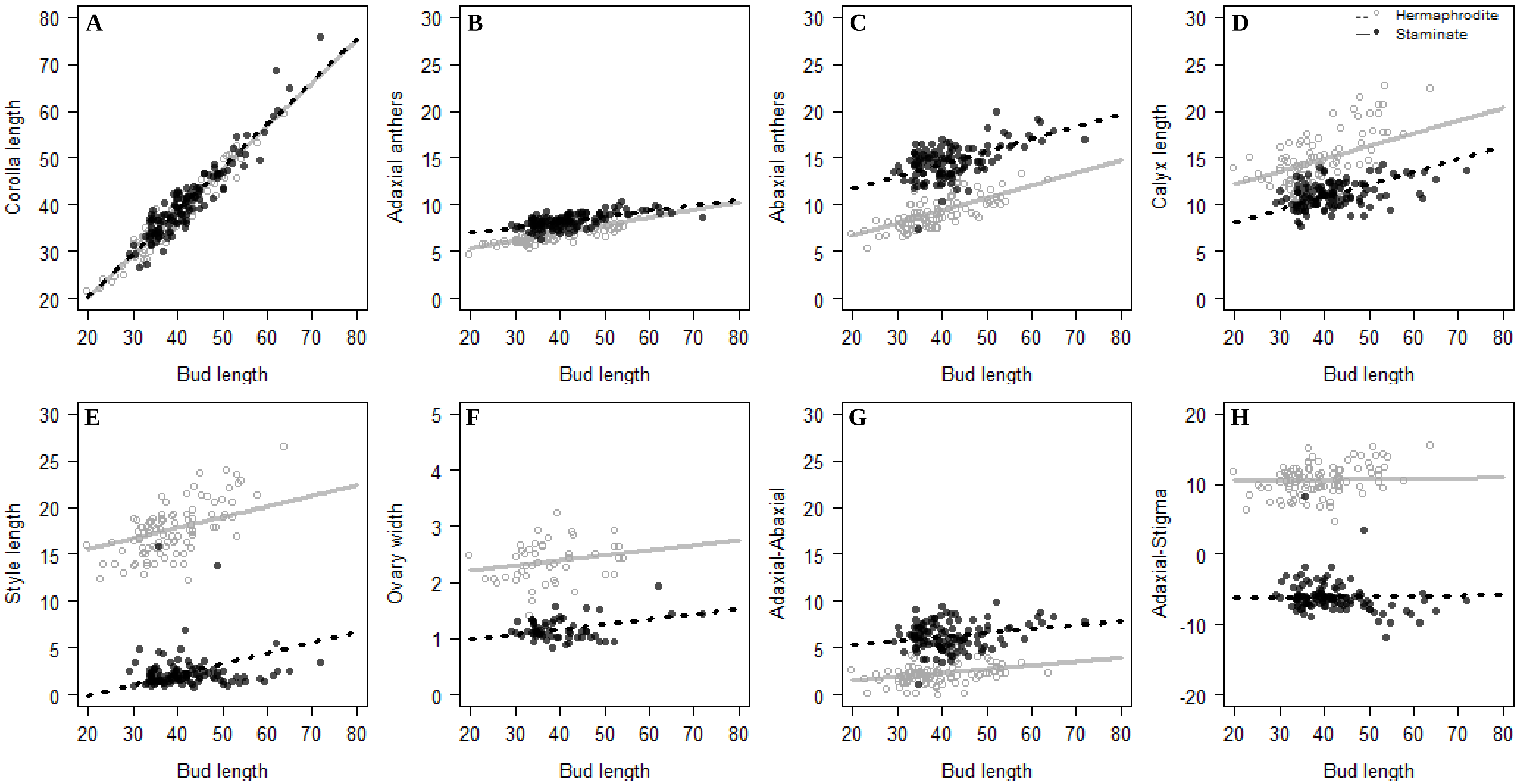


Figure 2.7. Allometric relationships among floral organs and corolla size of hermaphrodite and staminate flowers of *S. houstonii*. Open symbols and full lines denote hermaphrodite flowers, closed symbols and doted lines denote staminate flowers. See Table 2.3 for model coefficients.

Table 2.3. Allometric relationships of floral traits for hermaphrodite (H) and staminate (S) flowers. Coefficients are derived from linear mixed effect models for each floral organ.

Response variable	**Fixed and random variables**	**Estimate/ Variance***	**SE/ SD***	***t***	***P***	***N***	**Graphs in text**
Corolla length	Intercept	1.86	1.08	1.7	0.085		Fig. 2.7A
	Corolla width	0.91	0.03	32.9	<0.001	225	
	Sex (Staminate)	0.38	0.38	1.0	0.318		
	Collection (Glasshouse)	0.96	0.48	2.0	0.045		
	Plant ID (random)	1.47	1.21			110	
Adaxial anthers heigth	Intercept	4.25	0.44	9.7	<0.001		Fig. 2.7B
	Corolla width	0.07	0.01	6.2	<0.001	223	
	Sex (Staminate)	1.17	0.12	9.7	<0.001		
	Collection (Glasshouse)	1.24	0.25	4.9	<0.001		
	Plant ID (random)	1.11	1.06			109	
Abaxial anthers height	Intercept	4.08	0.72	5.7	<0.001		Fig. 2.7C
	Corolla width	0.13	0.02	7.2	<0.001	222	
	Sex (Staminate)	4.97	0.24	20.5	<0.001		
	Collection (Glasshouse)	0.44	0.33	1.3	0.193		
	Plant ID (random)	1.04	1.02			109	
Calyx length	Intercept	9.50	1.10	8.7	<0.001		Fig. 2.7D
	Corolla width	0.14	0.03	4.8	<0.001	221	
	Sex (Staminate)	-4.08	0.34	-11.9	<0.001		
	Collection (Glasshouse)	-0.42	0.54	-0.8	0.436		
	Plant ID (random)	3.71	1.93			107	
Style height	Intercept	13.32	0.80	16.6	<0.001		Fig. 2.7E
	Corolla width	0.11	0.02	5.4	<0.001	215	
	Sex (Staminate)	-15.65	0.31	-50.1	<0.001		
	Collection (Glasshouse)	0.30	0.34	0.8	0.384		
	Plant ID (random)	0.16	0.40			106	
Ovary width	Intercept	1.50	0.17	8.9	<0.001		Fig. 2.7F
	Corolla width	0.01	<0.01	1.8	0.071	107	
	Sex (Staminate)	-1.23	0.06	-20.1	<0.001		
	Collection (Glasshouse)	0.54	0.08	6.8	<0.001		
	Plant ID (random)	0.01	0.10			43	
Distance adaxial-abaxial anthers	Intercept	0.74	0.55	1.4	0.179	215	Fig. 2.7G
	Corolla width	0.04	0.01	2.8	0.006		
	Sex (Staminate)	3.87	0.20	19.3	<0.001		
	Collection (Glasshouse)	-0.58	0.24	-2.4	0.019		
	Plant ID (random)	0.29	0.53			106	
Distance adaxial anthers-stigma	Intercept	10.37	0.88	11.7	<0.001	215	Fig. 2.7H
	Corolla width	0.007	0.02	0.3	0.770		
	Sex (Staminate)	-16.71	0.33	-50.2	<0.001		
	Collection (Glasshouse)	-0.48	0.38	-1.3	0.209		
	Plant ID (random)	0.46	0.68			106	

*SE and estimate for fixed variables, SD and variance for random variables.

2.4.4 Pollen quantity and size

Pollen quantity differed significantly among the anthers of hermaphrodite and staminate flowers (Table 2.4, 2.5). Each feeding anther of both hermaphrodite and staminate flowers bore the least amount of pollen, while each pollinating anther of staminate flowers bore twice as much as an individual feeding anthers (Figure 2.8, Table 2.4). Overall, staminate flowers bore 831,601±72,757 pollen grains, which approximately 1.5 times more pollen than hermaphrodite flowers (529,534±30,926 pollen grains). The number of pollen grains in one hermaphrodite flower was not statistically different from the pollen borne on three pollinating anthers of a staminate flower (Table 2.5). Pollen size was statistically similar among anthers of both floral sexes and pollen from both feeding and pollinating anthers had an average diameter of 24 mm approximately (Table 2.5).

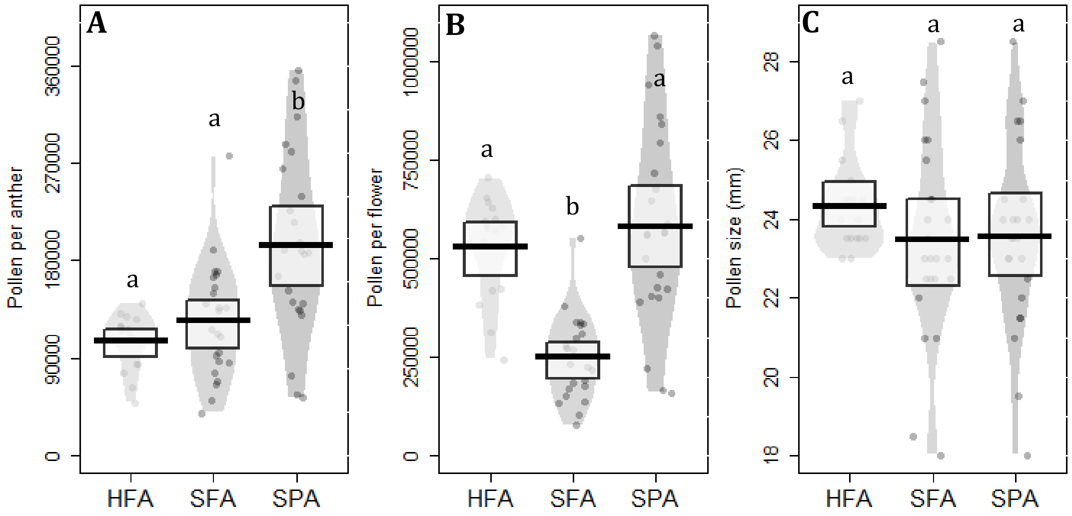


Figure 2.8. Pollen measurements of hermaphrodite and staminate anthers of *S. houstonii*. Black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas represent density and each individual point is an observation from a single anther type. HFA-feeding anthers of hermaphrodite flowers, SFA-feeding anthers of staminate flowers, SPA-pollinating anthers of staminate flowers. Different letters on figures denote significant differences among the anthers (see Table 2.4 for coefficients).

Table 2.4. Summary statistics of pollen measurements of hermaphrodite and staminate anthers. Mean and standard error are shown for each anther type. Feeding anthers in staminate correspond to anthers in the adaxial position and pollinating anthers in the abaxial position in the flower. Note that hermaphrodite flowers have five feeding anthers and staminate flowers two feeding anthers and three pollinating anthers.

	Anthers of hermaphrodite flowers	**Anthers of staminate flowers**	
	Feeding	Feeding	Pollinating
Pollen grains per anther	105,907 ± 6,185	124,451 ± 10,756	194,233 ± 17,082
Pollen grains per flower	529,534 ± 30,926	248,903 ± 21,511	582,698 ± 51,246
Pollen grains size	24.3 ± 0.3	23.5 ± 0.5	23.5 ± 0.5

Table 2.5. Coefficients of statistical models describing the relationships of pollen quantity per anther and per flowers and pollen diameter with anthers type of *S. houstonii*.

Response variable	**Fixed and random variables**	**Estimate/ Variance***	**SE/ SD***	***t***	***P***	***N***	**Graphs in text**
Pollen per anther	Intercept	100053.3	14109.6	7.1	<0.001		Fig. 2.8A
	Anther (Staminate feeding anthers)	25245.3	17196.6	1.5	0.147	65	
	Anther (Staminate pollinating anthers)	95026.5	17196.6	5.5	<0.001		
	Plant ID (random)	207300e4	45532			37	
Total pollen per flower	Intercept	514159.3	41744.6	12.3	<0.001		Fig. 2.8B
	Anther (Staminate FA)	-266576.8	51543.4	-5.2	<0.001	65	
	Anther (Staminate PA)	67218.2	51543.4	1.3	0.195		
	Plant ID (random)	157400e5	125450			37	
Pollen diameter	Intercept	24.53	0.52	47.4	<0.001		Fig. 2.8C
	Anther (Staminate FA)	-1.07	0.64	-1.7	0.099	65	
	Anther (Staminate PA)	-1.01	0.64	-1.6	0.120		
	Plant ID (random)	2.27	1.51			37	

*SE and estimate for fixed variables, SD and variance for random variables.

2.5 Discussion

2.5.1 Determination of unisexuality

Floral organ initiation in *S. houstonii* primordia was similar to what has been described for other *Solanum* species (Sekhar & Sawhney, 1984; Diggle, 1991b; Ronse De Craene, 2010). Buds from basal and distal positions were bisexual at initiation, until they reached 3-4 mm of length and differences in floral organs growth arose. At this bud length, ovaries and styles of buds at distal positions of the inflorescence ceased growing, while gynoecia of buds at the most basal positions continued growing in a similar pattern as the other floral organs. These findings indicate that buds at basal positions maintained their bisexuality while buds at distal positions became unisexual, staminate, at this stage of development.

For other *Solanum* species, it has been demonstrated that after gynoecial arrest, sex determination is achieved due to ovule abortion (Diggle, 1991a,b, 1993). For instance, histological studies on hermaphrodite and staminate buds of *Solanum hirtum* showed that after ovary growth ceased at 5 mm length, the ovule development continued normally. But when staminate buds reach 9-10mm in length, the integument and embryo sac of ovules collapsed causing necrotic ovules (Diggle, 1991a,b). Although, in this study, I did not focused in determining when ovule development occurred in each sex, the early arrest of gynoecium suggests ovules could have had an abnormal development like in *Solanum hirtum.* In support of this conjecture, staminate flowers have a reduced number of ovules that are smaller in size than ovules of hermaphrodite flowers (41±10 on staminate flower vs ~250±28 on hermaphrodite flowers). In any case, staminate flowers at distal positions of the inflorescences bear unfertile gynoeciums, which suggest ovule abortion occurs at some point during sex determination.

The results presented here indicate that only the first bud to develop in the inflorescence maintained bisexuality. These findings suggest floral sex determination of *S. houstonii* could be fixed at certain positions in the inflorescence, starting from the second position and continuing through the end of the inflorescence. In andromonoecious species, this pattern of expression in known as strong andromonoecy and in some species is often maintained despite fluctuations in resource availability (Whalen & Costich, 1986; Diggle & Miller, 2004, 2013). For instance, in *Solanum quitoense* and *S. palinacanthum*, both species with strong andromonoecy, Diggle and Miller (2013) found only *S. palinacathum* is capable to adjust its sex expression (the proportion of staminate flowers produced) under treatments that reduce resource availability for fruit production. Whether *S. houstonii* is able to produce more hermaphrodite flowers remains unknown; however, the early arrest of the gynoecium and the expression of hermaphrodite and staminate flowers in certain positions of the inflorescence suggest that sex expression in *S. houstonii* is fixed. Further studies investigating the patterns of sex expression under different resource levels will be able to confirm the potential of flowers from distal positions to become hermaphrodite (see Chapter 3).

2.5.2 Patterns of development of anther dimorphism: heteranthery

Heteranthery is the occurrence of two or more types of stamens in the same flower (Barrett, 2002a). It has evolved within *Solanum* several times by convergent evolution; however, the patterns of development and genetic basis within this genus are still unknown (Lester *et al.*, 1999; Bohs *et al.*, 2007; Vallejo-Marín *et al.*, 2010). In species that present separation of sexes and heteranthery, investigating the how heteranthery arise is critical to further understand how labile the development of the system is. In *S. houstonii,* heteranthery occurred in distal buds only, by the enlongation of two abaxial anthers after buds reached 2-4mm of length. At this point, abaxial anthers of distal buds enlarged faster than the adaxial anthers, but followed a similar pattern of growth as the style of basal buds. Such patterns of development have been documented for other species with heteranthery, such as *Senna* and *Chamaecrista*, in which stamen differentiation occurs at early stages of development (Tucker, 1996; Jesson *et al.*, 2003; Marazzi & Endress, 2008; Nogueira *et al.*, 2018).

In *S. houstonii*, anther dimorphism developed in buds from distal positions that had their gynoecia arrested and became unisexual, suggesting heteranthery is fixed to certain positions in the inflorescences in a similar way to unisexuality. This pattern is not very common in *Solanum* as heteranthery is often expressed in all flowers from a given species. For instance, in *S. vespertilio* and *S. lidii* both hermaphrodite and staminate flowers are heterantherous (Dupont & Olesen, 2006; Anderson *et al.*, 2014). As the genetic pathway for the development of heteranthery and unisexuality in *Solanum* is unknown, it remains unclear whether the expression of these two traits is associated or if they are developmentally constrained. In *S. houstonii*, it seems likely that heteranthery and unisexuality are developmentally constrained.

2.5.3 Morphological differentiation on hermaphrodite and staminate flowers

From the quantitative analyses of hermaphrodite and staminate flowers, I found flowers of *S. houstonii* were morphologically different, not only in anther, style and ovary size but also in traits such as corolla and calyx length. Hermaphrodite flowers presented radially symmetric corollas with large styles, calyxes and ovaries, while staminate flowers possessed large bilaterally symmetrical (zygomorphic) corollas and two sets of anthers, two short adaxial anthers that function as feeding anthers and three long abaxial anthers that function as pollinating anthers (Papaj *et al.*, 2017). This combination of characters of hermaphrodite and staminate flowers is not frequently observed in *Solanum*, especially radial symmetry and zygomorphy of the corolla within the same plant. Usually, in *Solanum*, radial symmetry is the rule, but zygomorphy has arisen several times (Lester *et al.*, 1999; Knapp, 2002; Bohs *et al.*, 2007). For instance, in species from the Canary Islands and some of the section Androceras (both subgenus *Leptostemonum*), zygomorphy is very common and has been associated with the presence of anther dimorphism and characters that promote pollinator attraction (Lester *et al.*, 1999; Dupont & Olesen, 2006; Levin *et al.*, 2006; Bohs *et al.*, 2007). In sister species of *S. houstonii,* from the section *Elaeagnifolium*, zygomorphy is rare (Knapp *et al.*, 2017). The developmental analyses suggest that zygomorphy of staminate flowers in *S. houstonii* occurs due to the enlargement of pollinating (abaxial) anthers.

In many andromonoecious species that possess a labile sex expression in response to changes in resource availability, investing resources towards the production of large hermaphrodite flowers and small staminate flowers is a major advantage as it ensures the production of fruits of higher quality (Anderson & Symon, 1989; Vallejo-Marín & Rausher, 2007b; Anderson *et al.*, 2014). However, in few andromonoecious species allocating resources towards the female function than to the male function is not always a major advantage (Bertin, 1982; Podolsky, 1993; Barrett, 2002a,b; Vallejo-Marín & Rausher, 2007b). Here, I found *S. houstonii* possesses staminate flowers that were not only larger than hermaphrodite flowers that but also had greater amounts of pollen and hermaphrodite flowers with a smaller corolla than staminate flowers but larger styles and ovaries. These results suggest that in *S. houstonii* species rather than re-allocating resource from the male function towards the production to larger hermaphrodite flowers, there might be a trade-off between the size of hermaphrodite flowers and the pollen production of staminate flowers. In some andromonoecious species and other *Solanum* species, investing in large male function is explained often by the hypotheses that staminate flowers increase pollinator attraction and that greater amounts of pollen increases the chances of pollen reaching the stigma (Bertin, 1982; Whalen & Costich, 1986; Podolsky, 1993; Barrett, 2002a; Vallejo-Marín & Rausher, 2007b; Anderson *et al.*, 2014). These are two of the main hypotheses used to understand the maintenance and evolution of staminate flowers and have been supported by studies that show staminate flowers are preferred by pollinators and/or donate more pollen that reaches the stigma than hermaphrodite flowers (Bertin, 1982; Whalen & Costich, 1986; Huang, 2003; Quesada-Aguilar *et al.*, 2008; Luo *et al.*, 2012).

The results shown here are the first characterisation of andromonoecy in *S. houstonii*, which not only provide relevant data of sex determination and development of floral adaptations common in andromonoecious species in *Solanum,* but provides a framework of the functional role of staminate flowers at pollen donation. Further investigations on the functional role of staminate flowers that examine pollinators preference to staminate flowers and that measure pollen contribution for seed production could be helpful to investigate the mechanisms that maintain the production of staminate flowers and the evolution of andromonoecy in *Solanum*.

2.6 Conclusions

The developmental pattern of sex determination in *S. houstonii* is characterised by the early arrest of gynoecia. Here, I showed that all floral buds are bisexual at initiation and achieve unisexuality due to gynoecium arrest when buds are 3-4 mm of length. I demonstrated bisexuality is maintained in buds that developed in the first position of the inflorescence, while the remaining buds of the inflorescence developed unisexuality. In addition, buds that were unisexual also presented anther dimorphism that in mature flowers is known as heteranthery. The fact that heteranthery was only developed in buds at distal positions of the inflorescence that were also unisexual, is consistent with unisexuality and heteranthery being developmentally constrained to certain positions of the inflorescence. Furthermore, the changes of floral organ growth observed during bud development were translated to mature flowers in which I found a strong morphological differentiation. Hermaphrodite flowers possessed larger calyxes, ovaries, styles and herkogamy, traits that are associated with fruit production and pollen reception. In contrast, staminate flowers possessed larger corollas, heteranthery and a larger amount of pollen, traits that are often associated with pollinator attraction and pollen donation. The results of this study provide the first data on sex determination in *S. houstonii*, and valuable information on developmental mechanisms of sex determination in species that exhibit a fixed proportion of hermaphrodite and staminate flowers.

Chapter 3

Sex expression

Chapter 3. Fixed sex expression of andromonoecy in *Solanum houstonii*

3.1 Abstract

Andromonoecy, the presence of hermaphrodite and staminate (female-infertile) flowers in the same individual, can be phenotypically plastic due to changes in resource availability, or fixed at certain positions in the inflorescence due to architectural constraints. These types of expression are well characterised in the genus *Solanum*, in which many species present weak andromonoecy that often is phenotypically plastic or strong andromonoecy that is often constrained at certain positions of the inflorescence. However, fixed phenotypes in this genus are rare, as andromonoecy is sometimes considered a way to maximise fitness by optimal allocation of reproductive resources to male and female functions. *Solanum houstonii* is an andromonoecious species that produces a relatively low proportion of hermaphrodite flowers in an apparently fixed pattern of sex expression. Nevertheless, little is known about this species' reproductive system, and whether it is capable of adjusting its floral expression in response to changes in resource availability. In this chapter, I investigated whether *S. houstonii* possesses a plastic phenotype with respect to sex expression. To achieve this, I characterised the sex expression of individuals from natural populations and plants grown under controlled conditions. I tested the effect of floral position in the inflorescence and the effect of increased and decreased resource availability. My results indicated the ratio of production of hermaphrodite and staminate flowers in natural populations was of two hermaphrodite flowers per eight staminate flowers and that at the inflorescence level, hermaphrodite flowers have the highest probability to be produced in the first position of the inflorescence, while staminate flowers are expected at subsequent positions. In addition, individuals under treatments of increased and decreased resources demonstrated similar patterns of sex expression. Altogether, these results are consistent with that flower sex being constrained by architectural effects in the design of the inflorescence, suggesting *S. houstonii* has lost its plasticity to produce hermaphrodite flowers in subsequent positions. I discuss that this pattern of expression may be a strategy to maximise resources available for the production of large fruits.

3.2 Introduction

Andromonoecy, the breeding system in which plants possess hermaphrodite and staminate (female-sterile) flowers, occurs in approximately 4000 species, which are ~2% of all flowering plants (Yampolsky & Yampolsky, 1922; Miller & Diggle, 2003). The study of andromonoecy has focused on understanding its adaptive significance, evolution and diversification (Bertin, 1982; Whalen & Costich, 1986; Diggle, 1993; Miller & Diggle, 2003; Diggle & Miller, 2004; Vallejo-Marín & Rausher, 2007a,b; Dai & Galloway, 2012; Granado-Yela *et al.*, 2017). One of the main hypotheses proposed for the evolution of andromonoecy suggests this sexual system evolves as a reproductive energy-saving strategy, as it assumes staminate flowers are less costly than hermaphrodite flowers, and resources saved by producing them can be reallocated towards fitness-enhancing traits of both male and female function (Primack & Lloyd, 1980; Bertin, 1982; Solomon, 1985; Vallejo-Marín & Rausher, 2007b). As a result of this strategy, in environments where resources for plant growth are reduced, some andromonoecious species exhibit variation in their sex expression (relative production of staminate flowers) as a strategy to maximise resource reallocation towards fruit production (female fitness)(Diggle, 1993; Korpelainen, 1998; Miller & Diggle, 2003). Because of the latter, andromonoecy is frequently considered a form of adaptive phenotypic plasticity (Miller & Diggle, 2003).

Regardless of the particular advantages of a plastic phenotype, many andromonoecious species exhibit a fixed proportion of hermaphrodite and staminate flowers (non-plastic sex expression) that is not attributed by resource reallocation (Miller & Diggle, 2003; Diggle & Miller, 2004). In these species, sex determination may be due to developmental constraints imposed by inflorescence architecture (Diggle, 1995, 1997), leading to inherent features of the inflorescence that have predictable patterns of variation associated with position (Diggle, 1995). In species with unisexual flowers, causes of these effects on flower sex can help to determine the developmental pathways of sex determination and therefore provide a better understanding of the evolutionary dynamics to achieve unisexuality. However, little attention has been paid to these positional effects in inflorescences of species with unisexual flowers, such as in taxa with andromonoecy, gynomonoecy or monoecy (Miller & Diggle, 2003; Reuther & Claßen-Bockhoff, 2013; Granado-Yela *et al.*, 2017).

In the large genus *Solanum* (Solanaceae), andromonoecy is well documented, especially in the subgenus *Leptostemonum* (Whalen & Costich, 1986; Levin *et al.*, 2006; Stern *et al.*, 2011; Diggle & Miller, 2013). Andromonoecious species in *Solanum* are characterised for being weakly andromonoecious, if plants produce relatively few staminate flowers or as strongly andromonoecious, if they produce a relatively high number of staminate flowers (Whalen & Costich, 1986). Experimental analyses have demonstrated that species that are weakly andromonoecious decrease their production of hermaphrodite flowers but increase their production of staminate flowers when resources for plant growth are scarce, suggesting the production of staminate flowers is phenotypically plastic for increasing resource demands (Solomon, 1985; Diggle, 1991a, 1993; Miller & Diggle, 2003; Diggle & Miller, 2013). In this species, staminate flowers are smaller than hermaphrodite ones, and when produced as a plastic response occur at distal positions of the inflorescence (Diggle, 1993; Miller & Diggle, 2003; Diggle & Miller, 2004). For instance, in *Solanum hirtum,* it has been shown fruit set developing treatments represent a significant drain of resources for further plant growth and reproduction, which reduces the relative proportion of hermaphrodite flowers produce per inflorescence and increase the proportion of staminate flowers at distal positions of the inflorescence (Diggle, 1993, 1994; Miller & Diggle, 2003). On the contrary, species with strong andromonoecy have a non-plastic phenotype and possess flowers that do not differ in size (Miller & Diggle, 2003; Diggle & Miller, 2004). However, evidence supporting this difference is scarce, as this has only been documented for few species of the *Lasiocarpa* and *Acanthophora* sections (subgenus *Leptostemonum*) (Miller & Diggle, 2003; Diggle & Miller, 2004, 2013).

Solanum houstonii Martyn is a perennial shrub endemic to Mexico of the subgenus *Leptostemonum*, section *Eleaegnifolium*, characterised for being andromonoecious. Previous studies showed each inflorescence is able to produce one hermaphrodite and several staminate flowers and that these flowers differed in their morphology, with staminate flowers having a larger corolla and anthers than hermaphrodite flowers (Knapp *et al.*, 2017; see results Chapter 2). The biased production of staminate flowers indicates this species is strongly andromonoecious, however, whether the flower dimorphism is the result of resource allocation rather than a fixed expression of sexual dimorphism remains to be tested.

Here, I investigated the patterns of sex expression of *S. houstonii*, in order to determine the ability of this species to adjust its staminate flower production in response to changes in plant resource availability. I measured sex expression in natural populations, in plants grown under controlled conditions and in plants with experimentally increased or decreased resource availability for plant growth. These manipulations should increase (treatment with fertiliser addition) or decrease (treatment with fruit developing) the probability of finding hermaphrodite flowers within inflorescences compared with the control treatment. Additionally, I performed another resource manipulation to determine whether the removal of the first flower, which is often hermaphrodite (see Chapter 2; Knapp *et al.*, 2017), had an effect on the probability to produce hermaphrodite flowers in basal positions of the inflorescence. Specifically, I asked the following questions: 1) how does sex expression vary in natural populations? 2) Does flower position in the inflorescence have an effect on sex expression? 3) Does increased or decreased resource availability affect sex expression at the inflorescence level? 4) Does the removal of the basal flower, often characterised as hermaphrodite, affect the sex expression in the inflorescence?

3.3 Methods

3.3.1 Study species and plant material

The shrub *Solanum houstonii* Martyn is a perennial species that occurs in the arid habitats of Mexico (Knapp *et al.*, 2017). This species is characterised by strong andromonoecy for possessing in each inflorescence one basal hermaphrodite flower and up to 35 staminate (female sterile) flowers. Inflorescences are scorpioid-cymes that bear up to five open flowers at a time, each lasting two days in natural populations and up to five days under controlled conditions. Flowers have five lilac or purple petals and five yellow or purple poricidal anthers, and are heterostylous, heterandrous, nectarless and markedly dimorphic (Knapp *et al.*, 2017). Hermaphrodite flowers are characterised for their five straight anthers of similar size, long style (three times bigger than anthers) and their prickly calyx. Staminate flowers, on the contrary, are short styled, and possess two morphologically and functionally distinct set of anthers (Knapp *et al.*, 2017; Papaj *et al.*, 2017). Two short adaxial anthers provide pollen for visiting insects and function as feeding anthers and three longer, curved abaxial anthers (similar in size to the style of in hermaphrodite flowers, Knapp *et al.*, 2017), known as pollinating anthers, presumably contribute to pollen reaching the stigmas of hermaphrodite flowers (Papaj *et al.*, 2017). *Solanum houstonii* individuals flower all year, especially during the rainy season between June and September, and are distributed in dry and humid semi-deciduous forest and disturbed areas across Mexico (Nee, 1993; Knapp *et al.*, 2017).

3.3.2 Sex expression measured in natural populations

To characterise the sex expression of individuals from natural populations, in January 2016 and September 2017, I sampled 65 populations across Yucatan, Mexico (supplementary material, Table s2.1). These populations were located along the highways of Yucatan state and separated by at least 1 Km. In each population, I selected one to 23 individuals (mean ± SE=2±0.5 individuals per population) and quantified the number of hermaphrodite and staminate flowers displayed in each, plant height and the two maximum diameter lengths. I calculated plant size using the ellipsoid volume formula ($v = \frac{4}{3} h \cdot d1 \cdot d2$, where *h* is height, *d1* is diameter 1 and *d2* is diameter 2) that fit each plant height and maximum diameter lengths (Thorne *et al.*, 2002).

3.3.3 Sex expression measured in individuals grown under control conditions

In the spring of 2017, I conducted an experiment in the pollinator-free glasshouse at the University of Stirling to examine sex expression of *S. houstonii* plants under controlled conditions at the inflorescence level of plants. I used ten individuals belonging to four accessions (seed families) of the same population (Table s3.1) and characterised sex and position on the inflorescence of flowers produced. I selected four branches per individual and on each branch ten sequential inflorescences, in which I characterised the sex of the first ten flowers to develop and reach anthesis.

The plants used for this experiment were grown from seed on October 2016. All seeds were pre-treated with 2000ppm of gibberellic acid (GA_3, Sigma-Aldrich, Dorset, UK) overnight. Seeds were sown in a mix of All Purpose Growing Medium (William Sinclair Horticulture PLC, Lincoln, UK) and Perlite Standard (Sinclair). Plants were kept in a pollinator free glasshouse at the University of Stirling at 16-22°C with a natural daylight cycle of 16h light and 8h dark, supplemented with compact-fluorescent lamps.

3.3.4 Sex expression measured in individuals grown under treatments with differences in resource availability

To determine whether the sex expression shown at inflorescence level changes in response to resource availability, I experimentally manipulated resources available for plant growth. The treatments consisted of the following: 1) plus fertiliser, in order to give enough resources to the plant for growth and flower production and increase the probability of producing hermaphrodite flowers, 2) plus fruit, to reduce resources available to plant growth and reduce the probability of producing hermaphrodite flowers, and 3) no-manipulation (control). For the fertilisation treatment I prepared Tomorite plant feed (NPK: 4-3-8; Levington, UK) according to manufacturer instructions and applied it to the plants every week until soil reached its saturation point. In the plus fruit treatment, I hand-pollinated hermaphrodite flowers of each plant in the treatment using a mix of cross pollen and allowed them to set fruit. I performed an additional resource manipulation to determine whether the removal of the first flower, which is often hermaphrodite (Knapp *et al.*, 2017; personal observation), had an effect on the sex expression per inflorescence by increasing the probability to produce hermaphrodite flowers in basal positions of the inflorescence. The removing treatment (4) consisted of removing floral buds at the first position (the most basal) of the inflorescence when buds reached 2mm of length. This size was chose to ensure that buds from the other positions in the inflorescence were at a stage at which unisexuality had not yet been determined (see chapter 2).

Plants for this experiment were grown from seed on May 2018 as explained in section 3.3.3 of this chapter. Approximately 80 individuals from 26 accessions (hereafter seed families) and ten populations (Table s3.1) were grown in the pollinator free glasshouses at the University of Stirling and allocated to each treatment (20 individuals per treatment). All treatments were initiated when plants reached 20cm of height and started producing inflorescences (approximately at ten to twelve weeks old). After six weeks of applying treatments, I started characterising the sex and the position in the inflorescence of flowers produced in at least 2 inflorescences per plant. However, due to the low flower production during this experiment, I subsequently adjusted my target to characterise sex expression in at least 20 inflorescence per treatment (mean±SD=2.4±1.6 inflorescences measured per individual).

5.3.5 Statistical analyses

To investigate the variation in floral sex expression of *S. houstonii*, I performed generalised linear mixed effect models (GLMM, in recognition of the hierarchical nature of our data Pinheiro & Bates, 2000; Granado-Yela *et al.*, 2017), specifying a binomial error distribution and logit-link error. First, to determine the influence of plant size on variation in the sex of the flowers (sex expression) produced in individuals from natural populations over two years of surveys and the influence of plant size in the sex expression, I built a model that included flower sex as a binary response variable (hermaphrodite=0, staminate=1). Fixed variables were year and plant size and random variables were population and plant ID. Then, to determine the effect of the position on the inflorescence on the flower sex of plants grown under controlled conditions, I fitted a model that included sex as a binary response variable, the positions in the inflorescence as a fixed variable and the random variables inflorescence, branch and plant IDs. Position in the inflorescence was a categorical predictor with two levels: first position or non-first position, which best captured the clear effect of sex on the first position, which I noted during sampling.

To determine how increasing or decreasing the resources available for plant growth affects sex expression, I fitted a model that included sex as the binary response, fixed variables treatment and inflorescence position and random variables inflorescence and seed family IDs. Similar to the model for testing the effect of position on the sex of flowers, I used a two-level categorical predictor (first and non-first) for position. Additionally, to assess whether removing a basal flower influenced sex expression in the remaining positions of the inflorescence, I fitted a model with sex as the binary response, two separate categorical fixed effects, one for resource manipulation treatment and one for basal flower removal treatment, and the random effect seed family IDs. Resource treatment was a two-level factor that specified whether plants received a treatment of increased or decreased resources, and basal flower removal treatment was a two-level factor that specified whether the basal flower was removed or not. Because I was interested in the effect of flower removal on the phenotypes of the remaining flowers in the inflorescence, for this model, I disregarded the phenotypes of the first position in the treatments without flower removal.

All models were fitted using the function *glmer* from the *lme4* package (Bates *et al.*, 2015) using *R* software v 3.4.0 (R Development Core Team, 2013). Additionally, to estimate the intraclass correlation coefficient for the random variables of each model, I used the *icc* function of the *sjstats* package for *R* (Ludecke, 2019). Models were validated by visually assessing diagnostic plots to confirm normality of residuals.

3.4 Results

3.4.1 Sex expression in individuals from natural populations

The number of flowers observed in each individual in natural populations ranged from 1 to 96 (mean±SD: 9.8±13.7). Approximately 78% ($CI_{95\%}$: 70, 86%) of flowers were staminate in each individual and 22% were hermaphrodite ($CI_{95\%}$: 14, 29%; Figure 3.1). My model to determine the effect of year and size on the number of hermaphrodite and staminate flowers revealed that predictors were non-significant (Table 3.1, Figure 3.1).

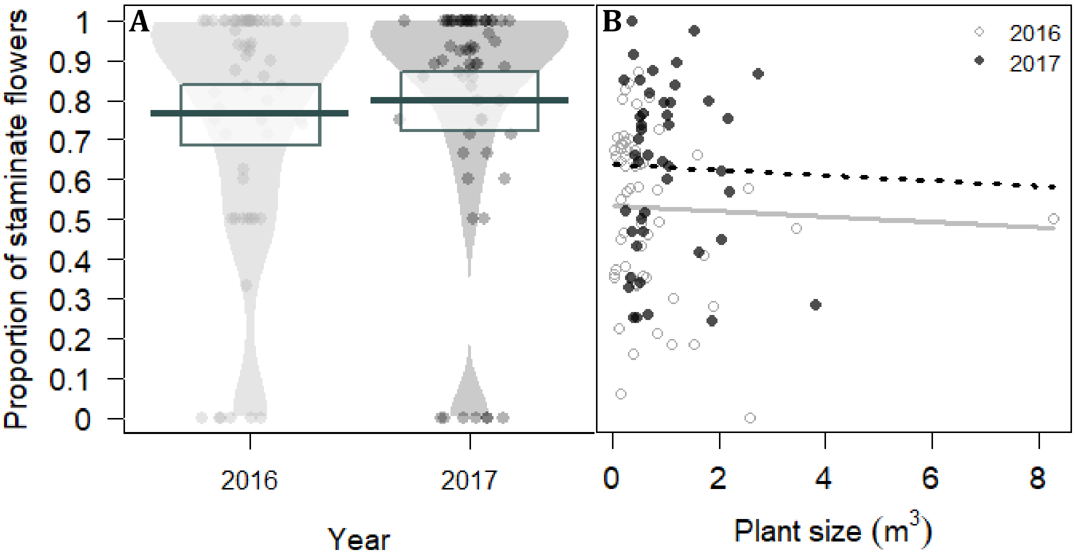


Figure 3.1. Proportion of staminate flowers of individuals from natural populations of Yucatan in two different years of sampling (A) and its relationship with plant size (B). In A, black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an observation from a single population.

Table 3.1. Coefficients of the model to test the effect of plant size and year on flower sex (hermaphrodite or staminate) of individuals from natural populations. Results show the fixed and random effects of generalised mixed effect model with binomial error distribution.

Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Intercept	-1.749	0.209	-8.363	<0.001
Year (2017)	-0.557	0.443	-1.259	0.208
Plant size	0.037	0.079	0.462	0.644
Random effects	**Variance**	**S.D.**	**ICC**	
Individual	0.020	0.140	0.006	
Population	0.278	0.527	0.079	

3.4.2 Sex expression in plants grown in under control conditions: variation per individual and inflorescence position

Flower position had a strong effect on floral sex (Table 3.2). The probability of developing staminate flowers in the first position was 0.0 ($CI_{95\%}$: 0.0, 0.02), while developing staminate in later positions was 0.96 ($CI_{95\%}$: 0.95, 0.97). Although my model suggested no differences in sex among inflorescence positions past the first, I found two individuals that produce hermaphrodite flowers in some later positions. These individuals belonged to the same seed family (07s211B, Figure 3.2). The hermaphrodite flowers produced were morphologically different to the hermaphrodite flowers in the first positions of the inflorescence, because of the presence of pollinating anthers that were similar in size to the stigma (Figure 3.3).

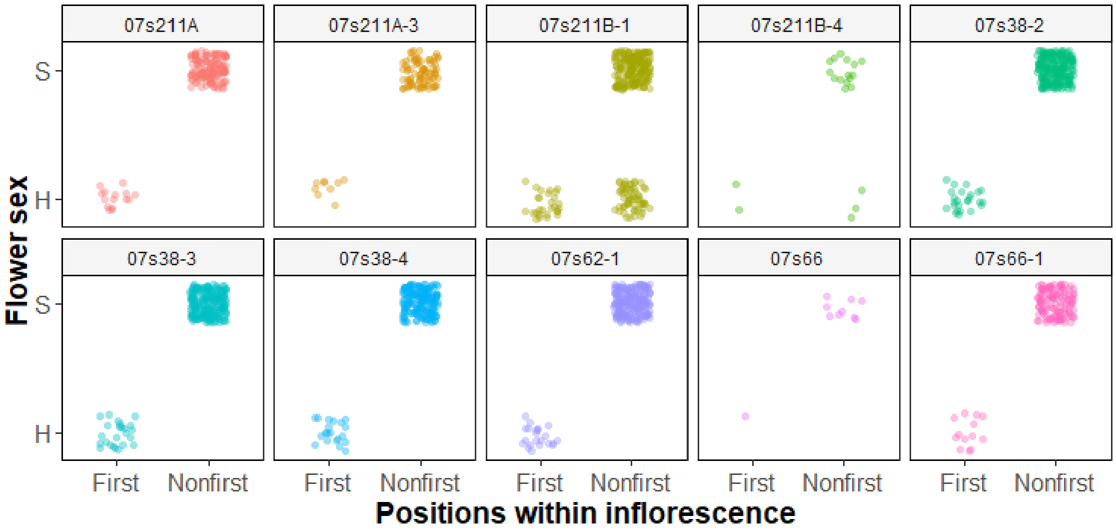


Figure 3.2. Hermaphrodite and staminate flowers grown in the first and the other position in the inflorescences of ten individuals of *S. houstonii* grown under control conditions.

Table 3.2. Effect of inflorescence position on the number of hermaphrodite and staminate flowers produced per inflorescence in individuals of *S. houstonii* grown under controlled conditions. Fixed and random factors are derived from the generalised fixed effect model with binomial distribution.

Fixed effects	**Estimate**	**S.E.**	***z***	***p***
Intercept	2165.0	170.7	12.69	<0.001
Position (Non-first)	-2179.8	170.7	-12.77	<0.001
Random effects	**Variance**	**S.D.**	**ICC**	
Branch	1.5	1.2	0.01	
Inflorescence	1.5	1.2	0.01	
Plant ID (random)	148.6	12.2	0.96	

Figure 3.3. Hermaphrodite flowers produced in different positions of the inflorescence of individual 07s211B-1 grown under controlled conditions at the glasshouse at the University Of Stirling. Note that the hermaphrodite in the most basal position (towards the right) bears feeding anthers (FA) and has a strong separation between stigma anthers, while the flower in the second position (to the left) possesses pollinating anthers (PA) and a short separation between anthers and stigma.

3.4.3 Sex expression under resource limitation treatments

Flower position had a strong effect on the sex of each flower produced in all treatments (Table 3.3). The probability of the first flower to be staminate in all treatments was 0.00 ($CI_{95\%}$: 0.00, 0.13), while the probability of the flowers in subsequent positions to be staminate was 0.97 ($CI_{95\%}$: 0.93, 0.99) for the control treatment, 0.96 ($CI_{95\%}$: 0.94, 0.98) for the fertiliser treatment and 0.98 ($CI_{95\%}$: 0.94, 0.99) for the fruit treatment. The resource availability treatments did not differ significantly from the control in sex expression (Table 3.3). There were individuals capable of producing hermaphrodite flowers at different positions of the inflorescence among the treatments (Figure 3.4A and supplementary information Figure s3.1), but overall the probability of these flowers to be produced in secondary positions of the inflorescence was 0.03 ($CI_{95\%}$:0.02, 0.05).

Compared to the treatments with increased and decreased resource availability, the removal of the most basal flowers did not significantly increase the production of hermaphrodite flowers in the subsequent positions in the inflorescence, which was contrary to was expected (Table 3.3, Figure 3.4B). Overall, the probability of producing staminate flowers in the basal flower removal treatment was 0.99 ($CI_{95\%}$: 0.97, 1.00) flowers, while with the probability of producing staminate flowers in the treatments without basal flower removal was 0.97 ($CI_{95\%}$: 0.95, 0.98).

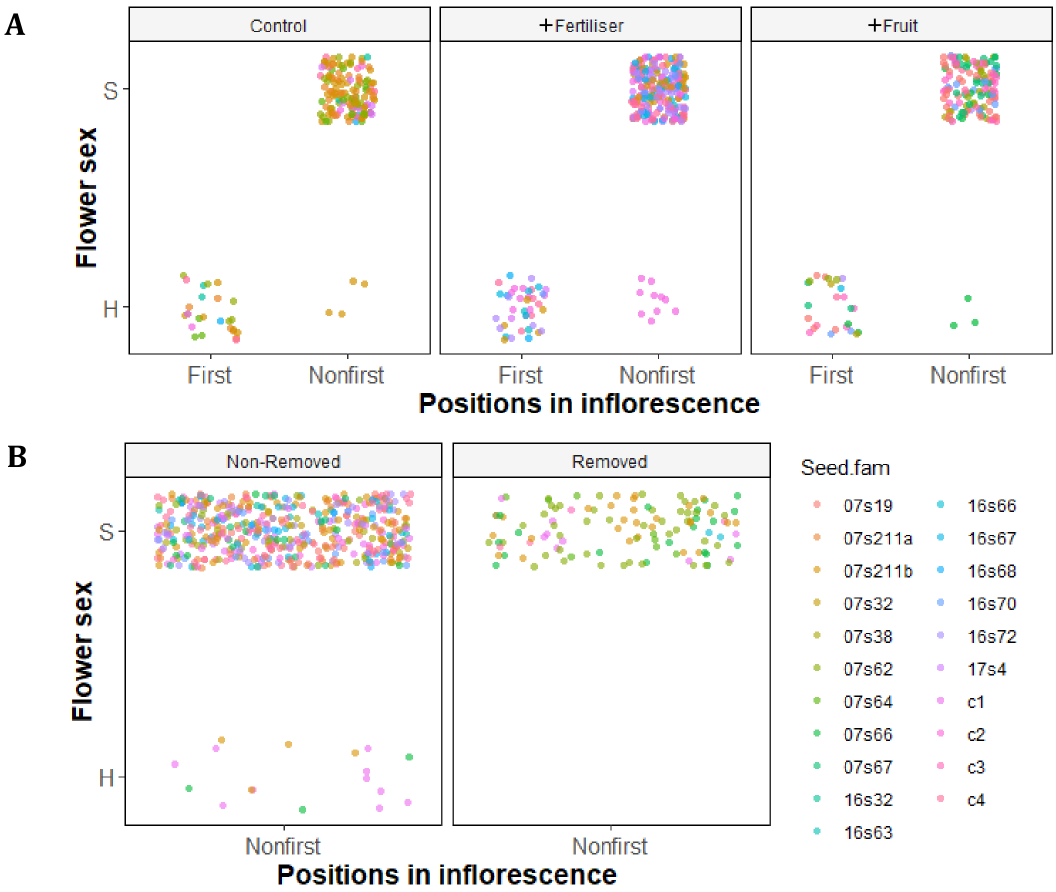


Figure 3.4. Hermaphrodite and staminate flowers produced in the first and subsequent positions in the inflorescences of individuals under treatments with increased (+Fertiliser) and decreased (+Fruit) resources availability and control (A). Hermaphrodite and staminate flowers produced in treatments with the basal flower removed (Removed) and treatments without the basal flower removed (Non-removed) (B).

Table 3.3. Coefficients of models 1) to test the effect on the sex of flower of treatments: plus fertiliser, +fruit and control and of flower position in the inflorescence. And 2) to test the effect on the sex of flower of removing the basal flower on the inflorescence Fixed and random effects shown are the result of generalised fixed effect model with binomial distribution.

	Fixed effects	**Estimate**	**S.E.**	***z***	***p***
1) Treatments	Intercept	-259.60	112.07	-2.316	0.021
	Fertiliser	0.87	0.88	0.988	0.323
	Fruit +	0.53	2.97	0.179	0.858
	Position (Non-first)	268.76	112.07	2.398	0.017
	Random effects	**Variance**	**S.D.**	**ICC**	
	Seed family	42.47	6.52	0.917	
	Inflorescence	0.57	0.75	0.012	
2) Basal flower removed	Intercept	40.49	147.81	0.274	0.784
	Basal f. removed (No)	-31.73	147.81	-0.215	0.830
	Resource manipulation (Yes)	0.83	0.08	0.979	0.328
	Random effects	**Variance**	**S.D.**	**ICC**	
	Seed family (random)	37.91	6.157	0.920	

3.5 Discussion

The production of staminate flowers is often considered a form of phenotypic plasticity to ensure enough resources are available for plant growth and reproduction (Primack & Lloyd, 1980; Bertin, 1982; Diggle, 1993). In *Solanum*, this has been widely documented (Solomon, 1985; Diggle, 1993; Miller & Diggle, 2003; Diggle & Miller, 2004, 2013). However, species like *Solanum houstonii* do not respond to changes in resource availability and are considered non-plastic. The results found in this study demonstrated *S. houstonii* has a male-biased sex expression in natural populations that does not depend on the overall plant size. Considering plant size might reflect the resources available for plant reproduction, these findings indicate sex expression of *S. houstonii* in natural populations is maintained irrespective of resource availability. Furthermore, my results from plants grown under controlled conditions and under treatments with decreased and increased resource availability showed there are strong positional effects at inflorescence level that determine the sex of flowers produced and that are maintained regardless of the resources available for plant growth and reproduction. These findings suggest that *S. houstonii* possesses a fixed pattern of sex expression that is maintained by the production of one hermaphrodite flower at the most basal (first) position of the inflorescence and staminate flowers in subsequent positions. Diggle (1995, 1997) suggested that predictable variation patterns are attributed to positional effects inherent to the architecture of each inflorescence. These architectural effects are mediated by developmental constrains that are taxon and organ specific and that determine both the sex and size of flowers, such as the amount of vascular tissue supplying the organs or the presence of identity genes that determine organ abortion (Diggle, 1995, 1997; Boualem *et al.*, 2008; Diggle *et al.*, 2011).

My results also indicated that few individuals were capable of producing hermaphrodite flowers in secondary positions of the inflorescence regardless of the treatment to which they were allocated. I found a single seed family with individuals capable of producing more than one hermaphrodite flower in the inflorescences. However, the morphology of these hermaphrodite flowers was very different from the hermaphrodite flowers from the most basal positions, as they possessed long pollinating anthers like staminate flowers (see Figure 3.2). Because these hermaphrodite flowers with pollinating anthers were only present in very few individuals from reduced resource availability treatment and control, is possible there is a genetic component to plasticity that makes only some genotypes plastic, and therefore, my models were unable to detect whether their presence was a plastic response due to differences in resource availability. In any case, the presence of some individuals capable of producing more than one hermaphrodite flower per inflorescence raises few hypothesis regarding the origin and maintenance of *S. houstonii* fixed expression.

One hypothesis that could explain the individuals that produce hermaphrodite flowers at secondary positions of the inflorescence is that a plastic phenotype is ancestral in *S. houstonii*. This notion is supported by previous studies in *Solanum* demonstrating species with fixed sex expression evolved from plastic species as a mechanism to ensure enough resources are allocated to the female function (Miller & Diggle, 2003; Diggle & Miller, 2004, 2013). Diggle and Miller (2013) found that fixed sex expression of species of section *Lasiocarpa* (subgenus *Leptostemonum*) is derived from an ancestor that possessed a plastic phenotype. This fixed expression in section *Lasiocarpa* is hypothesised to occur by the initial fixation of staminate flower in earlier (basal) positions of the inflorescence. In *S. houstonii*, a similar scenario could have occurred, and the presence of plastic individuals could mean that these individuals are in an early stage of fixating the production of staminate flowers. In these individuals, there is a slow response in hermaphrodite flower production due to changes in resource availability. This could be verified if individuals with phenotypes producing hermaphrodite flowers in secondary positions were exposed to treatments with decreased resources for long periods of time, and showed no variation or a slow decrease the proportion of hermaphrodite flowers produced. Whether the ancestor of *S. houstonii* was plastic or not concerning sex expression phenotype remains unknown. Nevertheless, phylogenetic ancestral character reconstructions of sex expression have indicated that within a clade of four species (*Lasicarpa*), two of which possess plastic phenotypes and two fixed phenotypes, the ancestor was phenotypically plastic for the production of staminate flowers (Diggle & Miller, 2013). *Solanum houstonii* belongs to section *Elaeagnifolium¸* in which three species (*S. houstonii, S. mortonii* and *S. homalospremum*) have an extreme phenotype that produces one hermaphrodite flower per inflorescence and that is hypothesised to possess a fixed expression (Diggle & Miller, 2013) and two (*S. hindisianum* and *S. elaeagnifolium*) possess phenotypes able to produce several hermaphrodite flowers, that is suggested to be more plastic (Knapp *et al.*, 2017). Moreover, basal sections of

Elaeagnifolium include several weakly andromonoecious species, while more derived sections, such as the Old World solanums include various weakly, strongly andromonoecious, and dioecious species (Wahlert *et al.*, 2014; see Solanaceae Source, http://solanaceaesource.org). Plasticity in the other species of the *Elaeagnifolium* section and other sister clades has not been investigated, but if the hypothesis for phenotypic evolution of sexual expression suggested by Diggle and Miller is consistent among the clades of the whole subgenus *Leptostemonum*, then *S. houstonii* fixed expression is probably derived from a plastic phenotype.

A second hypothesis for the production of hermaphrodite flowers with pollinating anthers could be that these flowers are a strategy to ensure reproduction in the absence of pollinators. During the experiments in the pollinator free glasshouses, I observed that hermaphrodite flowers with pollinating anthers were able to self-fertilise and produce fruits. Because the distance between the style and pollinating anthers was small, self-fertilisation is likely to occur. In species with specialised pollination systems, in which pollinator service becomes unreliable, self-fertilisation is a mechanism of reproductive assurance (Fenster & Martén-Rodríguez, 2007; Barrett, 2013). *Solanum houstonii* is a highly specialised species, as it requires bees capable of vibrating its poricidal anthers in order to release pollen. Moreover, the hermaphrodite flowers from basal positions that characterise this species have herkogamy, a morphological adaptation that reduces self-fertilisation (Barrett, 2002b) but requires bees of a certain size to ensure pollen transfer the stigma. In environments in which pollinators are reduced, *S. houstonii* have very low chances to set fruits. Under these circumstances, possessing hermaphrodite flowers capable of self-fertilisation could be advantageous.

3.6 Conclusions

The evolutionary dynamics of plasticity and resource allocation in andromonoecious species vary widely. Some species are able to change their sex expression in response to changes in resource availability while others possess a non-plastic phenotype in which the production of hermaphrodite and staminate flowers is fixed. In this study, I demonstrated *S. houstonii* has a fixed pattern of sex expression, in which each inflorescence produces one hermaphrodite flower at the most basal (first) position and several (up to 35) in the subsequent positions. This fixed pattern of expression may be determined by developmental constraints in the inflorescence architecture. I showed sex expression of *S. houstonii* does not change in response to resource availability. However, the presence of a few individuals capable of producing hermaphrodite flowers at secondary positions in the inflorescences could suggest some individuals maintained a plastic phenotype to ensure the production of fruits in environments that lack pollinators or represent a previous phenotype capable of responding to resource availability.

Chapter 4

Functional significance of staminate flowers

Chapter 4. The role of staminate flowers on pollen donation and pollinator attraction

4.1 Abstract

The majority of flowering plants have hermaphrodite flowers, each of which has the potential to develop into a fruit. However, in approximately 4,000 species, individual plants produce both hermaphrodite and female-sterile flowers, i.e., they are andromonoecious. One of the main hypotheses for the evolution and maintenance of female-sterile (staminate) flowers in andromonoecious plants suggests these flowers enhance fitness by increasing pollen donation and pollinator attraction. Many studies that support this hypothesis have demonstrated staminate flowers bear more pollen of better quality, are preferred by pollinators and transfer pollen to the stigma more efficiently than hermaphrodite flowers. Nevertheless, other studies have found contrasting results, and show difference across flowers in pollen production, siring success or pollinator attraction. *Solanum houstonii* (Solanaceae) is a buzz-pollinated, plant that relies on pollinator visitation to maximise seed set. This species possesses morphological adaptations in its staminate flowers such as heteranthery and increased pollen amount that are presumed to enhance pollen donation and pollinator attraction. In this study, I examined the functional significance of staminate flowers in andromonoecious *S. houstonii*, in order to determine whether staminate flowers main role is to promote pollinator attraction, pollen donation and ensure siring success. To achieve this, I examined pollen viability and the potential to sire seeds for both hermaphrodite and staminate flowers. Additionally, I tested whether staminate flowers acted as better pollen donors than hermaphrodite flowers using experimental arrays of flowers and captive bumblebees as pollinators. My results indicated that both staminate and hermaphrodite flowers are capable of producing viable pollen and siring seeds. Although I found hermaphrodite flowers have a greater probability of having viable pollen, there is no difference among flowers sexes in the probability of germination and siring seeds, indicating both kind of flowers have a similar siring success. In addition, I found pollinators did not show a preference for staminate flowers and that staminate flowers were not better pollen donors than hermaphrodite flowers. These results provide little support for the hypothesis that staminate flowers disproportionately promote pollen donation and pollinator attraction.

4.2 Introduction

The majority of flowering plants have hermaphrodite flowers, each of which has the potential to develop into a fruit (Barrett, 2002a). However, about 10% of plants possess unisexual flowers that are female-sterile and are unable to sire fruits (Yampolsky & Yampolsky, 1922; Torices *et al.*, 2011). These unisexual plants rely on morphological adaptations to disperse pollen and ensure reproduction (Podolsky, 1993; Harder & Wilson, 1994; Barrett, 2002a; Renner, 2014). Several studies have shown how floral morphology plays an important role in plant reproduction as it can affect the way a flower contributes to pollinator attraction, pollen dispersal and pollen transference to the stigma (Harder & Wilson, 1994; Fetscher, 2001; Barrett, 2002b; Vallejo-Marín *et al.*, 2009; De Luca & Vallejo-Marín, 2013; Solís-Montero & Vallejo-Marín, 2017; Koski *et al.*, 2018b,a; Leibman *et al.*, 2018; Nogueira *et al.*, 2018). In species that bear female-sterile flowers (hereafter, staminate flowers), morphological adaptations that promote pollen donation and pollinator attraction (i.e. heteranthery, enantiostyly, zygomorphy) are presumed to be enhanced (Podolsky, 1993; Bohs *et al.*, 2007). In fact, the main hypotheses for the evolution and maintenance of systems with staminate flowers suggests these flowers provide a mechanism to maximise pollinator attraction and pollen dispersal or receipt (Primack & Lloyd, 1980; Coleman & Coleman, 1982; Podolsky, 1992).

Andromonoecy is a sexual system spread in 33 families, approximately 2% of flowering plants, in which individual plants produce both staminate and hermaphrodite flowers (Torices *et al.*, 2011; Cardoso *et al.*, 2018). This system is present approximately 4000 species and has evolved independently in several plant lineages (Miller & Diggle, 2003). It has been hypothesised that the evolution of andromonoecy is selectively advantageous because the production of staminate flowers is less costly than hermaphrodite flowers, and resources saved by producing staminate flowers can be reallocated to produce more fruits or to other fitness-enhancing characters (Primack & Lloyd, 1980; Bertin, 1982; Solomon, 1985; Emms, 1993). This hypothesis is known as the *resource allocation hypothesis* and has been supported in a few species by the observation that staminate flowers are smaller than hermaphrodite flowers (Dulberger *et al.*, 1981; Anderson & Symon, 1989; Diggle & Miller, 2004; Vallejo-Marín & Rausher, 2007b; Liao & Zhang, 2008). However, some studies have shown that staminate flowers are not always cheaper to produce, and staminate flowers can be of similar size to or larger than hermaphrodites (Huang, 2003; Narbona *et al.*, 2008; Dai & Galloway, 2012). A second mechanism proposed to understand the production of staminate flowers is by the *pollen donation hypothesis*. This hypothesis postulates that staminate flowers are more efficient at pollen donation than hermaphrodite flowers. This advantages could arise for several reasons: staminate flowers may increase pollinator attraction, staminate flowers may increase the probability of ovules fertilisation (i.e. producing more pollen), or staminate flowers may produce higher amounts of pollen of better quality (Podolsky, 1992, 1993; Harder & Barrett, 1996; Elle & Meagher, 2000; Barrett, 2002b; Huang, 2003; Quesada-Aguilar *et al.*, 2008; Dai & Galloway, 2012). Evidence to support this hypothesis is equivocal, as some studies have found staminate flowers are not always better at siring seeds (Cuevas & Polito, 2004; Sunnichan *et al.*, 2004; Luo *et al.*, 2009), produce less or equal amounts of pollen as hermaphrodite flowers and sometimes of lower quality (Solomon, 1985; Cuevas & Polito, 2004; Vallejo-Marín & Rausher, 2007b) or flowers are less effective in dispersing pollen grains (Podolsky, 1993). These two hypotheses are not mutually exclusive as staminate flowers could disperse pollen more efficiently than perfect flowers, either by redirecting resources from pistil development to pollen production or by developing an enhance morphology better suited for pollen dispersal (i.e. heteranthery) (Bertin, 1982; Whalen & Costich, 1986; Podolsky, 1993).

Solanum houstonii Martyn (Solanaceae) a perennial shrub with a natural distribution in the tropical dry forest and disturbed areas of Mexico (Knapp *et al.*, 2017). It flowers throughout the year and is visited by a variety of bees capable of buzz-pollinating (see results Chapter 5). *Solanum houstonii* is an andromonoecious species in which individual plants bear one hermaphrodite flower and several staminate flowers per inflorescence. Hermaphrodite flowers are the only ones able to produce fruit, and although they have anthers, their large separation between anthers and stigma (herkogamy) does not allow autonomous self-fertilisation to happen (see results Chapter 5). Staminate flowers, on the other hand, possess two morphologically and functionally distinct set of anthers (heteranthery) that produce greater amounts of pollen than hermaphrodite flowers (see results Chapter 2) and presumably contribute to pollen reaching the stigmas of hermaphrodite flowers (Knapp *et al.*, 2017; Papaj *et al.*, 2017). Because of these morphological adaptations, in hermaphrodite and staminate flowers, *S. houstonii* provides a good opportunity to investigate the pollen donation hypothesis and to test whether a enhance male function promotes siring success and better pollen transfer to the stigma.

Here I compared the ability of hermaphrodite and staminate flowers to sire fruits and to transfer pollen to the stigma, with the aim of determining the functional significance of staminate flowers in pollen donation and pollination attraction in *S. houstonii*. I asked specifically: 1) is pollen from staminate flowers better at siring fruits than pollen of hermaphrodite flowers? 2) Do pollinators prefer visiting staminate flowers over hermaphrodite flowers? And 3) Are staminate flowers more efficient at transferring pollen to the stigma than anthers of hermaphrodite flowers? I addressed the first question by performing pollen viability, *in vitro* germination analyses and hand-pollination crosses to measure fruit and seed set. To determine whether staminate flowers are better at pollinator attraction and pollen deposition, I performed controlled visitations using captive bumblebees (*Bombus terrestris audax*) in experimental arrays of hermaphrodite and staminate flowers, recording their preference and the number of pollen grains deposited on the stigma.

4.3 Methods

4.3.1 Plant material

To investigate the role of staminate flowers of *S. houstonii* on pollen donation and pollinator attraction, I grew plants in a pollinator-free glasshouses at the University of Stirling in October 2016. Seeds were collected by MVM and AKZC in Mexico between 2007 and 2016 (Table s4.1, supplementary material). I pre-treated the seeds with 2000ppm of gibberellic acid (GA_3, Sigma-Aldrich, Dorset, UK) overnight and sowed them in a mix of All Purpose Growing Medium (William Sinclair Horticulture PLC, Lincoln, UK) and Perlite Standard (Sinclair). Plants were kept in a glasshouse at 16-22°C with natural daylight cycle of 16h light and 8h dark, supplemented with compact-fluorescent lamps.

4.3.2 Pollen germination and viability

Pollen viability of hermaphrodite and staminate flowers was tested using two methods: 1) *in vitro* pollen germination in Brewbaker-Kwack medium and 2) aniline blue-lactophenol stain. I used these two methods as pollen staining can sometimes be subjective (because some unviable pollen grains absorb stain) and *in vitro* germination is sometimes sensitive to environmental factors (Kearns & Inouye, 1993). For each method I followed the protocol of Kearns and Inouye (1993). Counts of viable/germinated and non-viable/non-germinated pollen were taken after observing 200 grains. I performed each of the viability methods on the anthers of 40 flowers belonging to 18 accessions and 8 populations (Table s4.1). Each method was applied to pollen from feeding anthers of hermaphrodite flowers and to feeding and pollinating anthers of staminate flowers. I decided to test the viability of the two set of anthers of staminate flowers, as it has been suggested that flowers with heteranthery sometimes possess pollen of different viability as a reflection of the division of labour in their anthers (Müller, 1981; Luo *et al.*, 2009; Paulino *et al.*, 2016).

4.3.3 Siring success (fruit and seed set)

I measured siring success as the number of fruits and seeds produced by pollen of the different anther-types of *S. houstonii* flowers. I performed hand-pollinations using pollen from feeding or pollinating anthers of hermaphrodite and staminate flowers. All hand pollinations were performed on hermaphrodite flowers, since a preliminary experiment showed staminate flowers bear unfertile gynoecia (from 20 crosses all failed to set fruit). To test the siring potential of each anther type, I performed three different pollination treatments: 1) hand pollinations using pollen from feeding anthers of hermaphrodite flowers, 2) hand pollinations using pollen from feeding anthers of staminate flowers and 3) hand pollinations using pollen from pollinating anthers of staminate flowers. Each treatment was performed in 30 flowers in up to 16 individuals belonging to two populations (Table s4.1). Pollen was extracted from anthers of one flower different to the pollen recipient using an electric toothbrush and then applied to the stigma of hermaphrodite flowers using a toothpick. Fruit set was quantified after two weeks of pollination and seed set after three months, when fruits were mature.

4.3.4 Pollen transfer efficiency

To assess whether staminate flowers are preferred by pollinators and transfer pollen more efficiently to the stigma, I used a series of experimental arrays with hermaphrodite and staminate flowers and exposed them to captive bumblebees (*Bombus terrestris audax*). *B. terrestris audax* is distributed in the British Isles and thus does not co-occur with natural populations of *S. houstonii*; however, commercial colonies of *B. terrestris audax* are available and are used for pollination of crops that need to be buzz-pollinated such as tomato (*Solanum lycopersicum*). Moreover, individuals of *B. terrestris audax* show considerable size variation (thorax width 2.3-8.8 mm; Goulson, 2010) that overlaps with the size of the main pollinators of *S. houstonii* (see results in Chapter 5). My experimental design consisted of two types of experiments to test 1) the preference of bees between hermaphrodite and staminate flowers and 2) the efficiency of pollen transfer from staminate flowers or hermaphrodites to the stigma. All experiments were performed on a foraging arena of 100 cm x 60 cm x 35 cm (L x W x H) made of wood with a Perspex. For both experiments, I used two commercial *Bombus terrestris audax* colonies obtained from Biobest (Belgium) via Agralan Ltd (Swindon, UK).

To test the preference of pollinators among flowers, I performed a choice experiment using two hermaphrodite and two staminate flower of *S. houstonii*. Fresh flowers of *S. houstonii* were attached to wooden sticks with Blu Tack (Bostik Blu Tack) and offered to a single bee for a period of 5 minutes (n=68 bees). During this time, I collected data on the number of floral visits (bee touching any part of flower) on each hermaphrodite or staminate flower and sex of first flower visited.

To test for the efficiency of pollen transfer from staminate flowers or hermaphrodite to the stigma, I performed two type of experiments, one using hermaphrodite flowers as pollen donors and hermaphrodite flowers as pollen recipients (n=15 trials) and the other using staminate flowers as pollen donors and hermaphrodite flowers as pollen recipients (n=14 trials). For each trial, I used an array of ten fresh flowers, five acting as pollen donors and five as pollen recipients. Pollen recipients for both trials consisted in hermaphrodite flowers with anthers blocked with super glue (Gorilla Super Glue, Gorilla Glue Inc.) to stop pollen release. Glue was applied on the pores of the anthers and dried for 5 minutes. To avoid odour effects on bee behaviour, I also applied drops of glue on the side of anthers from pollen-donor flowers without blocking the anther pores. During each trial, I randomly arranged the five pollen donors and the five pollen recipients on five wooden sticks inside the foraging arena. I allowed six bees to visit the flowers for a period of five minutes and observed the number of visits performed to each flower. After visitation, bees were removed from the arena and kept separated from the colony until the day of observation was finished. After each trial, I collected the pistils of pollen recipient flowers (n=75 pistils for trials with hermaphrodite flowers as pollen donors and n=70 pistils for trials with staminate flowers as pollen donors), fixed them in formaldehyde-acetic acid (FAA) for two days and placed them on a slide with fuchsine-stained glycerol jelly (Kearns & Inouye 1993), for later analysis. To determine the pollen transfer efficiency, I quantified the total number of pollen grains on the stigma using a light microscope.

4.3.5 Statistical analyses

To test for differences in pollen viability of the anthers of hermaphrodite and staminate flowers, I fitted a generalised mixed effect model (GLMM) with a binomial distribution and logit-link error with either the pollen grains viable or pollen grains germinated as the response variable. Pollen grains viable or pollen grains germinated were two-vector response variables with the total number of pollen grains germinated/viable or non-germinated/non-viable. The type of anthers from which pollen was extracted (i.e. hermaphrodite feeding anthers, staminate feeding anthers or staminate pollinating anthers) was fitted as a fixed effect and Plant ID as a random effect. Additionally, to control for overdispersion I included an observation-level random effect (OLRE) where each data point receives a unique level of a random effect (Hinde, 1982). *Post hoc* Tukey comparisons were used to test statistically significant differences among anther-types.

To determine the effect of anther-type on fruit set and seed set of *S. houstonii*, I used GLMM with binomial distribution for fruit set and with Poisson distribution for seed set. Fruit set was binary response variable (fruit set= 1 and failed fruit=0) and seed set a continuous response variable. In both models, I fitted as fixed effect the type of anther used (i.e. hermaphrodite feeding anthers, staminate feeding anthers or staminate pollinating anthers) and as random effects plant ID of the pollen donor, plant ID of the pollen recipient and an OLRE (to account for overdispersion). *Post hoc* Tukey comparisons were used to test statistically significant differences among anther-types.

To test the effect of flower sex on the number of visits by bees, I fitted a GLMM with Poisson distribution. I fitted the number of visits performed by bees as a response variable, flower sex as fixed effect and plant ID, bee colony and OLRE as random effects. *Post hoc* Tukey comparisons were used to test statistically significant differences between hermaphrodite and staminate flowers. Additionally, to test for bee preference of staminate flowers on their first visit, I used a binomial test and specified the total number of times hermaphrodite and staminate flowers were visited for the first time, a probability of 0.5 and a two sided hypothesis test. For this test, I used the *binom.test* function of *R*.

I fitted a linear mixed effect model with Gaussian distribution to study predictors of the number of pollen grains on the stigma (response variable), with sex as a two level fixed factor and the total number of visits performed to each flower sex were fitted as fixed effects and plant ID and trial number as random effects. In addition, to test for the effect of the treatment of closed and open anthers on the total number of visits, I performed a GLMM with Poisson distribution for the two experiments in which I used hermaphrodite or staminate flowers as pollen donors. In each model, the total number of visits was the response, with the type of treatment used (open or closed anthers) as fixed effect and plant ID, trial and OLRE as random effects. *Post hoc* Tukey comparisons were used to test statistically significant differences between among treatments.

All statistical analyses were conducted in *R* software v 3.4.0 (R Development Core Team, 2013). All generalised mixed effect models (GLMM) were performed with the function *glmer* from the *lme4* package (Bates *et al.*, 2015) and *post hoc* Tukey comparisons were fitted using the *multcomp* package for R (Hothorn *et al.*, 2008). Additionally, to estimate the intraclass correlation coefficient (ICC) for the random variables of each model, I used the *icc* function of the *sjstats* package for *R* (Ludecke, 2019). Models were validated by visually assessing diagnostic plots to confirm normality of residuals.

4.4 Results

4.4.1 Pollen germination and viability

Pollen viability differed between the all the anther types of hermaphrodite and staminate flowers, however, the two anther types in staminate flowers had pollen with statistically indistinguishable viability (Table 4.1). On average, pollen from hermaphrodite flowers had a 0.86 ($CI_{95\%}$=0.82, 0.90) probability of being viable, which was higher than pollen from staminate flowers. Feeding anthers of staminate flowers had a probability of 0.78 ($CI_{95\%}$=0.73, 0.82) and pollen from pollinating anthers of 0.77 ($CI_{95\%}$=0.72, 0.82) (Figure 4.1A). On the contrary, pollen germination was statistically the same among all anthers of hermaphrodite and staminate flowers (Table 4.1). On average, pollen from hermaphrodite flowers had a 0.81 ($CI_{95\%}$=0.73, 0.88) probability to germinate, while pollen of feeding anthers of staminate flowers had probability to germinate of 0.88 ($CI_{95\%}$=0.82,0.94) and pollen of pollinating anthers a probability of 0.85 ($CI_{95\%}$=0.80,0.91) (Figure 4.1B).

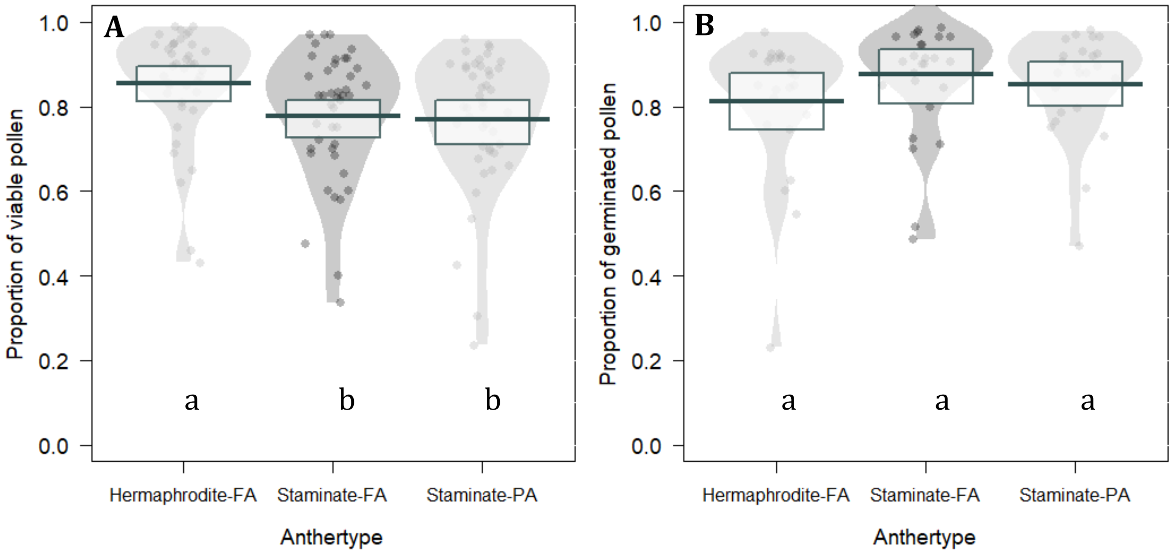


Figure 4.1. Proportion of viable (A) and germinated pollen grains (B) of feeding and pollinating anthers of *S. houstonii* flowers. Similar letters denote no statistical differences between pollen grains germinated/viable from the different anther types. Multiple comparisons derived from Tukey test. Black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an observation from a single population. FA-feeding anthers, PA-anthers.

4.4.2 Pollen siring success (fruit and seed set)

Fruit and seed set showed no statistical differences among the anther types of hermaphrodite and staminate flowers used for hand pollination (Figure 4.2, Table 4.1). The fruit siring probability of anthers from hermaphrodite flowers was 0.75 ($CI_{95\%}$=0.55,0.87), while the probability of feeding and pollinating anthers from staminate flowers was 0.73 ($CI_{95\%}$=0.55,0.86). The mean (±SE) number of seeds sired by hermaphrodite anthers was 156±13, by feeding anthers of staminate flowers 143±13 and by pollinating anthers of staminate flowers 120±19 (Figure 4.2B).

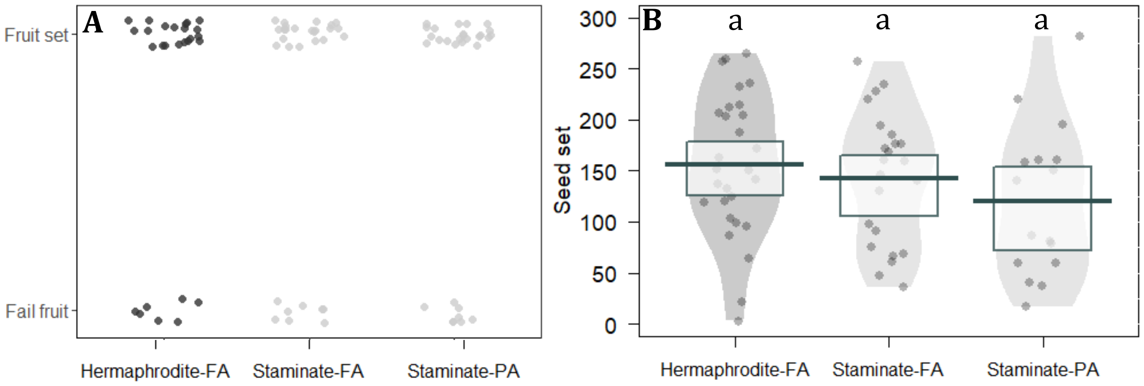


Figure 4.2. Fruit set percentage (A) and seed set (B) sired from pollen of feeding and pollinating anthers of hermaphrodite and staminate flowers. Similar letters denote no statistical differences between seed set sired from the different anther types. Multiple comparisons derived from Tukey test. In B, black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an observation from a single population. FA-feeding anthers, PA-flowers pollinating anthers.

Table 4.1. Coefficients of models to test the effect of anther-type on pollen viability, germination, fruit and seed set. Results show the fixed and random effects of generalised mixed effect models with binomial or Poisson distribution. FA-feeding anthers, PA-pollinating anthers.

	Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Pollen viability (Binomial)	Intercept	-2.19	0.17	-12.83	<0.001
	Anther-type (Staminate-FA)	0.56	0.20	2.75	0.006
	Anther-type (Staminate-PA)	0.66	0.20	3.30	<0.001
	Random effects	**Variance**	**S.D.**	**ICC**	
	Plant ID	0.13	0.36	0.03	
	OLRE	0.72	0.85	0.18	
	Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Pollen germination (Binomial)	Intercept	-1.47	0.29	-5.01	<0.001
	Anther-type (Staminate-FA)	-0.46	0.25	-1.84	0.066
	Anther-type (Staminate-PA)	-0.30	0.26	-1.17	0.249
	Random effects	**Variance**	**S.D.**	**ICC**	
	Plant ID	0.28	0.52	0.06	
	OLRE	0.72	0.85	0.17	
	Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Fruit set (Binomial)	Intercept	-1.04	0.52	-2.02	0.044
	Anther-type (Staminate-FA)	0.18	0.67	0.26	0.792
	Anther-type (Staminate-PA)	0.10	0.67	0.14	0.886
	Random effects	**Variance**	**S.D.**	**ICC**	
	Donor ID	<0.01	<0.01	<0.01	
	Recipient ID	0.59	0.77	0.15	
	OLRE	<0.01	<0.01	<0.01	
	Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Seed set (Poisson)	Intercept	4.95	0.14	34.93	<0.001
	Anther-type (Staminate-FA)	-0.10	0.18	-0.57	0.567
	Anther-type (Staminate-PA)	-0.13	0.23	-0.55	0.580
	Random effects	**Variance**	**S.D.**	**ICC**	
	Donor ID	0.04	0.21	0.12	
	Recipient ID	0.02	0.14	0.06	
	OLRE	0.29	0.54	0.81	

4.4.3 Pollinator preference and pollen transference efficiency

Pollinators had a probability to land first on staminate flowers of 0.62 ($CI_{95\%}$=0.49,0.74), which denotes a strong trend towards a preference for staminate flowers. However, the results of the binomial test showed no significant differences their first choice (p=0.077; n=63 bees). Additionally, I did not find an effect of sex on the total number of visits performed by pollinators (Table 4.2, Figure 4.3A).

The experiment to test the pollen transfer efficiency of pollinators when hermaphrodite or staminate flowers acted as pollen donors showed that flower sex did not have a statistically discernible effect on the total number of pollen grains deposited on the stigma (Figure 4.3B, Table 4.2). On average (±SE), when hermaphrodite flowers acted as pollen donors, pollinators deposited 32±4 pollen grains on the stigma and when staminate flowers acted as pollen donors, pollinators deposited 49±6 pollen grains on the stigma. Moreover, there was a tendency that with more floral visits to pollen donors, higher numbers of pollen grains were deposited on the stigma; however, the model to test the effect of this was non-significant (Figure 4.3B; Table 4.2).

In trials with hermaphrodite flowers as pollen donors, the mean (±SE) number of visits performed to pollen donors was 12±0.4 and to pollen recipients was 9±0.4. In trials with staminate flowers as pollen donors, the mean number of visits performed to pollen donors was 13±0.5 and to pollen recipients was 8±0.4 (Figure 4.4). Overall the number of visits to pollen donors and pollen recipients in both trials were statistically different (Table 2.3).

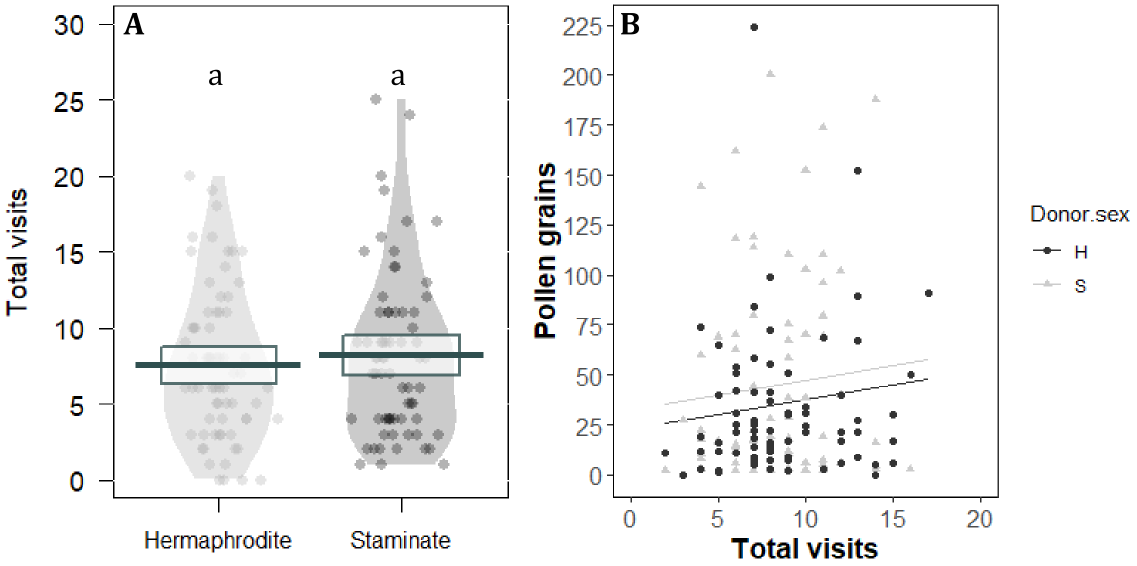


Figure 4.3. Total number of floral visits performed by captive bees *Bombus terrestris audax* on hermaphrodite and staminate flowers of S. *houstonii* (A) and the effect of total visits performed on hermaphrodite and staminate flowers on the number of pollen grains deposited on the stigma (B). Similar letters in A denote no statistical differences of the visits between hermaphrodite and staminate flowers. Multiple comparisons derived from Tukey test. In A, black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an observation from a single visit. H-hermaphrodite flowers and S-staminate flowers.

Table 4.2. Coefficients of models to test the effect of sex on the total visits and to test the effect of visits to hermaphrodite and staminate flowers and sex of flowers visited on the total number of pollen grains. Results show the fixed and random effects of generalised mixed effect models with Poisson and Gaussian distribution.

	Fixed effects	**Estimate**	**S.E.**	***z***	***p***
Total visits (Poisson)	Intercept	1.92	0.19	10.13	<0.001
	Donor sex (Staminate)	0.08	0.10	0.74	0.461
	Random effects	**Variance**	**S.D.**	**ICC**	
	Plant ID	0.09	0.30	0.18	
	Bee colony	0.05	0.22	0.10	
	OLRE	0.22	0.47	0.45	
	Fixed effects	**Estimate**	**S.E.**	***t***	***p***
Pollen grains (Gaussian)	Intercept	22.99	11.7	1.97	0.052
	Donor sex (Staminate)	9.57	10.9	0.88	0.387
	Total visits	1.45	1.0	1.41	0.162
	Random effects	**Variance**	**S.D.**		
	Plant ID	<0.01	<0.01		
	Trial	63.4	25.2		

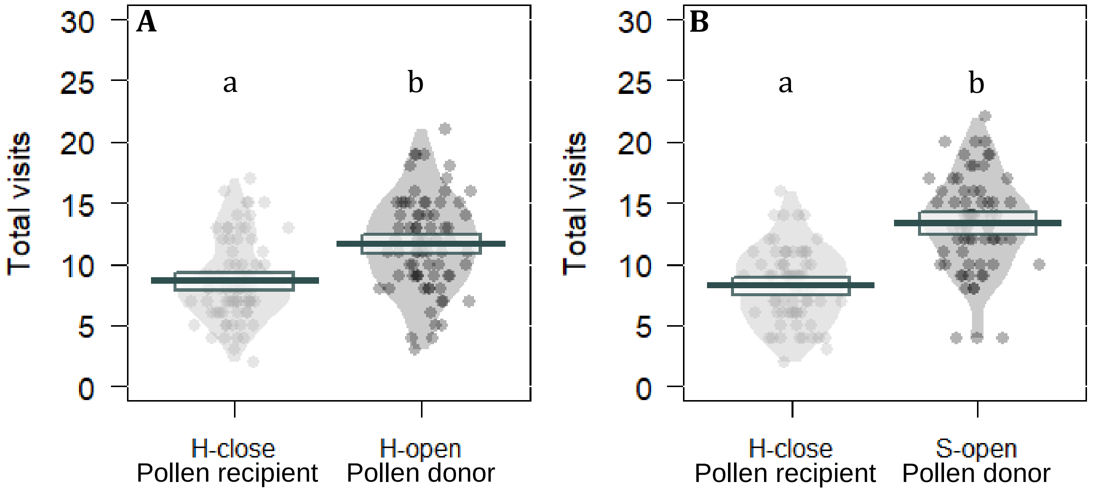


Figure 4.4. Total number of floral visits performed on flowers with closed and open anthers of *S. houstonii* for experiments using hermaphrodite (A) and staminate (B) flowers as pollen donors. Similar letters denote no statistical differences of the visits between hermaphrodite and staminate flowers. Multiple comparisons derived from Tukey test. Black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an observation from a single bee visiting. H-hermaphrodite flowers and S-staminate flowers.

Table 4.3. Models coefficients to test of the treatment of closed and open anthers on the total number of visits of the experiments using hermaphrodite or staminate flowers as pollen donors. Results show the fixed and random effects of generalised mixed effect model with Poisson distribution.

	Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Hermaphrodite flowers as pollen donors	Intercept	2.14	0.06	39.11	<0.001
	Donor treatment (Open)	0.30	0.06	0519	<0.001
	Random effects	**Variance**	**S.D.**	**ICC**	
	Plant ID	0.005	0.07	0.04	
	Trial	0.013	0.11	0.10	
	OLRE	0.005	0.07	0.04	
	Fixed effects	**Estimate**	**S.E.**	**z**	***p***
Staminate flowers as pollen donors	Intercept	2.11	0.04	48.17	<0.001
	Donor treatment (Open)	0.48	0.06	8.59	<0.001
	Random effects	**Variance**	**S.D.**	**ICC**	
	Plant ID	<0.01	<0.01	<0.01	
	Trial	<0.01	<0.01	<0.01	
	OLRE	0.01	0.09	0.09	

4.5 Discussion

4.5.1 Pollen viability and siring success

The results from this study to investigate the siring success of staminate flowers showed that both hermaphrodite and staminate flowers possess viable pollen, capable of germinating and siring seeds in similar amounts. The results of the viability analyses using pollen staining showed that pollen of hermaphrodite flowers have a greater viability than pollen of staminate flowers, while the results of the *in vitro* germinations showed germination, although non-statistically significant, was greater in pollen from staminate anthers. Pollen viability is an indirect test used to determine the quality of pollen grains by distinguishing between fresh and dead (non-viable) pollen grains, while *in vitro* pollen germination directly test the potential of each pollen grain to germinate in a period of time (Kearns & Inouye, 1993). Both germination and viability tests provide a measurement of pollen quality and often can provide contrasting results due to differences in the method accuracy as these methods are sometimes sensitive to environmental factors (Kearns & Inouye, 1993). In this study, I found opposite trends on pollen viability and germination, suggesting differences in pollen quality depending on the anther type they belong to; however, my results also showed pollen from all types of anthers had a high probability to germinate (~80%) and potentially sire seeds. In fact, the results of the hand-pollination experiments showed pollen from anthers of hermaphrodite and staminate flowers did not differ significantly in their potential to sire seeds. Similar results have been found in other andromonoecious species, in which neither *in vitro* pollen germination, pollen viability, nor the number of seeds sired of staminate flowers differed from hermaphrodite flowers (Cuevas & Polito, 2004; Sunnichan *et al.*, 2004; Zhang & Tan, 2009). Likewise, pollen germination and viability did not differ in other species of *Solanum* (Dulberger *et al.*, 1981; Anderson & Symon, 1989; Mione & Anderson, 1992; Knapp *et al.*, 1998; Anderson *et al.*, 2014). Although an enhanced pollen viability and siring success have been proposed and demonstrated as characteristics that promote pollen donation (Bertin, 1982; Emms, 1993; Podolsky, 1993; Huang, 2003; Dai & Galloway, 2012), evidence to support this is inconsistent, and suggest pollen viability and siring success should not be considered on their own to determine whether andromonoecy has evolved to increase pollen donation. For instance in *Solanum* and many buzz-pollinated plants, maintaining high amounts of pollen of high quality is important to ensure pollinators' visitation, as pollen is the only reward in these species. (Buchmann, 1983; Whalen & Costich, 1986; Knapp *et al.*, 1998; De Luca & Vallejo-Marín, 2013). Some studies have demonstrated pollen viability is associated with higher protein concentration (Yeamans *et al.*, 2014; Carr *et al.*, 2015; Ndem, 2018), and that this quality co-varies with higher visitation rates (Hanley *et al.*, 2008; Yeamans *et al.*, 2014; Carr *et al.*, 2015). The fact that *S. houstonii* bears viable pollen in both hermaphrodite and staminate flowers could suggest pollen in both flowers function is necessary to maximise pollinator visitation. Moreover, the lack of difference of siring success may indicate *S. houstonii* maintains pollen of high quality in hermaphrodite and staminate flowers in case this is transferred to the stigma.

4.5.2 The role of staminate flowers in pollen donation and pollinator attraction

One of the main hypotheses for the production of staminate flowers in andromonoecious species suggests these flowers are more successful at pollen donation and pollination attraction than hermaphrodite flowers (Bertin, 1982; Podolsky, 1993). *Solanum houstonii* is an andromonoecious species that produces a high number of staminate flowers and that possesses floral adaptations that presumably improve pollen dispersion and deposition on the stigma. Because of these characteristics, this species provides a good opportunity to test whether an enhanced male function promotes pollinator attraction and better pollen transfer to the stigma. The results from this study, using captive bumblebees as pollinators showed pollinators do not prefer visiting staminate flowers, in neither their first visit nor overall visitation, and the number of pollen grains transferred to the stigma did not depend on the sex of the flower used as pollen donor or the total number of visits performed by pollinators. These results provide little support to the hypothesis that staminate flowers in *S. houstonii* promote pollinator attraction and/or pollen donation. However, ruling out the potential of these flowers to act as better pollen donors may be premature, as results can be context dependant.

In andromonoecious *Solanum carolinense*, Quesada-Aguilar *et al.* (2008) found staminate flowers function primarily as pollen donors and hermaphrodite flowers as pollen recipients. Their study showed that pollinators extracted pollen more efficiently from staminate flowers, not only due to the pollinator morphology and behaviour but also due to the fact that the morphology of staminate flowers reduces sexual-interference (due to the absence of pistil), increasing the probability pollen reaching the stigma. Likewise, Elle and Meagher (2000) evidence in the same species that staminate flowers have a better siring success after visitation by natural pollinators. In contrast, another study of *S. carolinense* did not provide support for the pollen donation hypothesis as arrays of hermaphrodite flowers sired just as many seeds as staminate flowers after visitation of natural pollinators (Vallejo-Marín & Rausher, 2007b). These contrasting results indicate studies may be context dependent (i.e. differ in environmental conditions, such as pollinator availability), and pollinator composition could play an important role in determining the potential of one species to act as more efficient pollen donors. In this study, I used non-native pollinators to *S. houstonii* and performed the experiments under control conditions using the same amount of hermaphrodite and staminate flowers. However, in natural populations, pollinators tend to be of various sizes and morphologies, and *S. houstonii* presents a floral display with many staminate flowers. Under these circumstances, pollinator attraction could be bias towards one sex over the other and pollen export could be more successful from staminate flowers than hermaphrodite flowers.

One limitation of my study is that I used commercial *Bombus terrestris audax* as pollinators, a species that does not overlap with the range of distribution of *S. houstonii*. I adopted this approach as other studies have shown this species and other commercial bumblebees are efficient at extracting pollen of poricidal anthers and pollinating other species of *Solanum* (Messinger *et al.*, 2016; Papaj *et al.*, 2017; Solís-Montero & Vallejo-Marín, 2017; Arroyo-Correa *et al.*, 2018). However, it is possible *B. terrestris* was not an efficient pollinator of *S. houstonii* and, therefore, the results concerning pollen transfer to the stigma are not representative of what happens for other pollinator species. Bees able to buzz-pollinate differ in their behaviour during pollen extraction depending on plant species (Corbet & Huang, 2014; Switzer & Combes, 2017; Arroyo-Correa *et al.*, 2018). In fact, one study using the wild bee *Bombus impatiens* demonstrated one bee species changes its vibration behaviour depending on the plant species visited (Switzer & Combes, 2017). Others have shown morphological fit between pollinator and floral sexual organs influences the efficiency in pollen extraction and deposition on the stigma (Quesada-Aguilar *et al.*, 2008; Anderson, 2010; Corbet & Huang, 2014; Solís-Montero & Vallejo-Marín, 2017). These findings suggest flower morphology plays an important role in the behaviour of bees during buzz-pollination, and we should expect differences in pollinators behaviour when flowers possess different morphology.

The morphology of *S. houstonii* flowers depend on sex, as hermaphrodite flowers have short anthers and long pistils and staminate flowers two set of anthers of different lengths. The fact that I did not find staminate flowers were more attractive or better at depositing pollen grains on the stigma was not expected for two reasons. First, species with heteranthery are known to promote pollen transfer to the stigma (Barrett, 2002a; Luo *et al.*, 2008, 2012; Solís-Montero *et al.*, 2015). Studies have shown that the division labour of anthers into feeding and pollinating anthers can promote a placement of pollen on pollinator body, which improves pollen deposition on the stigma (Solís-Montero *et al.*, 2015; Mesquita-Neto *et al.*, 2017). Second, because pollinators tend to prefer flowers with more pollen, larger size and bilateral symmetry (Neal *et al.*, 1998; Fenster *et al.*, 2004; Fenster & Martén-Rodríguez, 2007; Gómez *et al.*, 2008; Mesquita-Neto *et al.*, 2017), I would expect staminate flowers of *S. houstonii* were more attractive to pollinators. Nevertheless, further studies must investigate the behaviour of different pollinators, native or non-native, on the flowers of *S. houstonii* before we can be confident about whether staminate flowers main role is pollen donation or pollinator attraction.

Other hypotheses that could explain the role of staminate flowers in andromonoecious species propose staminate flowers increase pollen receipt or reduce sexual interference. The first is known as *the increased pollen receipt hypothesis*, and propose that staminate flowers produce high amounts of pollen, and develop a morphology that enhances pollen dispersal and deposition on the stigma (Bertin, 1982; Whalen & Costich, 1986; Podolsky, 1993). Evidence supporting this hypothesis has been provided by Vallejo-Marin and Rausher (2007a) who used a multivariate selection analyses to show that the production of staminate flowers provides a selective advantage in the production of seeds. The second hypothesis is known as *the sexual interference hypothesis* and suggests staminate flowers help to reduce the problems in pollen removal and deposition. The separation of the male function in staminate flowers and female function in hermaphrodite flowers enhances the chances of pollinators visiting staminate flowers for pollen extraction and hermaphrodite flowers for pollen deposition. This reduces the chances of self-fertilisation to occur, although is highly dependent on the behaviour of the pollinator (Elle & Meagher, 2000; Barrett, 2002b; Quesada-Aguilar *et al.*, 2008). In *Solanum* *carolinense,* Quesada-Aguilar *et al*. (2008) found evidence of these two hypotheses. They supported the pollen receipt hypothesis by demonstrating staminate flowers deposited more pollen on the bodies of pollinators, specifically in body parts that touched the stigma, which increased the probability of pollen reaching the stigma. In addition, they showed the length of the pistil of hermaphrodite flowers interferes with pollen removal in hermaphrodite flowers, and because these flowers function as pollen recipients, which reduces the chances of self-fertilisation and supports the sexual interference hypothesis. The morphological characteristics of *S. houstonii* flowers, such as heteranthery and increased amount of pollen in staminate flowers and the large separation between anthers and stigma in hermaphrodite flowers, suggest that staminate flowers do more than merely donate pollen. Determining the reproductive success in natural populations and detailed analyses on the pollinator behaviour on both hermaphrodite and staminate flowers could help to propose alternative hypotheses for the origin, functional role and maintenance of staminate flowers in *S. houstonii*.

This study is the first to determine the relative siring ability of staminate flowers in *S. houstonii*. Although my results did not fully support the pollen donation hypothesis, ruling out the potential of *S. houstonii* staminate flowers to act as better pollen donors and increase pollinator attraction may be premature, as *S. houstonii* possess floral traits that have been shown to promote pollen dispersion and transfer to the stigma. However, I suggest that the role of staminate flowers could lie in other functions, such as those proposed by increased pollen receipt and the sexual interference hypothesis.

4.6 Conclusions

This study is the first investigation that test the relative capacity of staminate flowers of *S. houstonii* for pollen donation and pollinator attraction. Here, I did not find support for the hypotheses that staminate flowers sire more seeds, are more attractive to pollinators and promote better pollen deposition on the stigma. However, these results should remain tentative, as studies in other species (*Solanum carolinense*) have shown mixed support for the pollen donation and pollinator attraction hypotheses. Future work should also consider other hypotheses, such as increase pollen receipt and reduction of sexual interference. *Solanum houstonii* possesses morphological characteristics in staminate flowers (i.e. heteranthery, increased pollen amount) that suggest this species possesses a specialisation for pollen donation. Further studies analysing pollen deposition and removal using natural pollinators, as well as evidence that pollinators enhance reproductive success in natural populations, are key to evaluate comprehensively the pollen donation hypothesis.

Chapter 5

Reproductive success and pollination ecology

Chapter 5. Reproductive success and pollination biology of an andromonoecious shrub, *Solanum houstonii*, in southern Mexico

5.1 Abstract

The pollen transfer between flowers by animal pollinators is a critical event in the reproduction of most flowering plant species, affecting both the number and quality of seeds produced. Plants that use pollen as the main reward to attract floral visitors, often experience high levels of pollen theft, which can potentially have negative effects in seed production. In this study, I assessed the reproductive success of the buzz-pollinated, andromonoecious *Solanum houstonii,* a species that produces pollen as its only reward, in order to assess the extent to which the reproductive success depends on pollen and pollinator availability and to determine whether the incidence of pollen theft has a negative impact on its seed production. Firstly, I characterised the breeding system to determine the dependence of *S. houstonii* on pollinators to set fruits. Then, I quantified the incidence of pollen theft and pollinator visitation by performing 76 hours of floral observations in two natural populations. My results suggest *S. houstonii* is a self-compatible species highly dependent on pollinators to produce fruits. In natural populations, the incidence of pollen theft does not have a negative impact in seed production. However, the number of pollinator species is reduced. While pollen thieves were bees unable to buzz-pollinate, pollinators were bees able to produce high frequency vibrations and with a body size similar to the distance between stigma and anthers of hermaphrodite flowers. The incidence of pollen thieves in natural populations did not have a negative impact on the reproduction of *S. houstonii*, as pollinators were efficient in transferring pollen to the stigma for the production of seeds. In *S. houstonii* the separation of male and female sexual functions due to strong andromonoecy, coupled with a high production of pollen per flower, may allow floral visitors to steal pollen without reducing the plant's reproductive success much. The large number of staminate flowers, large amounts of pollen and floral traits that enhance pollen export and deposition (i.e. heteranthery and herkogamy) may also promote seed production in this species by improving pollinator attraction and pollen transference on pollinators' body, which increased the probability of reaching the stigma.

5.2 Introduction

Insect pollinators are essential for the reproduction of many flowering plants. More than 80% of wild plant species rely on insects, particularly bees, for fruit and seed production (Potts *et al.*, 2010; Ollerton *et al.*, 2018). However, pollinators may vary in their morphology, behaviour, frequency of floral visitation and efficiency during pollination (Wilson & Thomson, 1991; Hargreaves *et al.*, 2009). Many insects that visit flowers consume or collect pollen while providing little or no pollination service are known as pollen thieves (Inouye, 1980). In populations where pollen thieves are very abundant, plant reproductive success can be at risk (Hargreaves *et al.*, 2009; Harder & Aizen, 2010), especially in those populations in where plants depend completely on pollinators to ensure seed production (Hargreaves *et al.*, 2012).

The presence of pollen thieves in natural populations frequently cause plants experience a limitation on their seed production (Vaughton, 1996; Gross & MacKay, 1998; do Carmo *et al.*, 2004; Hargreaves *et al.*, 2010; Koski *et al.*, 2018a). Pollen limitation, the insufficient receipt of pollen, is often cause by pollinators that disperse too few pollen grains (Ashman *et al.*, 2004; Aizen & Harder, 2007). Pollen thieves can drastically reduce the amount of pollen available for fertilisation, pollen loads available for pollinators (do Carmo *et al.*, 2004) and the amount of pollen exported to stigmas (Gross & MacKay, 1998; Hargreaves *et al.*, 2010; Solís-Montero *et al.*, 2015; Koski *et al.*, 2018a). Furthermore, by stealing pollen, pollen thieves can alter the behaviour of legitimate pollinators, reducing their attractiveness to flowers or their visitation time (Gross & MacKay, 1998; Hargreaves *et al.*, 2009).

Pollen theft, thus, reduces the number of male gametes available for fertilisation, which can negatively affect siring opportunities and plant fitness (Hargreaves *et al.*, 2009). Some species often evolve anti-theft mechanisms. These mechanisms are often associated with the male function, because theft reduces siring success in particular. For example, some species increase their pollen production to compensate for losses, possess spatial or temporal separation of their sexes (i.e. monoecy, dichogamy), or possess floral adaptations that separates pollen function into feeding and pollinating, such as heteranthery (Jesson & Barrett, 2003; Hargreaves *et al.*, 2009). However, in some species that are pollen-rewarding only, such as buzz-pollinated plants, the presence of pollen thieves often leads to pollen limitation and negative impacts on fitness (Gross & MacKay, 1998; Hargreaves *et al.*, 2009). For instance, in *Melastoma* *affine*, a shrub that requires bees capable of buzzing to produce seeds, Gross and MacKay (1998) showed that the presence of bees unable to buzz caused a reduction in plant seed production. Similarly, in *Solanum rostratum,* a hermaphrodite species with heteranthery, Solis-Montero et al., (2015) found evidence of pollen limitation on natural populations and high incidence of pollen thieves. In *Campanula americana*, a species that separates female and male-function temporally (dichogamy), the presence of pollen thieves reduce the number of pollen grains on the stigma and the overall grains available for reproduction of a given population (Lau & Galloway, 2004; Koski *et al.*, 2018a). Further research is required to determine variation in these effects and to determine whether morphological adaptations for pollen production can reduce negative effects on plant reproductive success

In this study, I investigate the reproductive consequences of pollen theft on the buzz-pollinated *Solanum houstonii,* a species that produces pollen as its only reward and that possess separation of sexes into hermaphrodite and staminate flowers. *Solanum houstonii* is a perennial shrub widely distributed across Mexico characterised for possessing hermaphrodite and staminate (male) flowers within the same individual. Staminate flowers tend to bear high amounts of pollen (see Chapter 2) and the division of labour of anthers into feeding and pollinating (Papaj *et al.*, 2017), while hermaphrodite flowers have a strong separation between anther and stigma (herkogamy) that presumably reduces sexual interference and promotes pollen deposition on the stigma (see Chapter 4)*. Solanum houstonii* represents a good opportunity to investigate the reproductive consequences of pollen theft, as this species possesses traits that may promote pollinator attraction and increase siring success. Here I assess to which extent the reproduction in this species depends on pollinators, characterised the floral visits in populations from Yucatan, Mexico and determine whether the incidence of pollen theft reduces the reproductive success of *S. houstonii.* For this, I addressed the following questions: 1) does *S. houstonii* require pollinators to produce seed? 2) Are natural populations of *S. houstonii* pollen limited? 3) Which are main floral visitors? And what is their behaviour on flowers? 4) Does the presence of pollen thieves impact negatively the reproductive success of *S. houstonii*?

5.3 Methods

5.3.1 Study system

Solanum houstonii Martyn is a perennial shrub distributed in dry and humid semi-deciduous forest and disturbed across Mexico (Nee, 1993; Knapp *et al.*, 2017). This species is strongly andromonoecious, possessing in each inflorescence one basal hermaphrodite flower and up to 35 staminate (female-sterile) flowers. Flowers have five lilac or purple petals and five yellow or purple poricidal anthers, and are heterostylous, heterandrous, nectarless and markedly dimorphic (Knapp *et al.*, 2017). Hermaphrodite flowers have five straight anthers of similar size, a long style (three times longer than the anthers) and a prickly calyx. Staminate flowers are slightly larger, short styled, and are heterantherous, i.e. possess two morphologically and functionally distinct sets of anthers (Figure 1, Knapp *et al.*, 2017; Papaj *et al.*, 2017). Two short adaxial anthers provide pollen for visiting insects and function as feeding anthers and three longer, curved abaxial anthers (similar in size to the style of in hermaphrodite flowers, Knapp *et al.*, 2017), known as pollinating anthers, that presumably contribute to pollen transfer to the stigma of hermaphrodite flowers (Figure 1, Papaj *et al.*, 2017)*.* *Solanum houstonii* individuals flower all year, and especially during the rainy season between June and September. Little is known about the reproductive system and pollination biology of this species.

This study was conducted during September and October 2017 in two natural populations located in the Northwest and Centre of Yucatán, Mexico. Sierra Papacal was a population of 30 individuals found within a patch of secondary vegetation inside the facilities of the research campus Parque Científico de Yucatán (21°08'07.0"N, 89°47'04.3"W). San Isidro was a population of 35 individuals located 1Km from the locality San Isidro Ochil, in Homún, Yucatán. In this population, I found plants growing along the roadside of the highway from San Isidro Ochil to Tekit (20°37'45.9"N, 89°20'40.9"W).

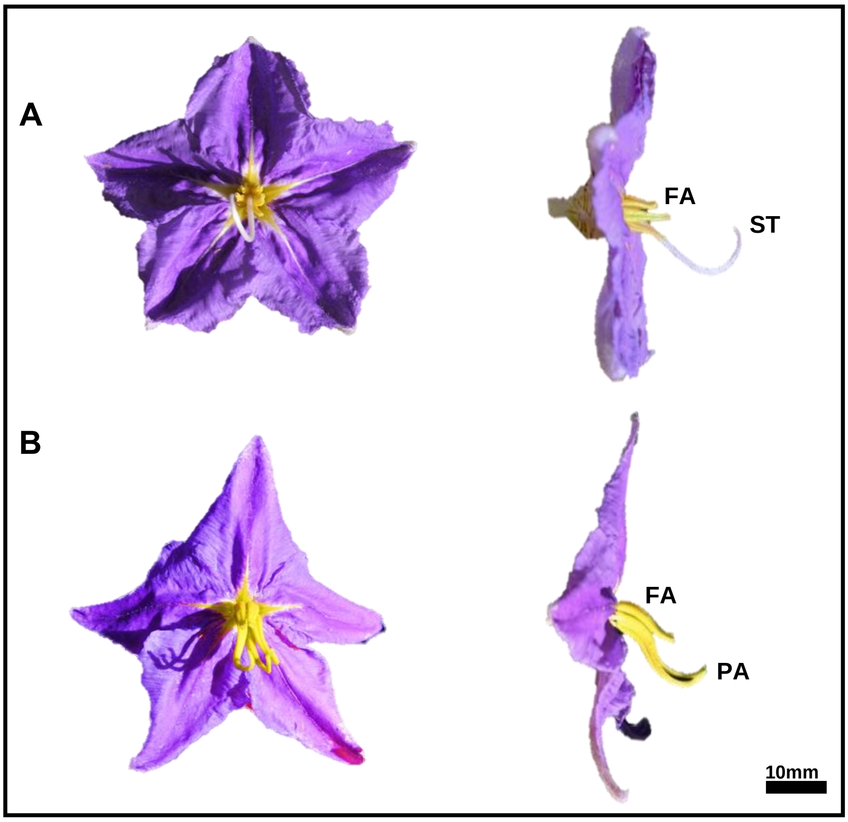


Figure 5.1. Floral morphology of hermaphrodite (A) and staminate (B) flowers of *S. houstonii*. FA-feeding anthers, PA-pollinating anthers, ST-stigma.

5.3.2 Breeding system

To determine the dependence of *S. houstonii* on pollinators I conducted open and hand-pollination treatments. I conducted two self-pollination treatments to determine self-compatibility: 1) self-pollination (SP) of hermaphrodite flowers using pollen from the same flower to hand-pollinate, and 2) geitonogamy with staminate flowers (GS), which consisted in hand-pollinations using pollen from a staminate flower within the same plant. Additionally, I conducted two cross-pollination treatments to determine the siring success of pollen of each flower (hermaphrodite and staminate): 3) cross-pollination with hermaphrodite flowers (CH), in which hermaphrodite flowers were hand-pollinated with pollen from hermaphrodite flowers from different individuals, and 4) cross-pollination with staminate flowers (CS), in which hermaphrodite flowers were hand-pollinated with pollen from staminate flowers from different individuals. I performed two additional treatments to assess the dependence of flowers on pollinators: 5) autonomous self-pollination (AP), which involved bagging flowers to exclude pollinators but to promote self-pollination, and 6) control or open pollination (C), free pollination to determine the contribution of pollinators to seed production. A final treatment to test for fruit production without fertilisation (apomixis) was conducted in a subset of hermaphrodite flowers. The apomixis test consisted in removing all anthers from a hermaphrodite flowers (emasculation) and keeping them bagged to determine whether they produce fruits spontaneously. I performed this treatment in five flowers per population and did not record the production of fruits in any case. In all treatments that required hand-pollination, pollen from all anthers of the flower sex mentioned was used; in treatments that required cross-pollination, pollen from all the anthers of at least five flowers belonging to different individuals on the population was collected and used.

All treatments were performed in San Isidro and Sierra Papacal in Yucatan, Mexico. Due to the reduced number of hermaphrodite flowers available per plant (see Chapter 3), I aimed to conduct each treatment in at least 30 flowers per population, in up to 25 different individuals. Fruit set was recorded two weeks after applying the treatments by determining whether fruit was formed (successful fertilisation) or a flower was dropped (unsuccessful fertilisation). Immature fruits were collected at the end of surveys and dried to count seed number.

5.3.3. Pollen limitation

In order to determine whether the populations were limited by pollen, I calculated the pollen limitation index (L) proposed by Larson & Barrett (2000): $L=1-(Po/Ps)$, where Po is the fruit/seed set in the open pollination (control) treatment and Ps is the fruit/seed set in the cross pollination treatment. Values of $L \leq 0$ indicate no pollen limitation. I calculated L for the average fruit set and seed set per population. To calculate Po for fruit and seed set I pooled the results of both cross-pollination treatments (cross-pollination with hermaphrodite and staminate flowers) because cross-pollination could occurred from both hermaphrodite and staminate flowers. In addition, I calculated 95% confidence intervals by bootstrapping with 1000 permutations in order to validate the pollen limitation index (L) using the *boot* package (Canty & Ripley, 2017).

5.3.4 Floral visitors, legitimacy and visitation rates

To describe the composition of floral visitors and their behaviour on flowers of *S. houstonii*, I conducted a total of 76 hours of observations in the two populations. In each population, five focal plants were marked and observed from 0830 to 1300h, in periods of 20 min. These observation intervals were established by conducting preliminary observations to determine the period of activity of floral visitors in *S. houstonii*. During each observation period I recorded the number of floral visits performed to each flower sex (hermaphrodite or staminate) by each individual visitor, the total number of flowers displayed by plants (floral display), the identity of the visitor, whether they buzzed or touched the sexual organs (FA-feeding anthers, PA-pollinating anthers, ST-stigma) and the body part they used to touch them (dorsal abdomen, ventral abdomen and thorax). Unidentified floral visitors were collected and identified in the laboratory by Humberto Moo-Valle, bee taxonomist at Universidad Autónoma de Yucatán.

To determine whether a floral visitor acted as a legitimate pollinator or a pollen thief (illegitimate visitor), I calculated legitimacy of each floral visitor based on each species behaviour on hermaphrodite flowers. I defined legitimate pollinators as those visitors capable of touching both female and male reproductive organs within flowers (stigma and anthers, respectively) during a single visit per individual. To determine a legitimate visit I used each species' average proportion of legitimate visits over the total number of visits (>50 % legitimate visits) or pollen thieves (<50% legitimate visits). I used 50% of legitimate visits as a threshold because more than 50% of all our visitors performed legitimate visits on hermaphrodite flowers. I excluded from the analysis visitors that did not perform visits to hermaphrodite flowers or contacted hermaphrodite flowers less than 10 times (see Table 5.3).

Additionally, I estimated visitation rates for both legitimate pollinators and pollen thieves to determine whether pollen thieves performed more visits than pollinators which could possibly had negative effects on *S. houstonii* seed production. Visitation rates were calculated as the total number of visits per plant per hour.

5.3.5 Statistical analyses

To determine the effect of treatments of each population on fruit and seed production of *S. houstonii*, I used a generalised mixed-effect model (GLMM), specifying a binomial distribution for fruit set, and a Poisson (or negative binomial) distribution for seed set. In each model, I used as a response variable either a binary variable of the fruit production or the number of seeds produced, treatment as fixed effect and Plant ID as a random effect. Treatment was a categorical variable with five levels: C treatment (control or open pollination), CH treatment (cross-pollination with hermaphrodite flowers), CS treatment (cross-pollination with staminate flowers), SP treatment (self-pollination) and GS treatment (geitonogamy using staminate flowers from the same individual). For each model, I used a data set that excluded autonomous self-pollination treatment (AP) because this treatment always failed to produce fruits. Additionally, to deal with overdispersion in the Poisson models I included an observation-level random effect (OLRE) where each data point receives a unique level of a random effect (Hinde, 1982). *Post hoc* Tukey comparisons were used to test statistically significant differences among treatments.

To determine the effect of floral display, type of visitor and population on the visitation rate I built GLMM. In cases when there was substantial overdispersion, I fitted alternative distributions such as negative binomial; when this still failed I used a negative binomial distribution with zero inflation and individual level random effects, using the function *glmmadmb* from the package *glmmADMB* (Skaug *et al.*, 2010). I determine the effect on visitation rate (my response variable) using a GLMM with negative binomial distribution and zero inflation, with fixed effects floral visitor type (pollinator or pollen thieve), population, floral display (total numbers of hermaphrodite and staminate flowers displayed per plant) and the interaction between floral display and type of visitor, and as random effects I included plant ID and date of observation.

To explore the visits performed by pollinators and pollen thieves in hermaphrodite and staminate flowers, I fitted two GLMM model with Poisson distribution for the visitation rate to each flower (my response variable). I used visitor type as a two level factor variable that included pollinators and pollen thieves, and population as fixed effects and the random effects plant ID and date of observation. Additionally, to control for overdispersion I included an observation-level random effect (OLRE) where each data point receives a unique level of a random effect (Hinde, 1982).

All statistical analyses were conducted using *R* software v 3.4.0 (R Development Core Team, 2013). Generalised mixed effect models (GLMM) with binomial or Poisson distribution were performed with the function *glmer* from the *lme4* package (Bates *et* *al.*, 2015) and models with negative binomial with the function *glmer.nb* from *lme4*. *Post hoc* Tukey comparisons were fitted using the *multcomp* package for *R*. Additionally, to estimate the intraclass correlation coefficient (ICC) for the random variables of each model, I used the *icc* function of the *sjstats* package for *R* (Ludecke, 2019). Models were validated by visually assessing diagnostic plots to confirm normality of residuals.

5.4 Results

5.4.1 Breeding system and pollen limitation

Solanum houstonii strongly depends on pollinators to produce fruits, as all flowers in both populations failed to produce fruits in the autonomous pollination treatment (Figure 5.2). Flowers from both self- and cross-pollination treatments successfully set fruits, suggesting *S. houstonii* is a self-compatible species. In San Isidro and Sierra Papacal, fruit set did not differ statistically among treatments (Table 5.1); however, the percentage of fruit set in San Isidro was higher than in Sierra Papacal (Figure 5.2). The number of seeds produced per fruit differed among treatments in each population. In San Isidro, fruits in the control treatment produce more seeds than the cross-pollination with hermaphrodite flowers (CH) and the geitonogamy (GS) treatments, but no more than the cross-pollination with staminate flowers (CS) and the self-pollination (SP) treatments, as I did not find significant differences (Table 5.1). Similarly, in Sierra Papacal, the fruits from the control treatment produced more fruits than all the remaining treatments except for the cross-pollination with staminate flowers treatment (CS) (Figure 5.2, Table 5.1) Furthermore, I did not find evidence of pollen limitation in fruit production or seed set in populations of *S. houstonii* surveyed (Table 5.2).

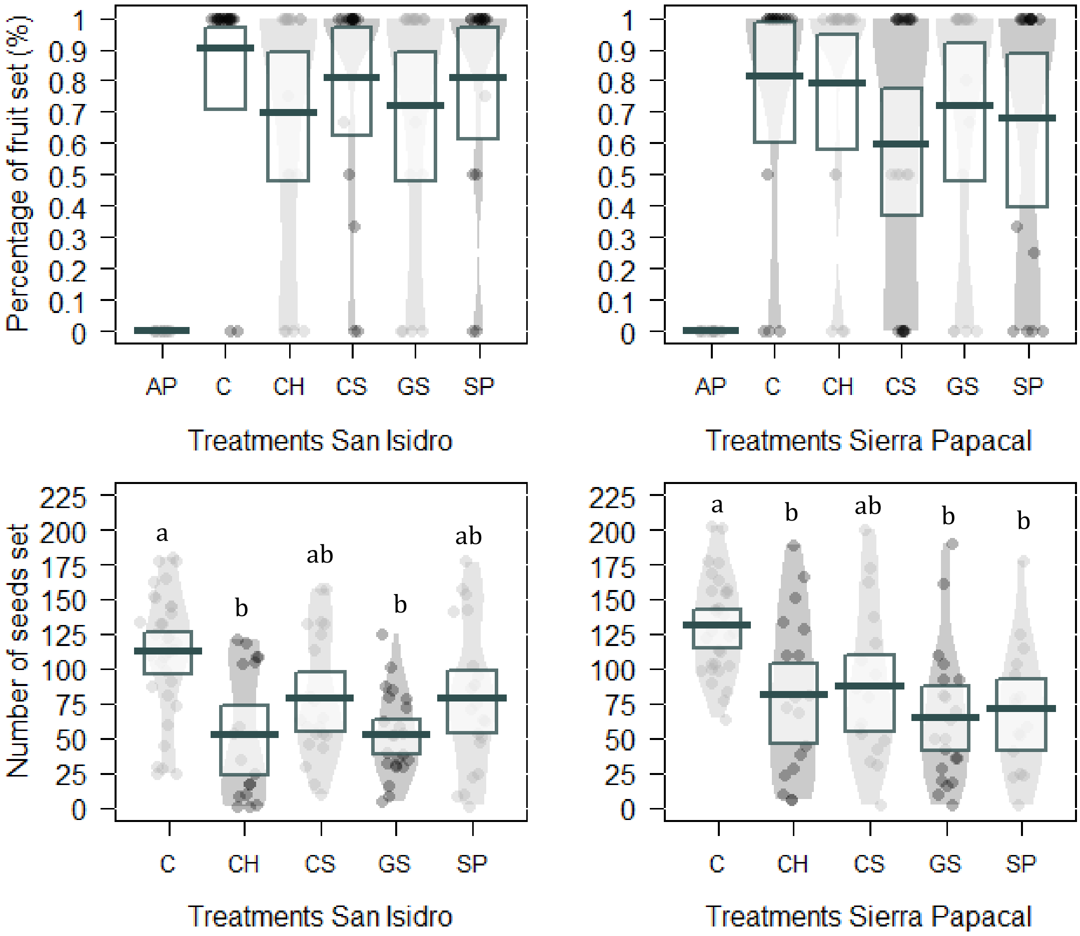


Figure 5.2. Percentages of fruit set and number of seed set per hand-pollination treatment among populations of *S. houstonii.* Different letters above treatments' name denote statistically significant pairwise differences ($p<0.05$). Note that black bars in each plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an individual fruit or seed. **OP**-open pollination, **AP**-autonomous self-pollination, **SP**-self-pollination of hermaphrodite flowers, **GS**-geitonogamous-pollination using pollen of staminate flowers, **CS**-cross-pollination using pollen of staminate flowers, **CH**-cross-pollination using pollen of hermaphrodite flowers.

Table 5.1. Model coefficients of the effects of treatment on fruit and seed production in two populations, San Isidro and Sierra Papacal, of *S. houstonii* in Yucatan. Results show the fixed effects from generalised mixed effect model with the distribution indicated.

Fruit set	**Fixed effects**	**Estimate**	**SE**	***z***	***p***
San Isidro (Binomial)	Intercept	-0.11	0.23	-0.46	0.647
	Treatment (CH)	-0.15	0.34	-0.45	0.654
	Treatment (CS)	-0.18	0.36	-0.51	0.607
	Treatment (GS)	-0.18	0.34	-0.53	0.595
	Treatment (SP)	-0.05	0.34	-0.16	0.875
	Random effects	**Variance**	**SD**	**ICC**	
	Plant ID	<0.01	<0.01	<0.01	
Sierra Papacal (Binomial)	Intercept	-0.18	0.25	-0.74	0.461
	Treatment (CH)	-0.07	0.37	-0.20	0.841
	Treatment (CS)	-0.31	0.38	-0.80	0.421
	Treatment (GS)	-0.11	0.37	-0.29	0.774
	Treatment (SP)	-0.31	0.38	-0.80	0.421
	Random effects	**Variance**	**SD**	**ICC**	
	Plant ID	<0.01	<0.01	<0.01	
Seed set	**Fixed effects**	**Estimate**	**SE**	***z***	***p***
San Isidro (Negative binomial)	Intercept	4.72	0.12	39.96	<0.001
	Treatment (CH)	-0.76	0.21	-3.61	<0.001
	Treatment (CS)	-0.35	0.20	-1.79	0.074
	Treatment (GS)	-0.76	0.19	-3.98	<0.001
	Treatment (SP)	-0.36	0.19	-1.87	0.062
	Random effects	**Variance**	**SD**	**ICC**	
	Plant ID	<0.01	<0.01	<0.01	
Sierra Papacal (Poisson)	Intercept	4.83	0.15	33.27	<0.001
	Treatment (CH)	-0.75	0.22	-3.39	<0.001
	Treatment (CS)	-0.57	0.22	-2.52	0.012
	Treatment (GS)	-1.01	0.22	-4.70	<0.001
	Treatment (SP)	-0.86	0.23	-3.68	<0.001
	Random effects	**Variance**	**SD**	**ICC**	
	Plant ID	0.08	0.28	0.13	
	OLRE	0.49	0.70	0.85	

Table 5.2. Fruit set (percentage of flowers maturing into fruits), seed set (mean number of seeds) and pollen limitation index (PL) from two populations of *S. houstonii* in Yucatan, Mexico.

		San Isidro		**Sierra Papacal**	
		Fruit set	Seed set	Fruit set	Seed set
Pollen supplementation	P_s	81.67	73.7(±9.8)	69.35	84.4(±13.9)
Open pollination	P_o	90.40	111.9(±7.2)	81.50	130.9(±6.50)
Pollen limitation index	L	-0.14(±0.003)	-0.63(±0.008)	-0.24(±0.005)	-0.62(±0.007)

Flowers were exposed to natural pollination conditions either with (pollen supplementation treatment (P_s), using hermaphrodites (CH) or staminates (CS) as pollen donors, both values were pooled) or without (P_o, open pollination) addition of supplemental outcross-pollen. The mean value of the pollen limitation index (L) was calculated for each population for fruit set and seed set using the formulas provided by Larson & Barrett (2000); the means and standard errors (generated by bootstrapping analysis with 1000 permutations) are reported.

5.4.2 Floral visitors

I recorded on average 52.5±7.4 and 57.4±6.8 visits per plant per hour in San Isidro and Sierra Papacal, respectively. Hymenopterans were the group with the highest number of species and percentage of visitation. (98% of visitation), but species composition varied among San Isidro and Sierra Papacal populations (Figure 5.3). I observed insects from other orders, such as Diptera, Coleoptera, Lepidoptera and Orthoptera, but with very low visitation percentage. Dipterans were observed on the anthers pores occasionally collecting pollen, but we never observed them contacting the stigma. Orders such Coleoptera and Orthoptera were observed eating some parts of the flower, and Lepidotera, was always observed landing on the corolla and elongating their proboscides for nectar probing. I focused on bees as the main floral visitors, as they were the most abundant and the only ones able to extract pollen and contact the reproductive organs during visits on *S. houstonii* flowers.

Species composition of bees differed between San Isidro and Sierra Papacal populations. In San Isidro, I observed eight species of bees, five of which were bees able to vibrate *S. houstonii* anthers (see Table 5.4). The most abundant species visiting *S. houstonii* flowers was *Centris analis* and the rarest *Trigona fulviventris.* Although I observed only one individual of *T. fulviventris* during my study, this species was very abundant on the site and was observed performing visits to other individuals of *S. houstonii*. In Sierra Papacal, I also observed eight species of bees, but only four were bees able to buzz (Table 5.4). The most abundant visitor in Sierra Papacal was *Augochloropsis metallica* and the rarest *Trigonisca maya*. In both populations, I observed two additional species of bees vibrating *S. houstonii* anthers. However, they were outside of my observation periods, and therefore, they were not included in our analysis or counts. These species were *Eulaema polycroma* and *Xylocopa muscaria* for Sierra Papacal and only the latter for San Isidro.

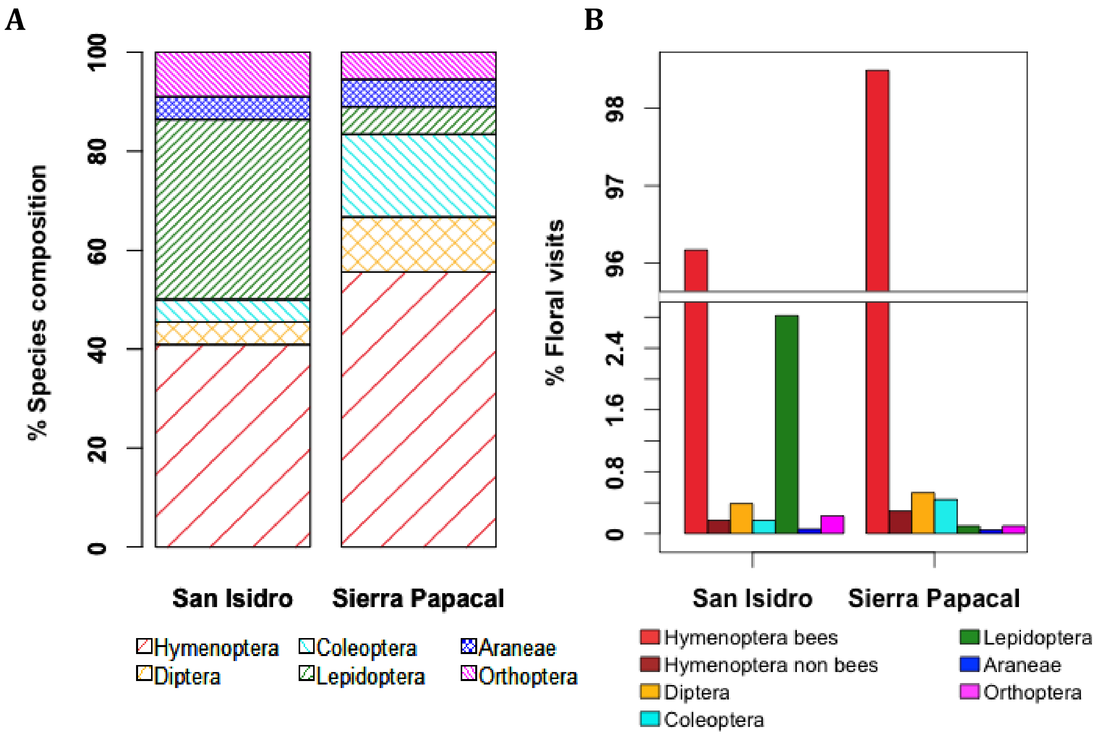


Figure 5.3. Relative composition of floral visitor families (A) and percentage of floral visits performed by each type of visitor family (B) in two populations surveyed in Yucatan, Mexico.

5.4.3 Pollinators and pollen thieves

From the twelve bees identified in this study, only *Xylocopa mexicanorum, Centris analis* and *Euglossa viridissima* were considered legitimate pollinators because more than 50% of the individuals were able to contact stigmas and anthers of *S. houstonii* hermaphrodite flowers (Table 5.4). Another set of bees that did not touch the stigma but performed more than ten floral visits to hermaphrodite and staminate flowers were considered pollen thieves (Figure 5.4). Pollen thieves included one anther-buzzing bee, *Augochloropsis metallica* and two non-buzzing bees, *Nannotrigona perilampoides* and *Trigona nigra* (Table 5.3). The remaining visitors were considered as occasional visitors to *S. houstonii* flowers (Figure 5.4). These bees performed none or less than ten visits to hermaphrodite flowers and varied in their behaviour. Two were bees capable of buzzing the whole flower, *Eulaema polychroma* (observed during non-observation periods only), or single anthers, *Melissodes baileyi*, and the remaining non-buzzing *Apis mellifera, Plebeia frontalis, Trigona fulviventris* and *Trigonisca maya* (Table 5.4).

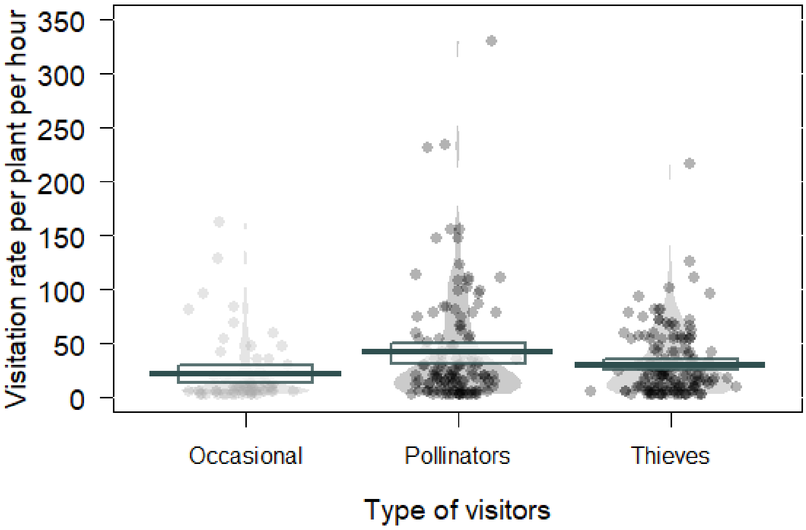


Figure 5.4. Visitation rate of the different types of floral visitors of *S. houstonii* in two natural populations in Yucatan. Visitation rate is expressed as the number of floral visits performed per plant per hour. Black bars in plot indicate mean, boxes the 95% confidence interval on the mean, shaded areas are density and each individual point is an observation from a single plant.

Table 5.3. Identity and characteristics of floral visitors of *Solanum houstonii* in two populations of Mexico. B-buzzing, H-hovering, He-herbivore, NP-nectar probing, OV-occasional visitor, PF-pollen forager, SP-scrabbling pollen.

Visitor	**Number of individuals**	**% Buzzing**	**Floral visits**	**% Floral visits**	**% Visits with buzz**	**Thorax width (mm)**	**Behaviour observed on flowers**
San Isidro							
Aranae							
Thomisidae 1	1		1	0.06	0		OV
Coleoptera							
Leptinotarsa sp.	3		3	0.17	0		He
Diptera							
Ornidia obesa	7		7	0.39	0	4.35	H/PF
Hymenoptera							
Apis mellifera	3	0	4	0.23	0		PF
Augochloropsis metallica	23	95.65	201	11.32	87.56	2.75	B/PF
Centris analis	98	97.96	982	55.29	96.13	3.99	B/PF
Euglossa viridissima	10	90.00	55	3.10	87.27	3.59	B/PF
Melissodes baileyi	30	80.00	131	7.38	87.79	3.79	SP/PF
Plebeia frontalis	64	0	316	17.79	0	1.35	SP/PF
Trigona fulviventris	1	0	2	0.11	0	1.91	SP/PF
Vespidae	3		3	0.17	0		OV
Xylocopa mexicanorum	2	100	17	0.96	100	8.55	B/PF
Lepidoptera							
Lepidoptera 1	3		4	0.23	0		NP
Lepidoptera 2	3		3	0.17	0		NP
Lepidoptera 3	7		11	0.62	0		NP
Lepidoptera 4	2		3	0.17	0		NP
Lepidoptera 5	11		13	0.73	0		NP
Lepidoptera 6	9		10	0.56	0		NP
Lepidoptera 7	2		2	0.11	0		NP
Lepidoptera 8	3		4	0.23	0		NP
Orthoptera							
Orthoptera 1	3		3	0.17	0		He
Orthoptera 3	1		1	0.06	0		He
Total	289		1776				
Sierra Papacal							
Aranae							
Thomisidae 2	1		1	0.05	0		OV
Coleoptera							
Curculionidae 1	5		5	0.24	0		He
Curculionidae 2	2		2	0.10	0		He
Leptinotarsa sp.	2		2	0.10	0		He
Diptera							
Ornidia obesa	4		4	0.20	0	4.35	H/PF
Syrphidae	5		7	0.34	0	4.09	H/PF
Hymenoptera							
Augochloropsis metallica	94	97.87	578	28.22	93.43	2.75	B/PF
Crematogaster sp.	2		2	0.10	0		NP

Euglossa viridissima	76	94.74	579	28.27	91.88	3.59	B/PF
Eulaema polycroma	1	0	1	0.05	0	7.41	PF
Melissodes baileyi	1	100	2	0.10	100	3.79	B/PF
Nannotrigona perilampoides	46	0	294	14.36	0	1.46	SP/PF
Polybia sp.	1		1	0.05	0		
Trigona nigra	39	0	426	20.80	0	1.83	SP/PF
Trigonisca maya	2	0	5	0.24	0	0.94	SP/PF
Xylocopa mexicanorum	28	100	135	6.59	98.52	8.55	B/PF
Lepidoptera							
Lepidoptera 3	1		2	0.10	0		NP
Orthoptera							
Orthoptera 2	2		2	0.10	0		HE
Total	312		2048		0		

Table 5.4. Classification of the pollinators and pollen thieves in *S. houstonii* natural populations. Legitimate pollinators were determined based on visits performed on hermaphrodite flowers only. If a visitor contacted both stigma and feeding anthers (FA, ST) more than 50% of the total number of visits, it was considered pollinator. Pollen thieves were visitors that touched the stigma and feeding anthers less than 50% of the visits. Occasional visitors were bees that performed less than 10 floral visits during our observation periods that could not be classified as pollinators or pollen thieves.

Visitor ID	**Individuals contacting hermaphrodite flowers**						**Individuals contacting staminate flowers**						**% Bees buzzing**	**Total bees**	**Body length**
	FA	FA, ST	Only petals	Total bees contacting	Total floral visits	% Bees contacting ST	FA	PA	FA, PA	Only petals	Total bees contacting	Total floral visits			
Pollinators															
Xylocopa mexicanorum	0	10	1	11	13	90.91	0	0	26	2	28	139	100	30	19.8±0.1
Centris analis	9	23	0	32	42	71.88	4	3	57	27	91	940	97.96	98	10.9±0.1
Euglossa viridissima	7	10	0	17	34	58.82	11	0	55	18	84	600	94.19	86	12.4±0.2
Total	16	43	1	50	89		15	3	138	47	203	1679		214	
Pollen thieves															
Augochloropsis metallica	12	5	0	17	19	29.41	10	15	74	13	112	760	97.44	117	8.6±0.3
Nannotrigona perilampoides	8	2	0	10	28	20	5	3	25	12	45	266	0	46	5.2±0.1
Trigona nigra	11	1	2	14	40	7.14	3	5	14	19	39	386	0	39	6.1±0.1
Total	31	8	2	41	87		18	23	113	44	196	1412		202	
Occasional visitors															
Apis mellifera	-	-	-	0	0	-	0	0	0	3	3	4	0	3	10.0±0.10

Table 5.4. Continued.

Visitor ID	**Individuals contacting hermaphrodite flowers**						**Individuals contacting staminate flowers**						**% Bees buzzing**	**Total bees**	**Body length**
	FA	FA, ST	Only petals	Total bees contacting	Total floral visits	% Bees contacting ST	FA	PA	FA, PA	Only petals	Total bees contacting	Total floral visits			
*Eulaema polycroma**	-	-	-	0	0	-	0	0	0	1	1	1	0	1	19.9±0.26
Melissodes baileyi	1	1	0	2	3	-	4	7	18	1	30	130	90.32	31	10.2±0.27
Plebeia frontalis	1	4	1	6	8	-	7	9	41	3	60	30	0	64	4.3±0.19
Trigona fulviventris	-	-	-	0	0	-	0	1	0	0	1	2	0	1	7.7±0.15
Trigonisca maya	-	-	-	0	0	-	0	0	2	0	2	4	0	2	2.0±0.06
Total	2	5	3	8	11		11	17	61	8	97	171		102	

*Observed buzzing flowers of *S. houstonii*, but never during visiting period

5.4.4 Behaviour and morphology of bees

Morphology and behaviour of pollinators and pollen thieves varied. Body size of bees observed in this study varied from 2 to 19.9 mm (Figure 5.5, Table 5.4). I found that bee morphology was an important characteristic to categorise bees in pollen thieves or pollinators, as bees able to buzz and that were larger than the distance between the anthers and stigma of hermaphrodite flowers contacted more times both anthers and stigma (Figure 5.5). Additionally, I observed pollinators exclusively buzzed feeding anthers of both hermaphrodite and staminate flowers and contacted both pollinating anthers and stigma with the same body part. For instance, during buzzing of staminate and hermaphrodite flowers *Centris analis* and *Euglossa viridissima* were able to contact pollinating anthers and stigma with the dorsal side of the abdomen, and during each floral visit *Xylocopa mexicanorum* positioned its body over both feeding and pollinating anthers of staminate flowers or over anthers and stigma in hermaphrodite flowers (Figure 5.6a-c).

Pollen thieves had a body size shorter than the distance between anthers and stigma. They were never observed contacting anthers and stigma at the same time. In the case of *A. metallica*, the only buzzing species observed among pollen thieves, bees that contacted the stigma did it by mistake, because they were observed buzzing the stigma in a similar form they buzzed pollinating anthers of staminate flowers (Figure 5.6d). The rest of pollen thieves never buzzed but collected pollen from open anther pores (Figure 5.6e-f).

In the case of *Eulaema polychroma*, one of the occasional visitors, I did not record buzzing during my observation periods, but I was able to observe individuals buzzing other flowers of *S. houstonii* and contacting both pollinating anthers and stigma with its ventral side of abdomen and thorax (Figure 5.6k). *Melissodes baileyi* and *Plebeia frontalis,* which sometimes contacted stigmas (Figure 5.6g-h), were very infrequent visitors of hermaphrodite flowers but frequent visitors of staminate flowers. During visitation, *M. baileyi* had a similar behaviour to *A. metallica*, while *P. frontalis* had similar behaviour to the other non-buzzing pollen thieves. The remaining visitors, *Apis mellifera*, *Trigona fulviventris* and *Trigonisca maya,* were not observed visiting hermaphrodite flowers. However, they were frequent visitors of staminate flowers and were collecting pollen from anthers in a similar way to the other pollen thieves, with the exception of *T. fulviventris*, which was observed piercing *S. houstonii* anthers to extract pollen (Figure 5.6i-j).

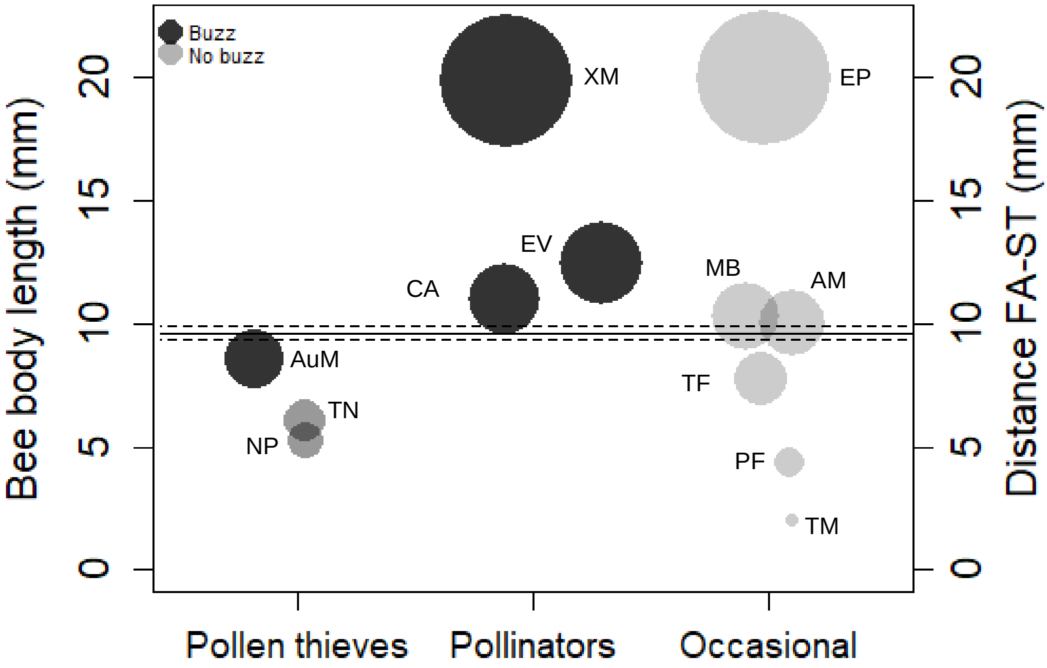


Figure 5.5. Body size of all bees observed visiting *S. houstonii* in natural populations. Line delimits mean and standard error of distance between feeding anthers and stigma of hermaphrodite flowers. Dot size corresponds to body size of each floral visitor (see Table 5.4 for detailed sizes). **AM**-*Apis mellifera*, **AuM**-*Augochloropsis metallica*, **CA**-*Centris analis*, **EP**-*Eulaema polychroma*, **EV**-*Euglossa viridissima*, **MB**-*Melissodes baileyi*, **NP**-*Nannotrigona perilampoides*, **TF**-*Trigona fulviventris*, **TM**-*Trigonisca maya*, **TN**-*Trigona nigra*, **PF**-*Plebeia frontalis*, **XM**-*Xylocopa mexicanorum*.

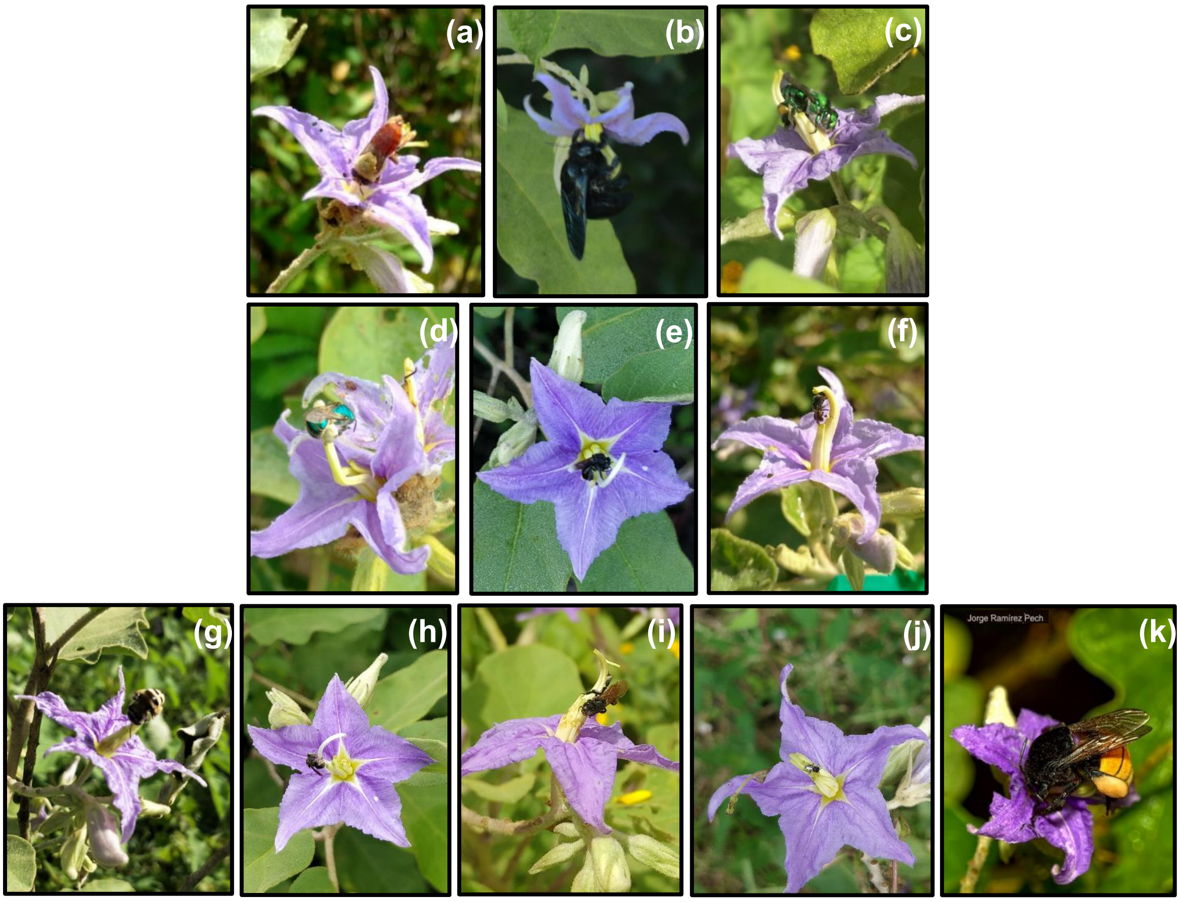


Figure 5.6. Floral visitors in *S. houstonii* flowers. **a**) *Centris analis,* **b**) *Xylocopa mexicanorum,* **c**) *Euglossa viridissima* were classified as legitimate pollinators. **d**) *Augochloropsis metallic*a, **e**) *Trigona nigra* and **d**) *Nannotrigona perilampoides* were classified as pollen thieves. **g**) *Melissodes baileyi*, **h**) *Plebeia frontalis*, **i**) *Trigona fulviventris*, **j**) *Trigonisca maya* and **k**) *Eulaema polychroma* were classified as occasional visitors. Pictures taken by AKZC and Jorge Ramirez-Pech.

5.4.5 Visitation rates of pollinators and pollen thieves

Overall legitimate pollinators performed 54% of the total floral visits observed in this study and pollen thieves performed 46%. The mean number of visits per hour per plant by pollinators was 47 ($CI_{95\%}$=35,56) and by pollen thieves 45 ($CI_{95\%}$=35,55). Visitation rate did not differ significantly between pollinators and pollen thieves, however, visitation rate differed among populations (Table 5.5). In Sierra Papacal, I recorded more floral visits accounting for 61% of the total visits observed in this study. In this population, pollen thieves performed slightly more visits (65%), and only *X. mexicanorum* and *Euglossa viridissima* were legitimate pollinators (Figure 5.7). In contrast, in San Isidro, the three main pollinators for *S. houstonii, C. analis*, *E. viridisima* and *X. mexicanarum,* performed the majority of floral visits for the population (84%) (Figure 5.7).

Pollinators and pollen thieves did not differ in their visitation rate on hermaphrodites or staminate flowers (Figure 5.8, Table 5.5). For both populations, the mean (±SE) number of visits per plant per hour performed by pollinators was 2±0.3 and by pollen thieves was 2±0.4. The mean number of visits performed per plant per hour on staminate flowers was 40±4 for pollinators and 30 ±2 for pollen thieves (Figure 5.8).

The mean number of flowers displayed per plant was 30±1, of which 2±0.1 were hermaphrodite and 28±1 were staminate flowers. Floral display had a positive effect on the overall visitation rate (Figure 5.9, Table 5.5).

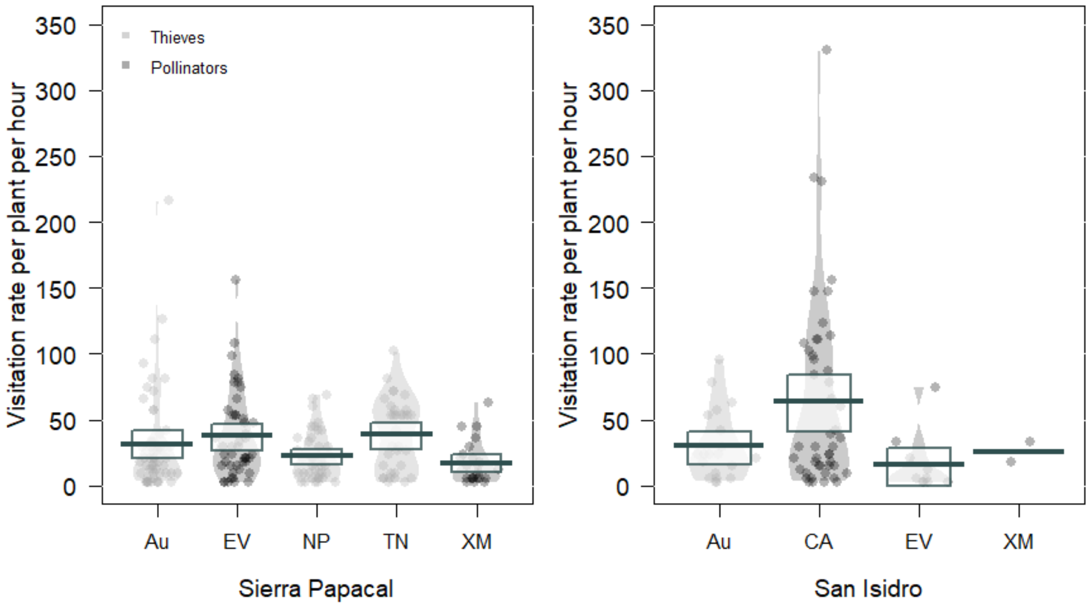


Figure 5.7 Visitation rate of pollinators and pollen thieves of *S. houstonii* in populations of Yucatan. Figures show visits per plant per hour performed in populations: San Isidro and Sierra Papacal. **Au**- *Augochloropsis metallica*, **CA**- *Centris analis*, **EV**- *Euglossa viridissima*, **NP**- *Nannotrigona perilampoides*, **TN**- *Trigona nigra*, **XM**- *Xylocopa mexicanorum*.

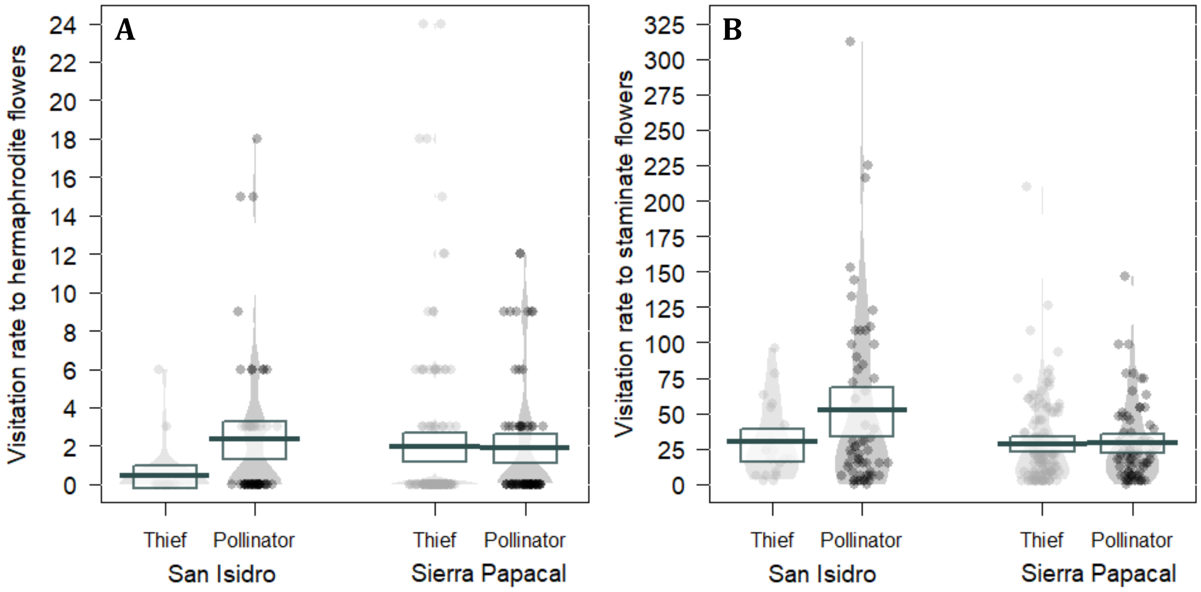


Figure 5.8. Visitation rate on hermaphrodite (A) and staminate (B) flowers performed by legitimate pollinators and pollen thieves in populations San Isidro and Sierra Papacal, Yucatan, Mexico.

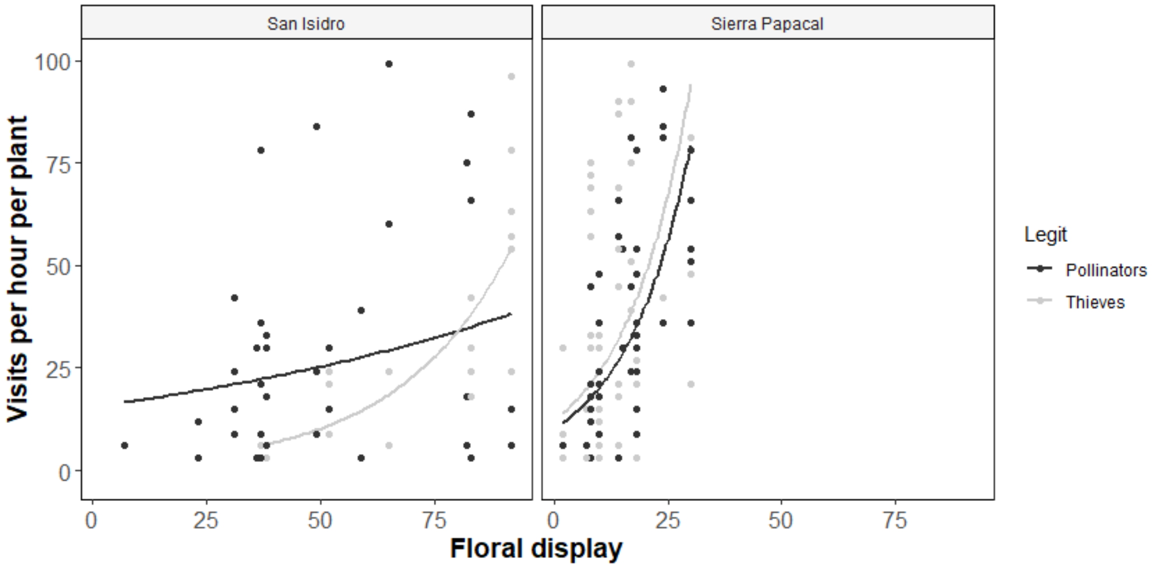


Figure 5.9. Effect of floral display on the visits per plant per hour (visitation rate) of pollen thieves and pollinators of populations in Yucatan. Predicted lines derive from the negative binomial model (Table 5.5).

Table 5.5. Model coefficients for the effect of floral display, type of floral visitor (pollinator or pollen thieve) and population (San Isidro and Sierra Papacal) on the visits performed per plant per hour (visitation rate) on hermaphrodite and staminate flowers. Results show the fixed effects from generalised mixed effect model with the distribution indicated.

	Fixed effects	**Estimate**	**SE**	***z***	***p***
Visitation rate (Negative binomial)	Intercept	2.02	0.46	4.42	<0.001
	Type of visitor (pollinator)	-0.34	0.20	-1.69	0.092
	Population (Sierra Papacal)	1.04	0.44	2.39	0.017
	Floral display	0.03	0.01	3.35	<0.001
	Type of visitor (pollinator)*Floral display	0.02	0.01	3.06	0.002
	Random effects	**Variance**	**SD**		
	Plant ID	0.18	0.45		
	Date	0.04	0.20		
	Fixed effects	**Estimate**	**SE**	***z***	***p***
Visits on hermaphrodite flowers (Poisson)	Intercept	-3.86	0.93	-4.16	<0.001
	Type of visitor (pollinators)	1.07	0.57	1.86	0.063
	Population (Sierra Papacal)	0.09	0.64	1.14	0.998
	Random effects	**Variance**	**SD**	**ICC**	
	Plant ID	<0.01	<0.01	0.00	
	Date	<0.01	<0.01	0.00	
	OLRE	0.16	3.98	0.81	
	Fixed effects	**Estimate**	**SE**	***z***	***p***
Visits on staminate flowers (Poisson)	Intercept	2.89	0.34	8.56	<0.001
	Type of visitor (pollinators)	0.08	0.14	0.60	0.551
	Population (Sierra Papacal)	-0.19	0.43	-0.44	0.662
	Random effects	**Variance**	**SD**	**ICC**	
	Plant ID	0.32	0.57	0.24	
	Date	0.08	0.29	0.06	
	OLRE	0.88	0.94	0.66	

5.5 Discussion

5.5.1 Behaviour and morphology of pollinators and pollen thieves on Solanum houstonii flowers

In this study, I classified floral visitors of *S. houstonii* into legitimate pollinators or pollen thieves depending whether they touched the reproductive organs of hermaphrodite flowers. My results suggest that bee body size and bee ability to produce high frequency vibrations were characteristics that separated pollinators from pollen thieves. For instance, I found pollinators were large body bees capable of buzzing feeding anthers and contacting the stigma of hermaphrodite flowers or contacting pollinating anthers of staminate flowers with the same body part. In contrast, I found pollen thieves had a smaller body size and varied in their ability to buzz. These differences in body size are likely to affect their ability to contact sexual organs during visitation. A bee with a larger body size is likely to contact female and male organs and to transfer pollen more efficiently. In species with a strong spatial separation of female and male organs (herkogamy), the presence of pollinators of a similar or larger size as this separation is important to ensure pollen transference. For example, some studies have demonstrated that an optimal match between floral visitors and flowers maximizes plant fitness and improve pollination accuracy during pollen deposition on the stigma (Kuriya *et al.*, 2015; Solís-Montero & Vallejo-Marín, 2017). Although in this study I did not measure pollinators' efficiency for pollen transfer, I registered a high fruit and seed production in the open pollination treatment in both populations (80-90%). Suggesting, pollinators are likely to perform efficient pollination services. Other studies in *Solanum* have found that similar species of pollinators to those found here are capable of producing high frequency vibrations to release large amounts of pollen while contacting the stigma and to trigger fruit production (Liu & Pemberton, 2009; Solís-Montero *et al.*, 2015; Solís-Montero & Vallejo-Marín, 2017).

In natural populations of *S. houstonii*, pollen thieves performed a little less than half of the floral visits. I registered three species of bees acting as pollen thieves; however, one was capable of buzzing single anthers of hermaphrodite and staminate flowers and performing more than half of the total visits on staminate flowers. Although these bees contacted the stigmas, their body size did not match with *S. houstonii's* floral morphology. I also found a subset of bees were occasional visitors of *S. houstonii flowers*, specifically to staminate flowers. These bees, however, were possibly also pollen thieves as they had a small body size and were observed collecting pollen from staminate flowers.

5.5.2 Consequences of pollen theft

In plants that require buzz-pollination, pollen theft is very common and can be reproductively costly (Gross & MacKay, 1998; Solís-Montero *et al.*, 2015; Caro *et al.*, 2017; Staines *et al.*, 2017; Rego *et al.*, 2018), because pollen thieves reduce the number of grains available for reproduction (i.e. removing them from stigmas) or because the presence of pollen thieves reduces the visitation rate of legitimate pollinators (Gross & MacKay, 1998; Hargreaves *et al.*, 2009). In this study with the andromonoecious *S. houstonii,* I did not find differences in the visitation rate between pollinators and pollen thieves, but I found a high number of floral visits performed by bees acting as pollen thieves. In fact, in one of the populations, visits by pollen thieves outnumbered those of legitimate pollinators. Despite this incidence of pollen theft, *S. houstonii*, I did not detect pollen limitation. The presence of efficient pollinators can sometimes outbalance the incidence of pollen thieves. In *Campanula americana*, for example, plant fitness, measured as seed set, was only affected when pollinators' visits were reduced. However, when pollen thieves and pollinators were abundant, plant fitness was unaffected (Lau & Galloway, 2004).

Some studies have found that pollen theft can also have negative impacts on male fitness (i.e. pollen siring success), especially for plants that exhibit temporal and spatial separation of sexes (Lau & Galloway, 2004; Case & Ashman, 2009; Hargreaves *et al.*, 2012; Koski *et al.*, 2018a). For instance, in species with temporal separation of sexes in female or male-phases, pollen thieves prefer visiting the male-phase and remove greater amounts of pollen than when they visit flowers in the female-phase (Lau & Galloway, 2004; Koski *et al.*, 2018a). In some cases, overall plant reproductive success is not affected as the production of pollen overpasses the number of ovules available to fertilise, however, in other cases, exploitation is so high that both male and female fitness are affected (Hargreaves *et al.*, 2009). *Solanum houstonii* is a species with separation of sexes into staminate and hermaphrodite flowers. In my results, high visitation rate of pollen thieves did not impose a negative effect in the female reproductive success; however, the effect on male-fitness was not very clear. I should note that in my study, I did not measure the effect of pollen theft on the male reproductive success directly, such as the relative number of pollen grains removed by pollen thieves from anthers or stigma. Instead, I measured the visitation rate performed to hermaphrodite and staminate flowers and found pollen thieves visits were not significantly rarer than visits by legitimate pollinators. However, there were other visitors, which were not included in the analysis due to the low proportion of visits, that were also observed collecting pollen and could negatively impact siring success. In any case, *S. houstonii* produces a high proportion of staminate flowers that probably has evolved as a strategy to produce high amounts of pollen to ensure pollen reaches the stigma.

In buzz-pollinated species that rely on pollen as reward, developing anti-theft mechanisms for the male function is essential to ensure siring success. High production of pollen, physical concealment of pollen and floral traits that promote a better transference of pollen to the stigma are anti-theft mechanisms that have evolved to compensate for pollen losses (Hargreaves *et al.*, 2009)*. Solanum houstonii* possesses a relatively high proportion of staminate flowers (see Chapter 2) and anther dimorphism in staminate flowers, both floral traits that possibly ensures fruit and seed production even in the presence of pollen thieves. For instance, the presence of heteranthery in staminate flowers may enhance pollen exportation towards the stigma, by improving pollen deposition on the pollinators' bodies. The division of labour hypothesis that explains the evolutionary significance of anther dimorphism suggests that the presence of two distinct sets of anthers reduces the conflict of the double function of pollen, as pollinators food and as gametes for fertilisation (Müller, 1882; Vallejo-Marín *et al.*, 2010; Mesquita-Neto *et al.*, 2017). In *S. houstonii*, the presence of large amounts of pollen and division of labour among anthers seems to be designed to ensure pollination and maintain reproductive success.

5.5.3 Relevance andromonoecy and floral morphology on reproductive success

In this study, I found fruit and seed set are highly dependent on pollinator availability, and in the two populations surveyed, female reproductive success is not limited by pollinators availability or pollen receipt. These results suggest this species strategy is highly effective to ensure seed production. *Solanum houstonii* is a species characterised by a relatively high proportion of staminate flowers (strongly andromonoecious) and for bearing flowers that are morphologically different in style and anther length (see Chapter 2 and 3). In other species, similar floral traits are considered strategies to promote pollinator attraction, pollen export, increase pollen receipt and/or reduce sexual interference between male and female function (Webb & Lloyd, 1986; Barrett, 2002b; Armbruster *et al.*, 2014; Mesquita-Neto *et al.*, 2017; Solís-Montero & Vallejo-Marín, 2017).

In this study, where I found many floral visitors collecting/stealing pollen, the female reproductive success was not impacted negatively, perhaps due to the great production of pollen and the efficient behaviour of pollinators. In some andromonoecious species, pollinators tend to prefer visiting staminate flowers because they possess large amounts of pollen (reward) (Whalen & Costich, 1986; Quesada-Aguilar *et al.*, 2008; Calviño *et al.*, 2014). For instance, some studies have shown a positive association between the relative high proportion of staminate flowers (maleness) and the frequency of pollinator visitation (Elle & Meagher, 2000; Calviño *et al.*, 2014). Pollinator visitation in staminate flowers may enhance female fitness as it increases the chances of pollen built up in pollinators' bodies and thus pollen be deposited in the stigma (Whalen & Costich, 1986; Quesada-Aguilar *et al.*, 2008). In the surveyed populations of *S. houstonii*, pollinators were very abundant and my results suggest they pollinate efficiently as fruit and seed set is relative high. Pollinators of *S. houstonii* usually are bees of large size, able to buzz the poricidal anthers of the flowers, and to touch the pollinating anthers and the stigma with the same body part. The efficiency during pollination, by these bees, may be achieve due to few factors: 1) a great floral display increases the chances of visitation, 2) the presence of sufficient pollen to ensure pollen export and deposition in the stigma, and 3) behaviour and bee morphology in both hermaphrodite and staminate flowers is similar. Whether native pollinators differ in their efficiency in transferring pollen to the stigma remains to be tested; however, the results from this study suggest the bee species observed in these populations play an important role in the female reproductive success of *S. houstonii*.

Other possible factors preventing decreased female reproductive success in the presence of pollen thieves are the morphological adaptations of hermaphrodite and staminate flowers. Staminate flowers are characterised for possessing heteranthery, the functional and morphological separation of anthers into feeding and pollinating (Cardoso *et al.*, 2018). Heteranthery is widely spread among angiosperms and is presumably a morphological adaptation that promotes the division of labour of anthers (into feeding and pollinating types) and enhances pollen export (Müller, 1882; Vallejo-Marín *et al.*, 2009; Mesquita-Neto *et al.*, 2017). Experimental work on heterantherous species has demonstrated that pollen exported from pollinating anthers is more likely to be transferred to stigmas, if a correspondence between pollen placement on pollinators body and stigma contact exist (Jesson & Barrett, 2003; Luo *et al.*, 2009; Vallejo-Marín *et al.*, 2010; Solís-Montero & Vallejo-Marín, 2017). For instance, in *Senna reniformis* and *Melastoma malabathricum*, there is a greater probability that pollen from pollinating anthers than from feeding anthers reaches the stigma mediated by the placement of pollen in pollinators body (Luo *et al.*, 2008; Mesquita-Neto *et al.*, 2017). In *S. houstonii*, heteranthery plays an important role on pollen export, as I found pollinators touched pollinating anthers and stigmas with the same body parts. During pollen extraction in staminate flowers, pollinators hold feeding anthers and started making vibrations to extract pollen, causing pollen from feeding anthers to be deposited on their ventral abdomen while pollen from pollinating anthers is deposited on their back. During visitation of hermaphrodite flowers, pollinators take a similar position to extract pollen and touch the stigma with their back. These behavioural observations suggest heteranthery in *S. houstonii* promotes pollen dispersion and deposition on the stigma. Moreover, the morphology of hermaphrodite flowers also plays an important role in the way pollen is deposited in the stigmas, as it seems to improve the pollinators' body placement on the flower during pollen extraction. Hermaphrodite flowers possess herkogamy, which is the separation of anthers and stigma, increases pollination accuracy and reduces interference between the male and female function (Webb & Lloyd, 1986; Barrett, 2002b; Armbruster *et al.*, 2014). For example, Armbruster *et al*. (2014) demonstrated spatial correspondence between anthers and stigma among flowers increases the accuracy and precision of pollinators pollen removal and deposition. In their study, they used *Parnassia epunctulata*, a species that besides herkogamy possess dichogamy, the temporal separation of female and male function, two floral characteristics that could limit plants reproductive success in the absence of accurate pollinators. The fact Armbruster *et al*. (2014) found high levels of pollinator accuracy demonstrated that systems with separate female and male function and herkogamy could benefit from efficient pollinators. In any case, herkogamy and heteranthery in *S. houstonii* could be a strategy to reduce interference between pollen removal and pollen receipt, floral traits that can enhance reproductive success in the presence of efficient pollinators.

5.6 Conclusions

In this study, I characterised the reproductive system and identified the main floral visitors of *S. houstonii* for the first time. I demonstrated that *S. houstonii* depends on pollinators to produce fruits, highlighting the role of pollinators in buzz-pollinated systems. In addition, I identified three species of bees acting as pollinators and three species of bees acting as pollen thieves. Overall, pollen thieves and pollinators did not differ in their visitation rate, but in one of the populations, pollen thieves had a higher visitation rate. Despite the incidence of pollen thieves, plant fitness was not negatively affected in any of the populations, as I did not find evidence of pollen limitation. The species I identified as pollen thieves were bees of small size unable to contact anthers and stigma at the same time. I found that bee body size and bee ability to produce high frequency vibrations were important characteristics to determine the legitimacy of pollinators in *S. houstonii*. These results highlight the role of andromonoecy and the floral adaptations exhibited by this species. The relatively high proportion of staminate flowers may function as a strategy to increase the pollen availability in natural populations which 1) reduces the effect of pollen theft and 2) maximises pollen deposition on the stigmas. Moreover, the separation between anthers and stigma in hermaphrodite flowers seems to function to reduce self-pollination and enhance pollen deposition from staminate flowers. Heteranthery in staminate flowers improves pollen export by depositing pollen on pollinator body, which maximises the chances of pollen reaching the stigma. Overall, the reproductive system of *S. houstonii* may reduce sexual interference and increase the probability of pollen of reaching the stigma.

Chapter 6

General discussion

Chapter 6. General discussion

The sex expression of andromonoecy in Solanum houstonii

The study of andromonoecy in *Solanum houstonii* suggests that the production of hermaphrodite and staminate flowers of this sexual system is fixed at certain positions of the inflorescence. This was confirmed, first, by the results of Chapter 2 where I found bisexuality is maintained only in buds from the most basal position of the inflorescence and, secondly, in Chapter 3 where I found no variation in the relative proportion of hermaphrodite flowers by characterisation of sex expression of individuals from natural populations and inflorescences.

In Chapter 2, I examined morphological differences between hermaphrodite and staminate flowers in order to determine when during development unisexuality is achieved. My results showed 1) all flowers are bisexual at initiation but only flowers at the most basal position maintain both male and female function, 2) unisexuality arise at early stages of development due to gynoecium abortion and 3) flowers have morphological adaptations intrinsic to their sex. In *Solanum,* sex determination plays an important role in the overall sex expression of species, as some studies have shown flowers that are bisexual at initiation have the potential to become hermaphrodite at some point during their development (Diggle, 1991a, 1993; Diggle & Miller, 2013). In *S. houstonii*, this scenario of labile expression seems unlikely as my results from Chapter 3 demonstrated the pattern of sex expression is biased towards the production of staminate flowers at several organisational levels. I found each inflorescence produces one hermaphrodite flower in the most basal position and several staminate flowers in distal positions (a maximum of ~35 staminate flowers per inflorescence), and among populations the ratio of hermaphrodite to staminate flowers was 1:4.

In general, this study shows that *S. houstonii* is a species with strong andromonoecy in which sex determination occurs early in development determining the pattern of sex expression. Sex expression is controlled by architectural effects in each inflorescence that regulate the developmental pathway of sex determination in each flower. As in other species of *Solanum* (Miller & Diggle, 2003; Diggle & Miller, 2013; Knapp *et al.*, 2017), *S. houstonii* possesses a strong andromonoecy with a fixed proportion of hermaphrodite flowers produced per inflorescence that is maintained among individuals and populations. The results presented in Chapter 3 further confirmed that sex expression did not to depend on resource availability, as I found that in natural populations and in treatments with decreased and increased resource availability, sex expression at the inflorescence level does not to change.

Previous studies in *Solanum,* have suggested that phenotypes with extreme sex expression may have evolved from genotypes with plasticity in their sex expression (Price *et al.*, 2003; Auld *et al.*, 2010; Diggle & Miller, 2013). These studies have suggested evolution may have occurred as a mechanism to maximise resource allocation towards the production of one hermaphrodite flower that produces larger fruits (which are putatively more expensive). Diggle and Miller (2013) demonstrated with phylogenetically-based ancestral state reconstructions that plasticity has been lost independently in some species from the sections *Acanthophora* and *Lasiocarpa* (*Solanum*). They explained the means to achieve a fixed phenotype is through genetic assimilation, a process by which a phenotype originally produced in response to an environmental condition later becomes genetically fixed through natural selection. In addition, their analyses demonstrated the common ancestor for the fixed phenotypes in the sections *Acanthophora* and *Lasiocarpa* was plastic. Diggle and Miller (2013) hypothesised a fixed genotype could have evolved by the initial fixation of staminate flower in earlier (basal) positions of the inflorescence, which became fixed as a strategy to maximise resource allocation to the female function. They concluded this hypothesis might be accurate, as species with fixed sex expression often possess large flowers with large ovaries and fruits that presumably are more expensive. Recently this hypothesis has been supported in a study performed by Torices *et al.* (2018), who found a correlation between highly specialised systems (with fixed sex expression, such as monoecy) and the investment of more resources in flowers that are produced early in the inflorescence. In the case of *S. houstonii*, it is possible that the fixed sex expression with only one hermaphrodite flower per inflorescence has evolved as a mechanism to ensure enough resources are allocated towards the female function for the production of high quality fruits. *Solanum houstonii* possesses fruits of approximately 2.5 cm of diameter, which is a large size for species within the same section (Knapp *et al.*, 2017). Moreover, the presence of large displays staminate flowers that possess heteranthery and large corollas suggest staminate flowers could be as expensive to produce as hermaphrodite flowers. Therefore, possessing a fixed phenotype to ensure enough resources are allocated to the production of high quality fruits regardless the resources available in the environment may be an advantageous solution for this species.

It remains unknown whether the strong andromonoecy in *S. houstonii* is evolutionarily derived from plastic phenotypes. However, in Chapter 3, I found evidence that a few individuals have the capacity to produce hermaphrodite flowers in secondary positions where staminate flowers usually develop. The question is how do these genotypes exist? One possible explanation is that these genotypes maintain some level of lability that is not expressed in others. In Chapter 3, I found a small proportion of individuals with a phenotype that produce flowers in secondary positions, but due to the small number of individuals, I was unable to detect whether the production of more hermaphrodite flowers was a plastic response to an increase of resource availability. Whether these individuals are plastic remains unknown, but the fact that I found them suggest fixed expression in *S. houstonii* could have been derived from plasticity and some individuals still maintain it, possibly as a strategy to ensure reproduction in changing environments. Reproductive assurance could be a mechanism that maintains plastic phenotypes in *S. houstonii.* In individuals that produced hermaphrodite flowers in secondary positions, the distance between anthers and stigma was equal and that these hermaphrodite flowers were likely to set fruit. In fact, in the pollinator-free glasshouse, this type of flowers produced fruit. In environments were pollinators are scarce, a mechanism leading to self-fertilisation could ensure reproduction. This has actually been shown for species with specialised pollination systems (Fenster & Martén-Rodríguez, 2007; Barrett, 2013)*. Solanum houstonii* is a highly specialised species, as it requires bees capable of vibrating its poricidal anthers in order to release pollen. Moreover, the hermaphrodite flowers from basal positions that characterise this species have herkogamy, a morphological adaptation that reduce self-fertilisation (Barrett, 2002b) but requires bees of a certain size to ensure pollen transfer the stigma. In environments in which pollinators are reduced, *S. houstonii* have a very low chance to set fruits. Under these circumstances, possessing hermaphrodite flowers able to self-fertilise could be advantageous.

The role of staminate flowers

The hypothesis of staminate flowers as better pollen donors than hermaphrodite flowers is one of the main hypotheses used to explain the evolution and maintenance of andromonoecy (Bertin, 1982; Podolsky, 1993; Vallejo-Marín & Rausher, 2007b; Quesada-Aguilar *et al.*, 2008). Some studies that have supported this hypothesis demonstrate the greater production, viability and siring success of staminate flowers (Elle & Meagher, 2000; Huang, 2003; Dai & Galloway, 2012), as well as their greater attractiveness to pollinators and their potential to reduce sexual interference (Elle & Meagher, 2000; Quesada-Aguilar *et al.*, 2008). My results suggest that one of the roles of staminate flowers of *S. houstonii* is to promote pollen donation, increase pollen receipt and reduce interference of the male and female function. The results of Chapter 2 demonstrate that staminate flowers bear higher amounts of pollen, are larger than hermaphrodite flowers and possess morphological adaptations (i.e. heteranthery) than enhance pollen dispersion and transference to the stigma. In addition, in Chapter 4, I found that siring success of the anthers of staminate flowers is as good as pollen of hermaphrodite flowers and that in the presence of pollinators that match the morphology of *S. houstonii* flowers, pollen transfer to the stigma is efficient from staminate and hermaphrodite flowers. Although, these results did not conclusively demonstrate that staminate flowers were better pollen donors than hermaphrodite flowers. These results, nevertheless, suggested other potential roles of staminate flowers, such as that staminate flower may increase pollen receipt and pollen build up on pollinators' bodies. Finally, the results of Chapter 5 showed *S. houstonii* is highly dependent on pollinators to produce fruits and that in natural populations there are pollen thieves and herbivores that could potentially reduce pollen availability. In spite of that, reproduction of *S. houstonii* was not limited by the amount of pollen or pollinators in two population of Yucatan. These findings suggest that the relatively high production of staminate flowers may be an effective strategy to ensure reproduction because these flowers provide enough pollen to feed all types of floral visitors, increase pollen export and pollen built up on pollinators' body. Moreover, the particular morphology of staminate flowers could play an important role in pollen deposition on pollinators' body.

Staminate flowers are characterised by possessing heteranthery, the functional and morphological separation of anthers into feeding and pollinating (Cardoso *et al.*, 2018). Heteranthery is widely spread among angiosperms and experimental work on heterantherous species has demonstrated that pollen exported from pollinating anthers is more likely to be transfer to stigmas, if a correspondence between pollen placement on pollinators body and stigma contact exists (Jesson & Barrett, 2003; Luo *et al.*, 2009; Vallejo-Marín *et al.*, 2010; Solís-Montero & Vallejo-Marín, 2017). In *S. houstonii*, heteranthery plays an important role on pollen export; in Chapter 5, I found pollinators touched pollinating anthers and stigma with similar body parts. During pollen extraction in staminate flowers, as pollinators held feeding anthers and started making vibrations to extract pollen, pollen from feeding anthers was deposited on their ventral abdomen while pollen from pollinating anthers was deposited on their back. During visitations of hermaphrodite flowers, pollinators acquired a similar position to extract pollen and touched the stigma with their back. This behaviour was observed in pollinators from natural populations in Yucatan and in bumblebees used during the experiments of Chapter 4, and suggest heteranthery of staminate flowers facilitates pollen deposition on pollinators' bodies, which promotes pollen export and deposition in the stigma. Furthermore, in Chapter 2, where I characterised the morphology of hermaphrodite and staminate flowers, I showed the length of pollinating anthers in staminate flowers is similar length styles of hermaphrodite flowers. The similarity in morphology between hermaphrodite and staminate flowers thus suggests pollinators with a matching morphology should be more efficient extracting and depositing pollen. For instance, in other species hermaphrodite flowers having a strong separation between anthers and stigma also experience more efficient pollen transfer to the stigma. In the andromonoecious *Solanum carolinense*, natural pollinators were more efficient at depositing pollen grains on the stigma than removing pollen from them when flowers had a large styles (Quesada-Aguilar *et al.*, 2008). These results not only highlight the importance of floral morphology in pollen deposition but also show that the separation between anthers and stigma reduces the chances of self-pollination.

Overall, my findings demonstrated that the functional significance of staminate flowers in *S. houstonii* is to promote pollen export, pollination attraction and increase the chances that pollen reaches the stigma. Moreover, the presence of pollinators matching the morphology of on both hermaphrodite and staminate flowers enhances the chances of pollen being transferred.

Evolutionary consequences of andromonoecy

The main hypotheses for the evolution of andromonoecy focus on the adaptive advantages of having staminate flowers to increase overall plant fitness. These hypotheses include 1) the *resource allocation hypothesis*, 2) the *pollen donation hypothesis*, 3) the *increased pollen receipt hypothesis*, and 4) the *male-female interference hypothesis* (Bertin, 1982; Whalen & Costich, 1986; Podolsky, 1993; Vallejo-Marín & Rausher, 2007a; Quesada-Aguilar *et al.*, 2008). The resource allocation hypothesis posits the production of energetically cheaper staminate flowers save resources that can be allocated to the female function or other fitness enhancing traits (Bertin, 1982, 2007; Solomon, 1986). The pollen donation and the increased pollen receipt hypotheses are not mutually exclusive as both suggest by producing large amount of pollen and big displays of staminate flowers, pollinator attraction is enhanced and so is pollen export and deposition on stigmas (Whalen & Costich, 1986; Podolsky, 1993; Elle & Meagher, 2000; Huang, 2003). However, the pollen donation hypothesis proposes that male fitness is also enhanced as a result of increased pollen removal, while the increased pollen receipt hypothesis suggests female fitness is enhanced through more efficient pollen deposition (Podolsky, 1993; Vallejo-Marín & Rausher, 2007a). The male-female interference hypothesis posits staminate flowers reduce interference during pollen removal and deposition, which also reduces the probability of self-fertilisation (Whalen & Costich, 1986; Diggle & Miller, 2004; Quesada-Aguilar *et al.*, 2008).

In this thesis, I investigated the functional significance of andromonoecy by assessing the lability of sex determination, examining the functional role of staminate flowers and evaluating the reproductive consequences of andromonoecy in natural populations of the Mexican endemic shrub *S. houstonii.* My thesis proposes several hypotheses for the evolution and maintenance of andromonoecy in this species. First, andromonoecy in *S. houstonii* is a mechanism to ensure the production of one large fruit per inflorescence regardless of the resources available for plant growth and reproduction. This was supported by the fact that I found sex determination of *S. houstonii* is fixed within the inflorescences and does not vary in response to resource availability (Chapters 2 and 3). This fixed pattern of expression has been hypothesised as a strategy to produce larger fruits of better quality (Miller & Diggle, 2007; Diggle & Miller, 2013; Torices *et al.*, 2018). Second, the production of a large display of staminate flowers increases pollinator attraction, which also increases pollen export and the probability of pollen reaching the stigmas. I found support for this hypothesis during my observations in natural populations, where I found: 1) plants display a relatively large proportion of staminate flowers that follows a ratio of 4:1 of staminate to hermaphrodite flowers (Chapter 3), 2) a positive relationship between floral display and pollinator attraction and 3) a relatively large proportion of pollinators visiting both hermaphrodite and staminate flowers (Chapter 5). In other andromonoecious species, such as *Besleria trifolia* and *Capparis spinosa* large displays of flowers likewise increase pollinator visitation, which also increase overall fruit set (Podolsky, 1992; Zhang & Tan, 2009). Third, staminate flowers and their relatively large proportion in each individual provide enough pollen to cope with pollen thieves and to increase pollen receipt and female fitness. This hypothesis can be supported by my results from Chapter 5, in which natural populations of *S. houstonii* have a relatively high reproductive success that was not limited by pollen availability or pollinators abundance. In natural populations, many floral visitors acted as pollen thieves, as they collected pollen without providing any pollination service to *S. houstonii*. Other species that experience pollen theft often have a negative impact in fruit and seed production (Hargreaves *et al.*, 2009, 2012; Koski *et al.*, 2018a). However, *S. houstonii* did not experience a reduced seed set due to the high production of pollen not only in each individual staminate flower but also in the overall staminate flowers display, and due to the presence of pollinators that were efficient at pollen removal and deposition. Lastly, staminate flowers and their characteristic floral morphology (anther dimorphism, also known as heteranthery) reduce interference during pollen removal and deposition. In natural populations and in the experiments using bumblebees as pollinators, I observed pollinators have behavioural patterns during pollen removal in both hermaphrodite and staminate flowers. During pollen removal in staminate and hermaphrodite flowers, pollinators hold the short-feeding anthers and produce high frequency vibrations to release pollen. Due to this position, pollen is deposited on the ventral thorax and abdomen of pollinators. When pollinators visit staminate flowers and performed the described behaviour pollen from the large-pollinating anthers is deposited on the upper side of pollinator abdomen, and when they visit hermaphrodite flowers they contact the stigma with the same body part. These behavioural patterns, together with the fact that stigma and pollinating anthers are of a similar size (Chapter 2), suggest pollen removal and deposition is efficient if pollinators possess a body size larger than the separation between anthers and stigma (and the separation between feeding and pollinating anthers in staminate flowers). In fact, pollinators that are efficient at pollen extraction also produce fewer visits but deposit a greater amount of pollen grains (Solís-Montero *et al.*, 2015; Solís-Montero & Vallejo-Marín, 2017).

In conclusion, my results suggest that: 1) the relatively large proportion of staminate flowers may increase pollen export and pollinator attraction, 2) staminate flowers may improve pollen receipt by producing high amounts of pollen exploited by florivores, pollinators and pollen thieves that in occur natural populations and 3) floral morphology of both hermaphrodite and staminate flowers may reduce sexual interference. My thesis provides the first detailed investigation of the reproductive system and pollination ecology of *Solanum houstonii,* as well as the first evidence of the evolutionary consequences of andromonoecy in this species.

Concluding remarks and future directions

Through the chapters of this thesis, I provided evidence of non-plastic sex expression of *S. houstonii*, as well as the role of staminate flowers in pollen export, pollinator attraction and in reducing sexual interference to ensure reproductive success. However, a few questions regarding the reproductive system of *S. houstonii* and its evolutionary implications remain unresolved.

I investigated the reproductive and pollination ecology of *S. houstonii* in two populations in Yucatan, Mexico, but this species has a wide distribution in Mexico. Mexico is biodiverse country, with a high diversity of bee species and ecosystems (Vergara & Ayala Barajas, 2002; Toledo, 2010). *Solanum houstonii* grows across a wide variety of habitats and elevations; it occurs in most of the arid habitats of Mexico from the Sonoran Desert zones in western Mexico, across the volcanic belt up to 2000 m to the limestone pans of the Caribbean coast (Knapp *et al.*, 2017). Thus, one of the main aspects that needs to be addressed to support the role of staminate flowers in maximising reproductive success is the characterisation of the reproductive system, pollination biology and reproductive success of *S. houstonii* in different environments and populations across Mexico. *Solanum houstonii* is a species that requires buzz-pollination and pollinators of a certain size to ensure reproduction. Moreover, in natural populations this species is visited by a great variety of pollen thieves and herbivores, which can have a negative impact in their reproductive success. By characterising the reproductive system and pollination ecology in different populations and identifying the pollen thieves or agents that reduce reproductive success, it would become clearer, whether the production of high amounts of staminate flowers is an efficient strategy to ensure reproduction.

Another aspect that would be useful to investigate in order to understand further the evolutionary consequences of andromonoecy is the genetic diversity and outcrossing rates of *S. houstonii.* Andromonoecy, heteranthery and herkogamy are some of the mechanisms proposed to avoid physical interference between the sexual functions and promote cross-fertilisation among individuals (Fetscher, 2001; Barrett, 2002b). Because *S. houstonii* is highly specialised in these traits, it is expected that outcrossing rates of individuals from natural populations is relatively high in the presence of pollinators. However, in the absence of pollinators, it is expected that populations are pollen limited and experience high levels of inbreeding, and in this case maybe the factors maintaining andromonoecy are different. Therefore, I highly recommend population genetics analyses across different regions of Mexico to test whether the highly specialised sexual system of *S. houstonii* maintains high outcrossing rates across populations and has evolved as a strategy to promote outcrossing.

Furthermore, studies on the genetic basis of sex determination can help to elucidate the transition pathways from hermaphroditism to unisexuality in *Solanum. Solanum houstonii* has proven to be a good model to study the patterns of sex expression of andromonoecy, as the majority of individuals possess a fixed phenotype characterised by the production of one hermaphrodite flower at the base position of each inflorescence, but others still maintain a plastic phenotype characterised by the production of hermaphrodite flowers in secondary positions of the inflorescence. Hence, transcriptomic analyses in each of the flowers of this species (i.e. hermaphrodite flowers of individuals with fixed sex expression, hermaphrodite of individuals with plastic phenotypes and staminate flowers) can determine the genes involved in the expression of each flower phenotype, which would provide a framework for clarifying the mechanistic pathways for the origin of unisexuality in *Solanum*. Moreover, *S. houstonii* is closely related to the dioecious species that occur in the Old World section (Echeverría-Londoño *et al.*, 2018) and can provide a good insight in the genetics of sex determination and in the evolution of the transition from bisexuality to unisexuality in *Solanum*.

Finally, a question that concerned me during my research, involves whether a fixed expression of andromonoecy is necessary intermediate step preceding the complete separation of sexes in different individuals (dioecy) in *Solanum*. This assumption is based on the hypothesis that in *Solanum* andromonoecy is the common ancestor of dioecy (Martine *et al.*, 2006, 2009) and on the resource allocation hypothesis. The latter hypothesis helps to understand the origin of fixed sex expression in andromonoecious species and has been also proposed as a mechanism for the origin of unisexual flowers in other plant species (Charlesworth & Charlesworth, 1978; Huang, 2003; de Jong *et al.*, 2008). For instance, it has been hypothesised that the origin of female and male flowers within individuals (monoecy) may be due to developmental changes that are induced as an strategy to maximise resource allocation to male and female function (de Jong *et al.*, 2008). Likewise, it has been suggested that monoecy is a transition pathway prior the evolution of dioecy (reviewed in Barrett, 2002a). The *monoecy pathway* for the evolution of dioecy is a hypothesis that posits unisexuality is achieved from a monoecious ancestor as a strategy to optimise resource allocation towards the production of female or male flowers. According to this theory, in changing environments where resource availability is scarce, the production of only female or staminate flower could be favoured by disruptive selection. For dioecy to occur in *Solanum* in a similar form, it would first require an initial separation of female and male functions in different flowers, which means the appearance of a monoecious phenotype. However, there are not yet any known species with monoecy within this genus. Therefore, I suggest andromonoecious species with a fixed sex expression phenotype precede an androdioecious phenotype, characterised by the presence of individuals with hermaphrodite or staminate flowers only. If an androdioecious phenotype precedes dioecy then I would expect the hermaphrodite function of some individuals to be lost to ensure enough resources are allocated to the female function only. In any case, in order to investigate whether the transition pathway to dioecy in *Solanum* has as an intermediate stage individuals with fixed sex expression, it is necessary to characterise the sex expression of andromonoecious species closely related to dioecious taxa. Additionally, performing an ancestral character reconstruction analyses could help to determine whether the common andromonoecious ancestor had a fixed phenotype.

This thesis provides the first evidence on the reproductive system of *Solanum houstonii*. Here I demonstrated: 1) in this species, the production of staminate flowers occurs at early stages of development and is influenced by inflorescence architecture; 2) laboratory experiments did not support a role for staminate flowers in pollen donation or pollination attraction, but highlighted the importance of performing experiments under different scenarios and using native and non-native species of pollinators. 3) In natural populations, *S. houstonii* reproductive success is not limited by pollen receipt, perhaps because andromonoecy reduces the negative effects of pollen theft by promoting pollinator attraction, pollen export, pollen deposition on pollinators body and increasing pollen transfer to the stigma of hermaphrodite flowers. My research provides evidence of the mechanisms involved in the production of staminate flowers and the reproductive success of a buzz-pollinated, andromonoecious species.

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Supplementary material

Table s2.1. Populations of *S. houstonii* surveyed and grown under control conditions.

Population	**Latitude**	**Longitude**	**State**
Greenhouse grown			
Cerro Colorado	18.47127	-97.35825	Puebla
Coxcatlan	18.174353	-97.119277	Puebla
El molino	18.4675	-97.3624	Puebla
Tehuacan-2015	18.315766	-97.360509	Puebla
Highway 15, Km81	25.339701	-107.951082	Sonora
Los Alamos intersection	26.9985	-108.930027	Sonora
Cuzama	20.72041	-89.38328	Yucatan
Carr-Abala-3	20.63676	-89.65543	Yucatan
Santa Clara-3	21.3702	-89.03168	Yucatan
Natural populations			
Champoton	19.328889	-90.740278	Campeche
Carr-Playa del Carmen	20.791639	-86.946667	Quintana Roo
Carr-Abala-1	20.64314	-89.67459	Yucatan
Carr-Abala-3	20.63676	-89.65543	Yucatan
Carr-Abala-7	20.62341	-89.60959	Yucatan
Carr-Cuzama	20.71674	-89.3227	Yucatan
Carr-Hunucma-1	21.05353	-89.92932	Yucatan
Carr-Hunucma-2	21.05937	-89.9369	Yucatan
Carr-Hunucma-3	21.06915	-89.94686	Yucatan
Carr-Hunucma-5	21.07825	-89.95623	Yucatan
Carr-Hunucma-6	21.08782	-89.96619	Yucatan
Carr-Hunucma-7	21.0982	-89.97684	Yucatan
Carr-Izamal-1	20.94527	-89.07614	Yucatan
Carr-Izamal-2	20.7084	-88.75525	Yucatan
Carr-Kimbila	20.927056	-89.134922	Yucatan
Carr-Motul-1	21.31635	-89.26222	Yucatan
Carr-Motul-2	21.30716	-89.26365	Yucatan
Carr-Motul-3	21.2597	-89.26741	Yucatan
Carr-Motul-4	21.12428	-89.28041	Yucatan
Carr-Mucuyche-1	20.61729	-89.6009	Yucatan
Carr-Mucuyche-2	20.60101	-89.59928	Yucatan
Chelenku cenote	20.713225	-89.328488	Yucatan
Chuburna	21.25359	-89.812	Yucatan
Cuzama	20.72041	-89.38328	Yucatan
Dzilam	21.39046	-88.90358	Yucatan
Homun	20.7139	-89.33287	Yucatan
Parque cientifico de Yucatan	21.1352652	-89.785224	Yucatan
San Isidro Ochil	20.629425	-89.344696	Yucatan
Santa Clara-2	21.37211	-89.02174	Yucatan
Santa Clara-3	21.3702	-89.03168	Yucatan
Seduma	21.32819	-89.2606	Yucatan
Tecnohotel	21.32338	-89.42334	Yucatan
Xaman-ik	21.32633	-89.4107	Yucatan

Table s2.2. Coefficients of models describing the growth of style and ovary in relation to bud length of *S. houstonii*. These models were ran with a linera mixed effect model with Gaussian distribution in a subsampled of buds of 0.1mm to 3 mm of length.

Response variable	**Fixed and random variables**	**Estimate/ Variance***	**SE/SD***	***t***	***P***	***N***
Style height	Intercept	<0.01	0.08	0.1	0.954	28
	Bud length	0.25	0.04	6.8	<0.001	
	Sex (Staminate)	-0.02	0.08	-0.2	0.836	
	Bud length:Sex(Staminate)	-0.07	0.05	-1.5	0.161	
	Plant ID (random)	0.002	0.04			4
Ovary width	Intercept	-0.69	0.06	2.7	0.015	28
	Bud length	0.16	0.03	5.1	<0.001	
	Sex (Staminate)	0.13	0.06	-1.7	0.099	
	Bud length:Sex(Staminate)	0.01	0.04	0.4	0.697	
	Plant ID (random)	0.002	0.05			4

*SE and estimate for fixed variables, SD and variance for random variables.

Table s2.3. Coefficients of the first three linear discriminants.

Floral traits	**LD1 (97.84%)**	**LD2 (1.81%)**	**LD3 (0.35%)**
Corolla length	0.013	0.122	-0.031
Corolla width	-0.039	-0.043	-0.076
Feeding anthers	-0.095	0.450	0.339
Pollinating anthers	-0.094	0.025	0.216
Style length	0.424	0.006	0.107
Distance between feeding and pollinating anthers	-0.126	-0.344	0.184
Calyx length	0.016	-0.138	0.112

Table s3.1**.** Accessions and populations of *S. houstonii* surveyed in natural populations and grown under control conditions.

Accessions	**Population**	**Latitude**	**Longitude**	**State collected**
Natural populations				
16s61	Carr-Abala-1	20.64314	-89.6746	Yucatan
16s62	Carr-Abala-1	20.64314	-89.6746	Yucatan
16s64	Carr-Abala-3	20.63676	-89.6554	Yucatan
16s65	Carr-Abala-3	20.63676	-89.6554	Yucatan
16s67	Carr-Abala-5	20.62654	-89.6291	Yucatan
16s69	Carr-Abala-7	20.62341	-89.6096	Yucatan
16s40	Carr-Cuzama	20.71674	-89.3227	Yucatan
16s41	Carr-Cuzama	20.71674	-89.3227	Yucatan
16s42	Carr-Cuzama	20.71674	-89.3227	Yucatan
16s50	Carr-Hunucma-1	21.05353	-89.9293	Yucatan
16s51	Carr-Hunucma-1	21.05353	-89.9293	Yucatan
16s52	Carr-Hunucma-1	21.05353	-89.9293	Yucatan
16s53	Carr-Hunucma-2	21.05937	-89.9369	Yucatan
16s54	Carr-Hunucma-2	21.05937	-89.9369	Yucatan
16s55	Carr-Hunucma-3	21.06915	-89.9469	Yucatan

16s58	Carr-Hunucma-5	21.07825	-89.9562	Yucatan
16s59	Carr-Hunucma-6	21.08782	-89.9662	Yucatan
16s60	Carr-Hunucma-7	21.0982	-89.9768	Yucatan
16s74	Carr-Izamal-1	20.94527	-89.0761	Yucatan
16s75	Carr-Izamal-1	20.94527	-89.0761	Yucatan
16s76	Carr-Izamal-2	20.7084	-88.7553	Yucatan
16s73	Carr-Kimbila	20.92706	-89.1349	Yucatan
16s12	Carr-Motul-1	21.31635	-89.2622	Yucatan
16s14	Carr-Motul-1	21.31635	-89.2622	Yucatan
16s15	Carr-Motul-1	21.31635	-89.2622	Yucatan
16s16	Carr-Motul-1	21.31635	-89.2622	Yucatan
16s17	Carr-Motul-1	21.31635	-89.2622	Yucatan
16s18	Carr-Motul-2	21.30716	-89.2637	Yucatan
16s19	Carr-Motul-2	21.30716	-89.2637	Yucatan
16s20	Carr-Motul-2	21.30716	-89.2637	Yucatan
16s21	Carr-Motul-2	21.30716	-89.2637	Yucatan
16s22	Carr-Motul-2	21.30716	-89.2637	Yucatan
16s23	Carr-Motul-3	21.2597	-89.2674	Yucatan
16s24	Carr-Motul-4	21.12428	-89.2804	Yucatan
16s70	Carr-Mucuyche-1	20.61729	-89.6009	Yucatan
16s71	Carr-Mucuyche-2	20.60101	-89.5993	Yucatan
16s80	Carr-Playa del Carmen	20.79164	-86.9467	Quintana Roo
16s81	Carr-Playa del Carmen	20.79164	-86.9467	Quintana Roo
16s29	Carr-Santa Clara	21.37787	-88.9785	Yucatan
16s46	Cenote Chelenku	20.72005	-89.347	Yucatan
16s01	Chuburna	21.25359	-89.812	Yucatan
16s02	Chuburna	21.25359	-89.812	Yucatan
16s03	Chuburna	21.25359	-89.812	Yucatan
16s25	Dzilam	21.39046	-88.9036	Yucatan
16s26	Dzilam	21.39046	-88.9036	Yucatan
16s27	Dzilam	21.39046	-88.9036	Yucatan
16s28	Dzilam	21.39046	-88.9036	Yucatan
16s44	Homun	20.7139	-89.3329	Yucatan
16s45	Homun	20.7139	-89.3329	Yucatan
16s08	Never	21.32871	-89.3922	Yucatan
16s09	Never	21.32871	-89.3922	Yucatan
16s34	Santa Clara-2	21.37211	-89.0217	Yucatan
16s35	Santa Clara-3	21.3702	-89.0317	Yucatan
16s36	Santa Clara-3	21.3702	-89.0317	Yucatan
16s38	Santa Clara-3	21.3702	-89.0317	Yucatan
16s39	Santa Clara-3	21.3702	-89.0317	Yucatan
16s10	Seduma	21.32819	-89.2606	Yucatan
16s11	Seduma	21.32819	-89.2606	Yucatan
16s04	Tecnohotel	21.32338	-89.4233	Yucatan
16s05	Tecnohotel	21.32338	-89.4233	Yucatan
16s06	Tecnohotel	21.32338	-89.4233	Yucatan
16s07	Xaman-ik	21.32633	-89.4107	Yucatan
17pc1-17pc16	Sierra Papacal	21.13527	-89.7845	Yucatan

17s01-17s21	San Isidro Ochil	20.62943	-89.3447	Yucatan
17ch03-17s21	Chunkanan	20.62398	-89.6096	Yucatan
Greenhouse 2017				
07s38	Los Alamos intersection	26.9985	-108.93	Sonora
07s211a	Los Alamos intersection	26.9985	-108.93	Sonora
07s211b	Los Alamos intersection	26.9985	-108.93	Sonora
07s62	Los Alamos intersection	26.9985	-108.93	Sonora
07s66	Los Alamos intersection	26.9985	-108.93	Sonora
Greenhouse experiments 2018				
07s19	Los Alamos intersection	26.9985	-108.93	Sonora
07s211a	Los Alamos intersection	26.9985	-108.93	Sonora
07s211b	Los Alamos intersection	26.9985	-108.93	Sonora
07s211b1	Los Alamos intersection	26.9985	-108.93	Sonora
07s266	Los Alamos intersection	26.9985	-108.93	Sonora
07s32	Los Alamos intersection	26.9985	-108.93	Sonora
07s38	Los Alamos intersection	26.9985	-108.93	Sonora
07s62	Los Alamos intersection	26.9985	-108.93	Sonora
07s64	Los Alamos intersection	26.9985	-108.93	Sonora
07s66	Los Alamos intersection	26.9985	-108.93	Sonora
07s67	Los Alamos intersection	26.9985	-108.93	Sonora
16s32	Carr-Santa Clara	21.37787	-88.9785	Yucatan
16s57	Carr-Hunucma-4	21.07579	-89.9535	Yucatan
16s63	Carr-Abala-2	20.63844	-89.6588	Yucatan
16s66	Carr-Abala-4	20.62756	-89.6398	Yucatan
16s67	Carr-Abala-5	20.62654	-89.6291	Yucatan
16s68	Carr-Abala-6	20.62557	-89.6186	Yucatan
16s70	Carr-Mucuyche-1	20.61729	-89.6009	Yucatan
16s72	Carr-Mucuyche-2	20.60101	-89.5993	Yucatan
16s72a	Carr-Mucuyche-2	20.60101	-89.5993	Yucatan
17s4	San Isidro Ochil	20.62943	-89.3447	Yucatan

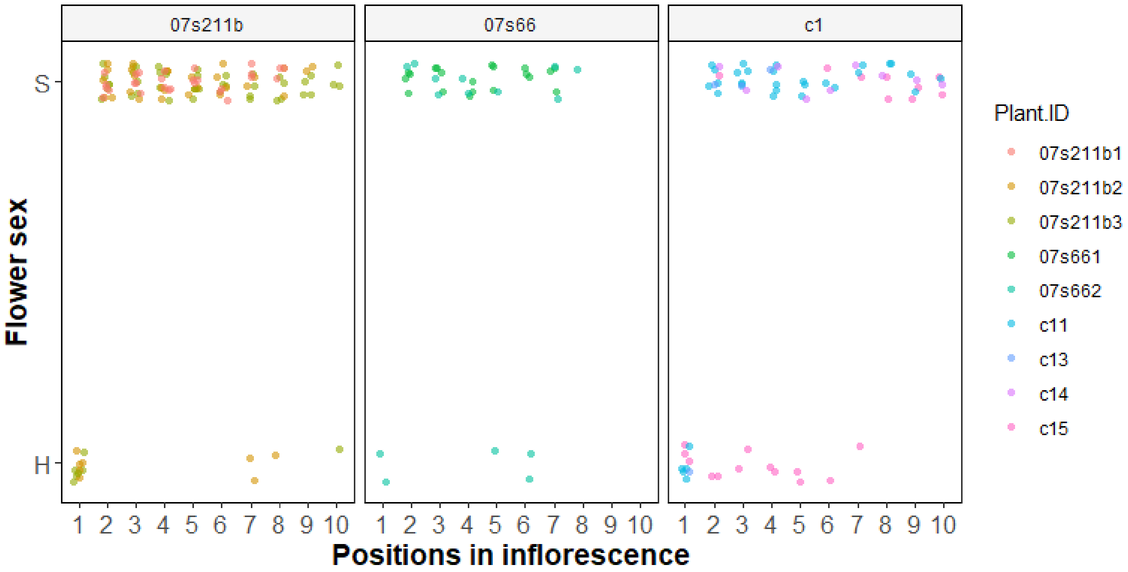

Figure s3.1. Hermaphrodite and staminate flowers produced in the first ten positions of the inflorescence of individuals belonging to seed families capable of producing hermaphrodite flowers in several positions of the inflorescence. Seed family 07s211b included two individuals capable of producing hermaphrodite flowers at different positions of the inflorescence (07s211b2 and 07s211b3). In seed family 07s66, both individuals were capable of producing hermaphrodite flowers at different positions of the inflorescence. In family c1, only one individual produced more than one hermaphrodite flower (c15).

Table s4.1. Populations of the seed families of *S. houstonii* used in the experiments of this study.

Population	**Latitude**	**Longitude**	**State**
Crosses			
Highway 15, Km81	25.339701	-107.951082	Sonora
Los Alamos intersection	26.9985	-108.930027	Sonora
Pollen germination and viability			
Carr-Abala-3	20.63676	-89.65543	Yucatan
Carr-Abala-5	20.62654	-89.6291	Yucatan
Carr-Cuzama	20.71674	-89.3227	Yucatan
Carr-Hunucma-2	21.05937	-89.9369	Yucatan
Santa Clara-3	21.3702	-89.03168	Yucatan
Carr-Cuzama	20.71674	-89.3227	Yucatan
Highway 15, Km81	25.339701	-107.951082	Sonora
Los Alamos intersection	26.9985	-108.930027	Sonora
Bee behaviour experiments			
Carr-Abala-3	20.63676	-89.65543	Yucatan
Carr-Abala-5	20.62654	-89.6291	Yucatan
Carr-Cuzama	20.71674	-89.3227	Yucatan
Carr-Hunucma-1	21.05353	-89.92932	Yucatan
Carr-Hunucma-2	21.05937	-89.9369	Yucatan
Carr-Hunucma-3	21.06915	-89.94686	Yucatan
Carr-Mucuyche-1	20.61729	-89.6009	Yucatan
Chelenku cenote	20.713225	-89.328488	Yucatan
Santa Clara-3	21.3702	-89.03168	Yucatan
Highway 15, Km81	25.339701	-107.951082	Sonora
Los Alamos intersection	26.9985	-108.930027	Sonora

Solanum houstonii Martyn
Anna K. Z. Carbonell