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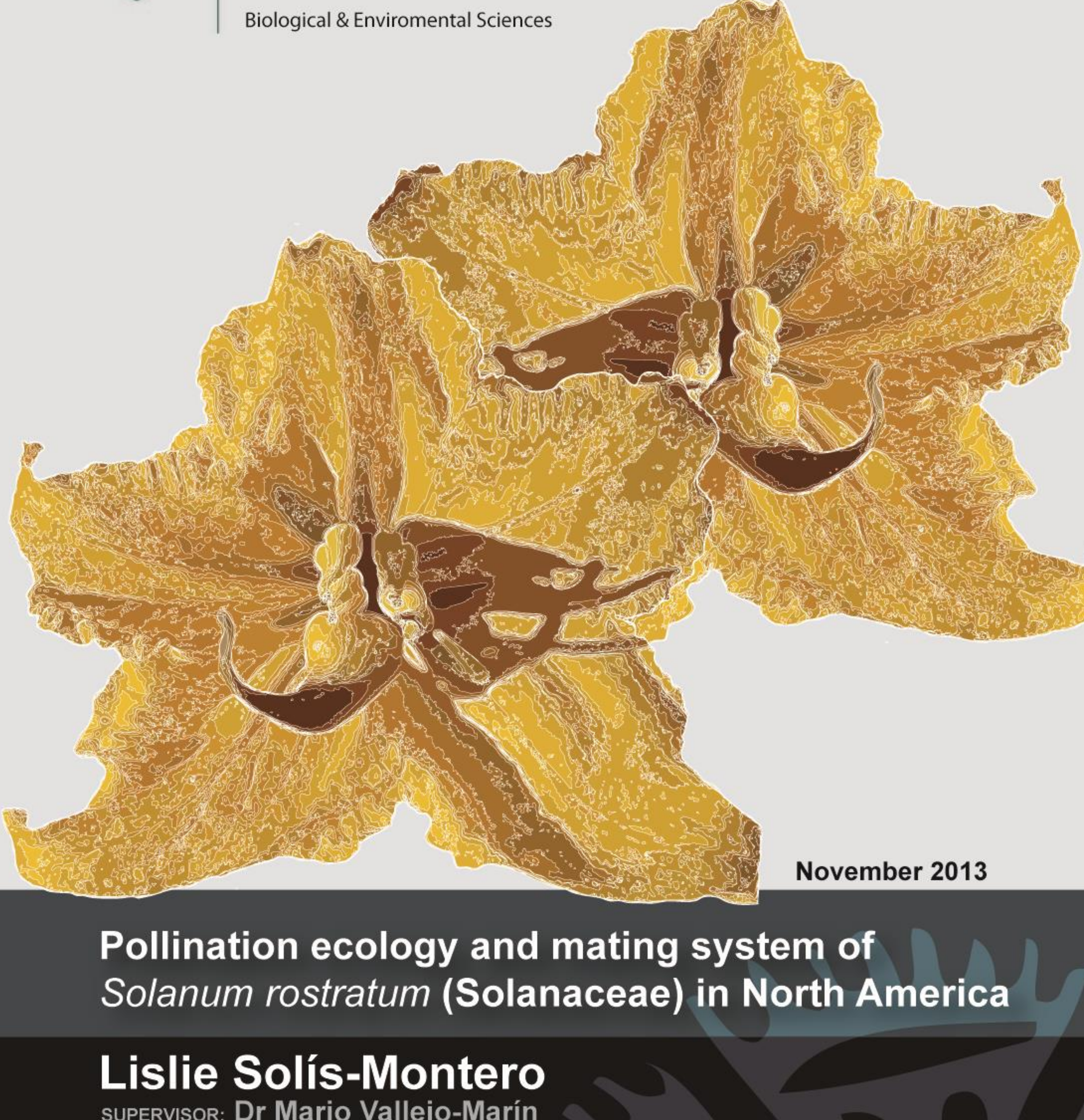

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Pollination ecology and mating system of *Solanum rostratum* (Solanaceae) in North America

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Declaration

I hereby declare that this thesis has been composed by myself and that it embodies the results of my own research. Where appropriate, I have acknowledged the nature and extent of work carried out in collaboration with others.

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Lislie Solís-Montero

Summary

Buzz-pollination or pollination by vibration occurs in several families of angiosperms including some important commercial crops such as potatoes and tomatoes. Buzz-pollinated flowers release pollen via small pores or slits on the anther's tip that require the use of vibrations by specialized pollinators, usually bees, to remove the pollen. Some buzz-pollinated species have elaborate floral morphologies including dimorphic anthers within the same flower (heteranthery), and mirror-image flowers (enantiostyly) where the style is reciprocally deflected to either the left or right side of the floral axis. The complex floral morphology and buzz-pollination syndrome seen in these species require a close physical interaction between the sexual organs of the flowers and the bodies of insect visitors. Despite the broad taxonomic distribution of buzz-pollination in angiosperms (more than 60 families are buzz-pollinated) relatively few studies have described the pollination ecology of these species under natural conditions. The main goal of the present work was to characterize the pollination biology, mating patterns and antagonistic interactions (e.g. pollen theft) in natural populations of a buzz-pollinated species. For this purpose, I studied *Solanum rostratum* (Solanaceae), a buzz-pollinated, self-compatible, annual weed with complex floral morphology (both enantiostylous and heterantherous flowers). This species usually grows in disturbed areas in its native range (Mexico) and has become invasive around the world. My research was divided into three components. First, I characterized the pollination and reproductive biology of natural populations in Mexico. I performed floral manipulations in six natural populations of *S. rostratum* to estimate fruit and seed set. In three of these populations, I carried out 115 hours of pollinator observations and quantified the incidence of pollinators versus pollen thieves. I also measured the efficiency of a subset of floral visitors in triggering fruit set after single visits. Second, I investigated whether morphological correspondence between the size of the pollinator's body and floral morphology influences pollen transfer. In experimental arrays, I exposed flowers of *S. rostratum* that varied in the distance between their sexual organs, to bumblebees (*Bombus terrestris*) of different sizes, and recorded pollen deposition and fruit and seed production. Finally, I determined the mating system (i.e. the proportion of self- and cross-fertilized offspring) of natural populations in Mexico and of introduced populations in the United States of America, using newly developed microsatellite markers. My results show that *S. rostratum* is visited by a wide range of bees of different sizes (0.9–9.8 mm in thorax diameter), but that only a small subset of these visitors act as pollinators. Most visitors act as pollen thieves, consuming pollen while effecting little or no pollination. I also found that correspondence between a pollinator's size and the separation of the *S. rostratum* sexual organs determines pollen deposition and fruit production; pollen deposition decreased when bees were small relative to the distance between the sexual organs visited the flowers. My genetic analyses show that natural populations of *S. rostratum* maintain a relatively high outcrossing rate ($t_m = 0.75 \pm 0.03$) across the native and introduced range. Furthermore, genetic diversity is reduced in invasive populations, but this is not accompanied by changes in mating system. My work shows that the morphological fit between the pollinator and the flowers is important in determining the dynamics of pollen transfer and fruit production in this buzz-pollinated plant. Distinguishing between pollinators and pollen thieves in buzz-pollinated plants is essential for understanding the evolution of buzz-pollination, as pollen theft could be a major selective force for these species.

Keywords: Apoidae, buzz-pollination, mating system, Mexico, pollen theft, *Solanum rostratum*.

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This thesis is dedicated to the astonishing **Mexican biodiversity** that make possible for biologists to tell its secrets.

"tha raon mo ghaoil fo choilltean dlùth

meirg-uilebheist san tràigh

bidh chruinne loisgte ruisgte brisg

mur toir a poball dion"

"the land I love is densely treed

rust-monsters scar the shore

the world will burn be brittle bare

if mankind won't conserve"

Salm an Fhearainn/Psalm of the land
Aonghas MacNeacail (1987)

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CHAPTER 1:

Introduction

1.1. INTRODUCTION

The flowers and inflorescences are the most diverse structures produced by angiosperms, a diversity that has arisen mainly as a result of the interactions between plants and their pollen vectors (Harder & Barrett 2006). Pollinators exert a differential selective pressure over floral traits (Fenster *et al.* 2004). In the case of plants that undergo strong selection due to pollinators, the pollinators influence the variability of floral traits and stabilize floral morphology (Berg 1959; Berg 1960). Then flower specialization evolves along different axes that are related to pollinator attraction and the interaction between pollinators and floral morphology (Armbruster & Muchhala 2009).

In order to attract pollinators, plants offer different types of reward (e.g. pollen, nectar and oils) and signal their location through floral advertisements (e.g. guides of nectaries and olfactory signals; Harder & Barrett 1996). While the nature of the reward influences which species of floral visitors are attracted, the floral advertisements promote learning in pollinators (Armbruster & Muchhala 2009). Pollinator attraction is also affected by the accessibility of a reward (Armbruster & Muchhala 2009), which for some plants is restricted to certain animals with specific behaviours or morphologies (Stang *et al.* 2009).

Different pollinators exert differential selective pressures on floral traits depending on the degree to which the pollinator morphology closely matches that of the flower morphology. For example, the long-tube flowers of *Narcissus papyraceus*, which possess nectar deposits hidden at the bottom of the floral tube, some populations of this species are pollinated by moths with long proboscides and others by flies with short proboscides. Furthermore, those populations pollinated by moths show a stronger correlation among floral traits than those population pollinated by flies because the length of the proboscis of moths closely match with the length of the floral tubes compare to flies with poor fit with the flowers (Perez-Barrales, Arroyo & Armbruster 2007). The degree to which a floral visitor fits the flower morphology also influences the efficiency of pollen transfer. In the case of specialized flowers, such as those with long corolla tubes, only a subset of floral visitors act as pollinators (Armbruster & Muchhala 2009).

Pollen transfer efficiency depends on the extent of contact between the pollinator with the plant's sexual organs. For example, hummingbirds are more efficient at transferring pollen when visiting *Penstemon barbatus*, a bird-pollinated plant, than when visiting *P. strictus*, a bee-pollinated plant, and this difference is partly due to there being a better fit between the visitor's morphology and the position of the plant's sexual organs within the flower (Castellanos, Wilson & Thomson 2003). Conversely, mismatches between the flowers and their visitors can preclude pollen deposition and result in the collection of rewards without providing pollination services (e.g. nectar or pollen theft; Hargreaves, Harder & Johnson 2009). For example, when the distance between the stigma and anthers in *Dalechampia magnoliifolia* blossoms is small, *Trigona* cf. *pallens* commonly touches the sexual organs and occasionally transfers pollen. However, when this distance is large this bee rarely makes contact with the stigma while still collecting pollen, and therefore acts as a pollen thief (Armbruster *et al.* 1989). In contrast to nectar-theft, which has been well documented, the occurrence of pollen theft has received little attention (Hargreaves, Harder & Johnson 2009).

Floral traits that typically facilitate pollen theft involve either spatial (herkogamy) or temporal (dichogamy) separation of sexual functions (Hargreaves, Harder & Johnson 2009). In the particular case of herkogamy, where sexual organs are separated, pollinators only make contact with one sexual organ at time or touch both but with different parts of their bodies, thus decreasing the pollen transfer efficiency. This problem is resolved in various ways in the different classes of herkogamy (Webb & Lloyd 1986). For example, in reciprocal herkogamy the male and female sex-organs are located in reciprocal positions between two or three floral morphs, which occur on the same or different plants (Barrett 2002; Webb & Lloyd 1986). This promotes precise pollen transfer between floral morphs without the cost associated with sexual interference and self-pollination (Barrett 2002), which is usually present in hermaphroditic plants that receive and export pollen in the same flower (Fetscher 2001). The evolution and function of reciprocal herkogamy have been well documented through study of heterostyly (style-length polymorphism). In contrast, enantiostyly (a kind of asymmetric polymorphism) is not yet well understood (Jesson & Barrett 2003).

1.1.1. Enantiostyly: the mystery of mirror-image flowers

Enantiostyly is the reciprocal deflection of the style to either the left or right side of the floral axis, resulting in mirror-image flowers (Jesson & Barrett 2002; Jesson & Barrett 2003; Webb & Lloyd 1986). Since the 19th century, enantiostyly has captured attention of many evolutionary biologists (including Darwin) that have been trying to understand the function and evolution of enantiostyly (Jesson & Barrett 2005). Enantiostylous species have been reported from at least ten unrelated families which indicates that this floral polymorphism has originated independently on numerous occasions in angiosperms (Jesson & Barrett 2003).

In most enantiostylous species an individual produces both left- and right-styled flowers, which is referred to as monomorphic enantiostyly. Furthermore, it is rare that an individual produces exclusively left- or right-styled flowers (dimorphic enantiostyly); this has only been recorded in three families (Barrett, Jesson & Baker 2000; Jesson & Barrett 2003). Phylogenetic evidence from monocots suggests that dimorphic enantiostyly arose from monomorphic enantiostyly, which itself evolved from a straight-styled ancestor (Jesson & Barrett 2003). However, it remains unknown in other taxa.

Enantiostyly has been interpreted as a mechanism by which the precision of cross-pollination is increased in bee-pollinated plants, by reducing pollination between flowers in the same plant (geitonogamy; Jesson & Barrett 2005). Some experiments have demonstrated that monomorphic enantiostyly reduces geitonogamous pollination compared with non-enantiostyly condition (Barrett, Jesson & Baker 2000; Jesson & Barrett 2005). However, dimorphic enantiostyly is the most efficient sort of enantiostyly, as it registers the lowest levels of geitonogamy (Barrett, Baker & Jesson 2000; Jesson & Barrett 2005). Despite advances in our understanding of the function and evolution of enantiostyly, yet little is known about the mating system (the contribution of self- and cross-fertilization to seed set; Barrett & Harder 1996; Charlesworth 2006) associated with this peculiar polymorphism under natural conditions.

Moreover, enantiostyly is usually associated with other traits such as the loss of nectaries and anther dimorphism (Jesson & Barrett 2003), and vibration pollen collection by bees (Barrett, Baker & Jesson 2000). This association with these traits suggests a pollination syndrome in which the position of the pollinator is important for the reproductive success of the plant (Barrett, Baker & Jesson 2000).

1.1.2. Heteranthery: morphological and functional anther dimorphism

Heteranthery (the presence of two or more types of anther in the same flower) is thought to have evolved, solving the potential conflict of using pollen both as the carrier of gametes for fertilization and as a reward to attract pollinators. The presence of two distinct morphological types of anther is considered to reflect the evolution of stamen morphology through specialization in the pollination and feeding functions (Vallejo-Marín *et al.* 2009). Feeding anthers are usually more attractive to pollinators that rely on bright colours and accessible placement when foraging (Luo, Zhang & Renner 2008). Vallejo-Marín *et al.* (2009) tested experimentally the “division of labour” hypothesis suggested by Müller (1883), where one set of stamens is specialized to reward pollinators and another set is specialized for fertilization. According to Müller’s hypothesis pollen-collecting bees would focus on feeding anthers to gather pollen, rather than on pollinating anthers, so that pollen from pollinating anthers is more successful in reaching stigmas than pollen from feeding anthers (Vallejo-Marín *et al.* 2009).

A recent theoretical model suggested that heteranthery can evolve when pollinators consume more pollen than a plant should provide in exchange for pollinator services. Furthermore, anther dimorphism causes a differential probability of pollen grooming between anthers types (Vallejo-Marín *et al.* 2009). Therefore, this model predicts that the evolution of heteranthery depends on pollinators acting as pollen thieves (Vallejo-Marín *et al.* 2010). However, pollen theft could affect plant fitness directly by reducing the amount of pollen available to ovules for fertilization, or indirectly by reducing the attractiveness of the flower, which affects the behaviour of legitimate pollinators that visit later (Hargreaves, Harder & Johnson 2009). One mechanism that may reduce the magnitude of the pollen theft is the restriction of access to pollen contained in poricidal anthers. This type of anther has small apical pores on the tip that keep the pollen hidden and relatively safe from pollen thieves because only the primary legitimate pollinators (usually buzzing-bees) are able to access the pollen by vibration (Buchmann 1983).

1.1.3. Poricidal anthers and buzz-pollination

Poricidal anthers are strongly associated with heterantherous plants and frequently involve buzz-pollination (Vallejo-Marín *et al.* 2010). The pollinators are usually bees that release pollen from the anthers through the vibration of their indirect flight muscles (Buchmann 1983). When a pollinator approaches enantiostylous and heterantherous flowers, it holds the feeding anthers and vibrates to extract the pollen. Then on one side of the pollinator's body the pollinating anther deposits pollen that will subsequently be deposited on the opposite floral morph (Whalen 1979).

Buzz-pollination is important for commercial crops including tomato (*Solanum lycopersicum*), eggplant (*S. melongena*), and red pepper (*Capsicum annum;* Raw 2000). Although buzz-pollinated flowers occur in species from 65 plant families *(*De Luca & Vallejo-Marín 2013), there are relatively few studies that have described, in detail, this buzz-pollination syndrome or have reported buzz-pollinators species (Arceo-Gómez *et al.* 2011; Bowers 1975; Buchmann & Hurley 1978; Buchmann 1983; De Luca *et al.*

2013; De Luca & Vallejo-Marín 2013; Duncan, Nicotra & Cunningham 2004; Gao *et al.* 2006; Harder & Barclay 1994; Kawai & Kudo 2009; Larson & Barrett 1999; Liu & Pemberton 2009; Michener 1962; Proenca 1992; Wanigasekara & Karunaratne 2012).

Buzz-pollinated plants usually have nectarless flowers, which offer pollen as the main or only reward to attract pollen-collecting insects (Buchmann & Hurley 1978; Buchmann 1983; Vallejo-Marín *et al.* 2010). Studies that described the pollination and reproductive biology of buzz-pollinated plants show that they are primarily visited by bees (Bowers 1975; Buchmann 1983; De Luca & Vallejo-Marín 2013; Duncan, Nicotra & Cunningham 2004; Gao *et al.* 2006; Kawai & Kudo 2009; Larson & Barrett 1999; Liu & Pemberton 2009; Michener 1962; Proenca 1992; Wanigasekara & Karunaratne 2012). Bees are the most commonly documented pollen thieves (Hargreaves, Harder & Johnson 2009), even though few studies have reported the incidence of pollen theft in buzz-pollinated plants (Bernhardt 1995; Snow & Roubik 1987).

Understanding the ecology and evolution of buzz-pollination systems is essential for gaining insight into aspects of pollination efficiency and the antagonistic relationships that arise from pollen theft in these specialized systems under natural conditions. To explore the pollination biology, mating system and the occurrence of pollen theft in a buzz-pollinated species with a specialized floral morphology in natural populations, I used as a model species *Solanum rostratum* (Solanaceae), a buzz-pollinated species with enantiostylous and heterantherous flowers.

1.1.4. Study species

Solanum is composed of approximately of 1400-1700 species, making it the largest genus in the Solanaceae and one of the largest genera of plants (Frodin 2004; Bohs

2005). This genus includes important crops such as tomato (*S. lycopersicum*), potato (*S. tuberosum*) and eggplant (*S. melongena*). *Solanum rostratum* Dunal is a self-compatible (Bowers 1975) annual herb that is incapable of vegetative reproduction and that grows in dry and disturbed habitats (Bassett & Munro 1985; Nee 1993). In North America, it is distributed from central Mexico where it is probably native (Whalen 1979) northwards to the Great Plains in the USA and in Canada as an invasive species (Bassett & Munro 1985; USDA 2013). However, *S. rostratum* is also currently spread around the world: in Asia, Europe, and Australia (The Global Biodiversity Information Facility 2013; Tropicos 2013; Whalen 1979).

Solanum rostratum bloom from the summer to the autumn in Mexico and the USA (Whalen 1979). Flowers open 30 minutes after dawn and remain open until late into the afternoon. The lifespan of the flowers is typically one day but bagged flowers continue opening for 4–5 days (Bowers 1975). Its bright yellow flowers do not secrete nectar, but do produce a fragrance (Bowers 1975) similar to the odour of peach fruit, and for this reason may has received its common name in Spanish, “duraznillo” (Nee 1993).

As with other *Solanum* species, the nectarless flowers of *S. rostratum* have five poricidal anthers (Stern, Weese & Bohs 2010; Todd 1882; Whalen 1979). Visitors of *S. rostratum* flowers often extract pollen from the poricidal anthers using vibrations (Bowers 1975) as with other buzz-pollinated species. Unlike most *Solanum* species, in which all the anthers within a flower are similar in size and shape and disposed in a central cone, *S. rostratum* is heterantherous with two sets of anthers (Bowers 1975; Müller 1883; Todd 1882; Vallejo-Marín *et al.* 2010). The first set, the feeding anthers, consists of four centrally-located yellow-coloured anthers which provide pollen for visiting insects (Fig. 1; Bowers 1975; Vallejo-Marín *et al.* 2009). The second set is a single larger anther the pollinating anther, which is sometimes darker-coloured (Fig. 1) and is usually ignored by visiting insects. The pollinating anther produces more than 50% of the total number of pollen grains produced by a flower and contributes disproportionately more to ovule fertilization (Bowers 1975; Todd 1882; Vallejo-Marín *et al.* 2009). Moreover, *S. rostratum* plants exhibit monomorphic enantiostyly, with 7-12 flowers that alternate in morph (left and right-styled; Fig. 1) along each inflorescence (Fig. 2; Whalen 1979; Todd 1882).

Pollinators of *S. rostratum* extract pollen from the feeding anthers by curling their bodies around the anther cone thus receiving pollen on their ventral surface. At the same time, the pollinating anther usually makes contact on the lateral or dorsal surface of pollinator’s abdomen, while the stigma makes contact on the opposite side of the pollinator (Bowers 1975). The sites of pollen deposition and collection alternate in the two enantiostylous floral morphs, promoting pollen transfer between flowers of different morph (Jesson & Barrett 2005). The necessity for buzzing-pollinators to come into close contact with the anthers, combined with differential pollen placement by the two types of anther on the visitor’s body, and the enantiostylous floral display increases the precision of both pollen deposition and receipt in *S. rostratum* (Jesson & Barrett 2005; Vallejo-Marín *et al.* 2010), resulting in a close interaction between flower and pollinator.

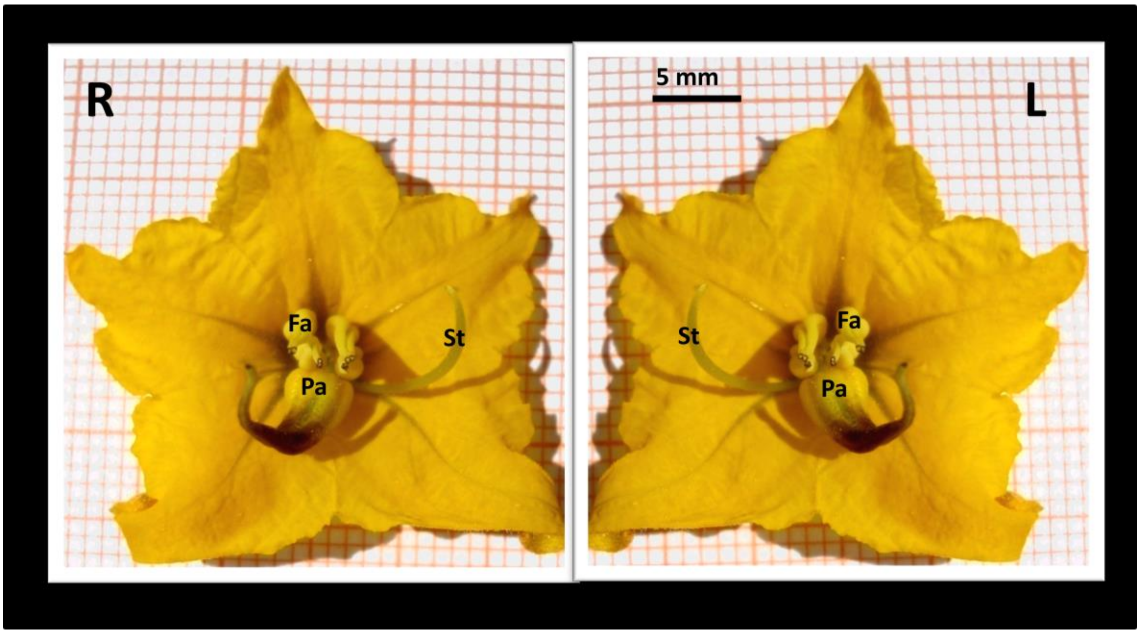

Fig. 1. The two floral morphs of *S. rostratum*: (R) right-styled and (L) left-styled. Fa = feeding anther, Pa = pollinating anther and St = style.

Despite the considerable research into this species and the dependency of this species on its pollinators in order to reproduce (Bowers 1975), few studies have described the pollination ecology of *S. rostratum* in North America. The few existing studies were done in the USA and Canada (Table 1) where the main pollinators observed were bumblebees (*Bombus spp*.). Furthermore, little is known about the pollination ecology in native populations of *S. rostratum* in Mexico (but see Table 1 García-Peña 1976). Mexico has a high diversity between 1800 and 2000 species of bees (Vergara & Ayala 2002). A population of a buzz-pollinated plant with a rich diversity of bees would provide a good opportunity for describing and quantifying the diversity of its pollinators and the incidence of pollen theft.

Fig. 2. Two flowers of alternate morph (left and right-styled) in an inflorescence of *S. rostratum* (foreground). At background an inflorescence with immature fruits at bottom and with flowers at the top.

In addition, when *S. rostratum* occurs as an introduced species, it provides the opportunity to investigate how the reproductive and mating system changes in novel environments with different pollinators. The ability of an alien plant to colonize a new environment depends significantly on its ability to reproduce in a novel pollination environment. For this reason, the reproductive system is considered to be an important trait in the life-history of a plant, because it can determine the demographic and genetic structure of invasive populations. Whether or not offspring arise from cross- or self-fertilization is important, because mates and pollinators may be limited during establishment or subsequent colonizing episodes (Barrett 2011).

Finally, *S. rostratum* is of agricultural interest as it is a host of pests that affect cultivated plants, not only in its introduced range but also in its native range. For example, in its native range this species is a natural host of the Colorado potato beetle (*Leptinotarsa decemlineata*), which is an important pest of potato plants (Brues 1940). In addition, the solanine produced in the leaves and fruit of *S. rostratum* is toxic for livestock, and can contaminate cereal grain (Parsons & Cuthbertson 2001). Understanding more about the pollination biology and mating system of this species would aid the development of suitable strategies for its management.

Table 1. Visitors to *S. rostratum* flowers in North America according to previous studies of reproductive biology.

Visitors	**Locality, Country.**	**Reference**
Bombus pennsylvanicus, B. scutellaris and *B. virginicus*	Texas, USA.	Harris & Kuchs 1902
Bombus sonorous, Caupolicana yarrowi, Centris atripes, C. caesalpiniae, Ptiloglossa jonesi, Protoxaea gloriosa and *Psaenythia mexicanorum*	Douglas, Cochise County, Arizona, USA	Linsley & Cazier 1963
Nomia tetrazonata, Protoxaea gloriosa and *Psaenythia mexicanorum*	Sonorita Creek near Patagonia, Santa Cruz County, Arizona, USA	Timberlake cited by Linsley & Cazier 1963
Ptiloglossa mexicana, Bombus pulcher, Colletes sp. Family: Halictidae and Subfamily: Anthophorinae	Pedregal de San Angél, D. F., Mexico	García-Peña 1976
B. pulcher, Colletes sp, Family: Andrenidae	Ciénega, Lerma and Río Mimbres, Durango, Mexico	Delgado Salinas cited by García-Peña 1976
Bombus americanorum, B. fraternus, Xylocopa sp., Anthophora sp., Augochlorella, Melissodes sp. and *Psaenythia sp.*	UOBS, Marshall County, Oklahoma, USA	Bowers 1975
SOUTH BASE: *B. americanorum, Augochloropsis metallica, Halictus selandonia, Augochlorella striata* and three species of *Dialictus*. LAKE THUNDERBIRD and LINDSEY ST.: *B. americanorum* and *Augochlorella*	Cleveland, Oklahoma, USA	
B. americanorum and *B. sonorous*	Uvalde, Texas, USA	
Colletes sp., A. metallica, Anthophora and *Bombus sp.*	Sonora, Sutton, Texas, USA	
Hemisia sp., A. metallica and *Bombus sp.*	Ft. Lancaster, Crockett, Texas, USA	
B. impatiens, B. nevadensis auricomus, B. americanorum and *Melissodes*	Cerro Gordo, Iowa, USA	
Bombus spp. (90% *B. impatiens*)	Toronto, Canada	Jesson & Barrett 2005

1.2. OBJECTIVES

The main goal of this research was to characterize the pollination biology, mating system and pollen theft occurrence in *Solanum rostratum*, a buzz-pollinated species that produces enantiostylous heterantherous flowers, under natural conditions. To achieve this goal, I studied the pollination ecology and mating system of natural populations of *S. rostratum* in North America, and conducted further studies in experimental arrays at the University of Stirling.

The four specific objectives of this research were as follows:

1. To characterize the pollination ecology of a buzz-pollinated herb, *Solanum rostratum*, to assess the extent to which seed production is limited by pollinator visitation, and to determine the diversity, behaviour and morphology of floral visitors, as well as their efficiency as pollinators.
2. To investigate whether the morphological fit between a pollinator's body and floral morphology influences pollen deposition in *S. rostratum*.
3. To develop genetic markers (microsatellites) using second generation sequencing in *S. rostratum*, as a tool to study its reproductive biology and genetic structure in natural populations.
4. To determine the mating system of *S. rostratum* across populations using the microsatellite markers developed, and to compare the outcrossing rates between the native and introduced range in North America.

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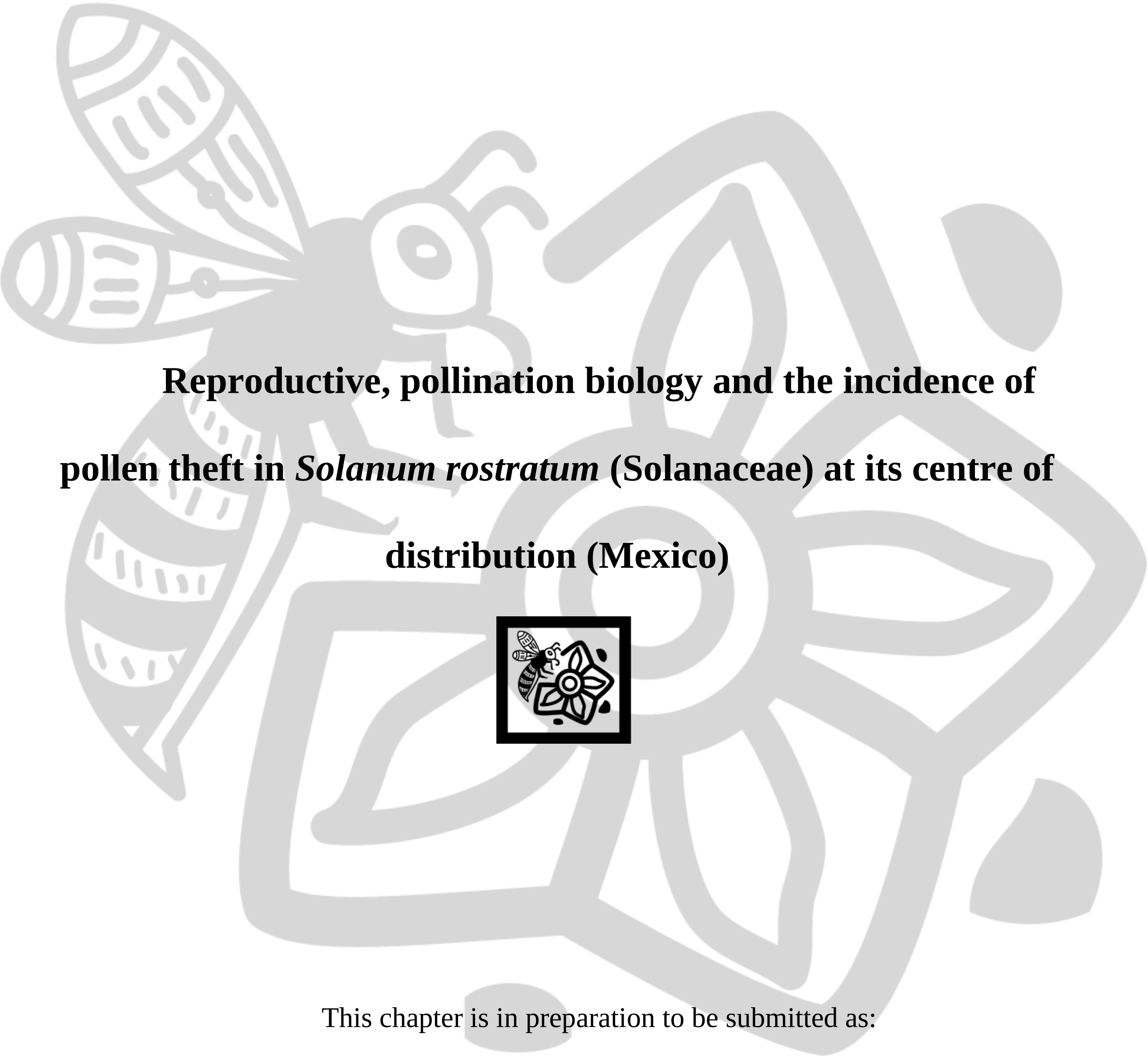
CHAPTER 2:



Reproductive, pollination biology and the incidence of pollen theft in *Solanum rostratum* (Solanaceae) at its centre of distribution (Mexico)

This chapter is in preparation to be submitted as:

Solís-Montero, L., C. H. Vergara & M. Vallejo-Marín. Strong reliance on pollinators for reproduction and high rates of visitation by pollen thieves in a buzz-pollinated plant. *Ecology*.

2.1. ABSTRACT

Buzz-pollinated plants are visited by pollen-eating insects, usually bees, which use vibrations to remove pollen from nectarless flowers. Mismatch between the flower and the visitor (i.e. due to a difference in size) precludes that visitor from making contact with the stigma, and who is thus functionally a pollen thief. To date, few studies have investigated whether or not visitors perform as pollinators or thieves based on size-matching between the visitors and the flowers in natural populations of buzz-pollinated species. In this study we characterized the reproductive ecology of the buzz-pollinated plant *Solanum rostratum*, to assess the extent to which fruit and seed set is limited by pollinator service. We determined the diversity, behaviour and morphology of floral visitors and characterized them according to their ability to produce fruits. We performed floral manipulations in six natural populations of *S. rostratum* to estimate the fruit and seed set (the percentage of flowers maturing into fruits and the number of seeds produced, respectively) in natural populations. In three of these populations, we carried out 115 hours of pollinator observations to quantify the incidence of floral visitors, and characterized them according to their behaviour and morphology. Finally, we measured the pollination efficiency under natural conditions of a subset of floral visitors based on fruit set. We found that *S. rostratum* is incapable of fruit production in the absence of pollinators. Fruit and seed set were limited by natural pollination services in all the populations studied. The majority of floral visitors were bees ranging from 0.9 to 9.8 mm in thorax diameter. Legitimate visitors that touched the stigma represented <20% of all floral visitors and were medium or large-sized buzzing bees. Illegitimate visitors that regularly failed to contact the stigma included buzzing and non-buzzing bees. These were significantly smaller in size and visited fewer flowers per bout, but stayed longer in each flower than legitimate visitors. The pollination efficiency experiment showed that mainly legitimate visitors produced fruits although some illegitimate visitors (e.g. *Apis mellifera*) occasionally made contact with the stigma resulting in low fruit production. Our study demonstrates the reliance of *S. rostratum* on insect visitation to set seed, and indicates that natural populations of this species experience moderate levels of pollen limitation. In addition, the majority of visitors to *S. rostratum* only act as pollen thieves.

Key words: Apoidea, buzz-pollination, Mexico, pollen limitation, pollen theft, *Solanum rostratum*.

2.2. INTRODUCTION

Buzz-pollination, pollination by insects that use vibrations to collect pollen, is widespread among the 20 000 species of flowering plants that release pollen through small pores or slits (Buchmann 1983; De Luca & Vallejo-Marín 2013). These plants usually have nectarless flowers which offer pollen as the main or only reward to attract pollen-collecting insects (Buchmann & Hurley 1978; Buchmann 1983; Vallejo-Marín *et al.* 2010). The principal visitors to plants with poricidal anthers are bees, which use high frequency vibrations produced with their indirect flight muscles to remove pollen from the anthers (Buchmann 1983).

Studies of pollination and reproductive biology of natural populations show that buzz-pollinated plants are visited primarily by bees of variable size (Bernhardt 1995; Duncan, Nicotra & Cunningham 2004; Gao *et al.* 2006; Kawai & Kudo 2009; Larson & Barrett 1999; Liu & Pemberton 2009). Some of these studies have shown that body size is an important characteristic that determines the ability of visitors to contact the stigma of visited flowers and thus be potential pollinators. For example, *Daniella revoluta* (Phormiaceae) is visited by mid-sized bees ranging from 5 mm to 13 mm in body length (e.g. *Lipotriches* and *Lasioglossum*) that usually make contact with the stigmas, whereas smaller bees are precluded from contacting the stigma (Duncan, Nicotra & Cunningham 2004). Moreover, *Solanum torvum*, an invasive species, is mainly visited by three bee species of different size (thorax width from 2.55 to 7.10 mm). The middle and large bees (*Xylocopa micans* and *Euglossa viridissima*) are efficient pollinators and induce the production of more fruits than smaller halictid bees. The low pollination efficiency of halictid bees has been interpreted as a result of the size-mismatch between flower and bee which often fails to contact the stigma (Liu & Pemberton 2009).

Bees visiting plants with poricidal anthers are not only diverse in their morphology but are also varied in their methods of pollen collection (Buchmann 1983). Typically, bees use vibrations (i.e. buzzing) to remove pollen which allow them to rapidly remove large quantities of pollen (Buchmann & Hurley 1978; Buchmann 1983). However, since many bees are able to use shivering for thermoregulation, these vibrations are not the result of their coevolving with flowers so as to obtain pollen, but instead have arisen for either behavioural or physiological reasons (Buchmann & Hurley 1978). Furthermore, non-buzzing bees (e.g. *Trigona*) access pollen by chewing the anther wall or simply glean pollen (e.g. *Apis mellifera*) previously extracted by other buzzing visitors (Buchmann 1983). Visitors to flowers with poricidal anthers are therefore varied in both morphology and behaviour.

The different morphology and behaviour of pollen-collecting bees may result in variation in their ability to transfer pollen from the anthers to the stigma, and therefore in their ability to effect pollination. For example, when small bees visit relatively large flowers they can remove pollen from poricidal anthers, but may fail to contact the stigma during the visit (Bernhardt 1995). If a bee deposits scarcely any of the pollen removed, or is unable to transfer it to conspecific stigmas then it is considered to be a pollen thief. Pollen theft can affect plant fitness directly by reducing the amount of pollen available for fertilizing ovules, or indirectly by reducing the attractiveness of robbed flowers, and thus affecting the visitation pattern of legitimate pollinators (Hargreaves, Harder & Johnson 2009).

Pollen theft is particularly widespread in bee-pollinated plants which usually offer pollen as the main reward. The pollen thieves can be classified into two types, habitual and conditional thieves, according to whether they consistently or occasionally fail to contact the sexual organs during a visit (Hargreaves, Harder & Johnson 2009). Habitual thieves generally fail to contact the stigma due to their size and/or behaviour. For example, bees that visit flowers of *Pseudobombax ellipticum* (Bombacaceae) primarily act as thieves because their small bodies and their patterns of foraging preclude them from making contact with the sexual organs while collecting pollen (Eguiarte, Del Rio & Arita 1987; Hargreaves, Harder & Johnson 2009).

In contrast, conditional thieves occasionally make contact with the stigma, and thus can deposit conspecific pollen. However, they usually fail to manipulate flowers legitimately, resulting in a low efficiency of pollen transfer. This occurs, for example, when honeybees (*A. mellifera*) visit protogynous flowers, where the stigma is receptive before the anthers expose pollen, in some cultivars of avocado (*Persea americana*). The small flowers of avocado lack a landing platform making flower manipulation difficult for mid-sized bees. However, nectar-collecting honeybees are efficient pollinators if they visit both stages of flowers: the pistillate flower that has a receptive stigma and the staminate flower that exposed pollen. Nevertheless, when honeybees are only collecting pollen they usually prefer the staminate flowers, and so do not deposit pollen in the pistillate flowers (Hargreaves, Harder & Johnson 2009; Isham & Eisikowitch 1993). The conditional thieves act as true thieves if plants are visited often by efficient pollinators, but they act as low-quality pollinators if they are the plants only visitor (Hargreaves, Harder & Johnson 2009). To date, few studies have attempted to quantify the incidence of pollen theft, or to systematically identify the characteristics that distinguish pollinators from pollen thieves in natural populations of buzz-pollinated plants.

In this chapter, we characterize the reproductive ecology of a buzz-pollinated herb, *Solanum rostratum* (Solanaceae), to assess the extent to which seed production is limited by pollinator visitation, and to determine the diversity, behaviour and morphology of floral visitors, as well as their efficiency to produce fruits. Specifically, we address five main questions: (1) Does *S. rostratum* depend on pollinators to reproduce? (2) Are natural populations of *S. rostratum* limited by pollen receipt? (3) What is the diversity of floral visitors and what is their efficiency as pollinators? (4) What is the proportion of pollinators to pollen thieves, and how do morphological and behavioural characteristics vary between these groups? (5) What are the reproductive consequences of variation in pollen theft frequency among populations? To answer these questions, we used the following methods.

2.3. METHODS

We studied six populations of *S. rostratum* from central Mexico between September 2009 and September 2011 (Table 1). We chose populations in different localities that were at least 43 km apart, to characterize the reproductive system of this species within its native range, with an overall distance of 470 km between the northernmost and southernmost populations sampled. We chose three of these populations to characterize the pollination biology. These three populations had different environmental conditions and were sampled in different years.

2.3.1. Reproductive biology of *S. rostratum*

2.3.1.1. Fruit and seed set

We characterized the reproductive system of six natural populations of *S. rostratum* using experimental manipulations carried out in Mexico between 2009 and 2011 (Table 1). In each population, we subjected individual flowers to the following four treatments as per Eckert *et al.* (2010): 1) **Emasculation** (**E**), where we removed the anthers before anthesis and used fine mesh bags to exclude pollinators. This treatment measured the ability to set seed without receiving pollen (agamospermy). 2) **Pollinator exclusion (Pe)**, where the anthers were left intact, but the flowers were covered with fine mesh netting before anthesis. This treatment was used to estimate the ability of plants to produce seed in the absence of pollinators. 3) **Pollen supplementation (Ps),** in which open pollinated flowers were supplemented with additional pollen extracted from the flowers of at least five individuals. Pollen was extracted from donor flowers using an electric toothbrush, collected in a 1.5 ml microcentrifuge vial and applied using a toothpick to the recipient flower. 4) **Open pollination (Op),** which consisted of unmanipulated flowers exposed to a natural pollination environment. Each of these experimental treatments was conducted on at least two flowers, on each of 15 individuals in six populations (2 x 15 x 6 = 180 flowers per treatment). Fruit set was recorded two weeks after applying the treatments. We recorded whether the flower had dropped (unsuccessful fertilization) or a fruit had begun to form (successful fertilization). If a fruit was forming, it was bagged to prevent seed loss after maturation and seeds were collected and counted approximately six weeks later.

Table 1. Description of six populations of *S. rostratum* studied in the centre of Mexico.

Pop. Code	Population	Latitude (N)	Longitude (W)	Elevation (m)	Population size	Date
CU	Ciudad Universitaria, Reserva del Pedregal, Distrito Federal.	19.32°	99.19°	2311	250 150	Sep-Dec 2009 Ago-Sep 2011
DHG	Dolores Hidalgo, Guanajuato.	21.17°	100.90°	1891	50	Sep 2011
LP	Libres, Puebla.	19.47°	97.67°	2373	1500	Jun-Aug 2011
SLG	San Luis la Paz, Guanajuato.	21.31°	100.51°	2002	50	Sep 2011
TEM	Teotihuacán, Estado de México.	19.68°	98.84°	2284	150	Sep 2011
TP	Zapotitlán de Salinas, Puebla.	18.33°	97.57°	1670	500	Oct-Nov 2010

2.3.1.2. Pollen limitation

To determine if natural populations were pollen limited, we used the pollen limitation index (L) proposed by Larson & Barrett (2000): L = 1 − (Op/Ps), where Op is the fruit or seed set in the open pollination treatment, and Ps is the fruit or seed set in the pollen supplementation treatment. We calculated the value of the index for each individual plant for fruit set (L_FS), seed set (L_SS) and pre-dispersal fitness (L_Wpre). We excluded the individuals that missed one of these two treatments (Op or Ps). We calculated the index for pre-dispersal fitness as L_Wpre = 1 − (Wpre_Op/Wpre_Ps), where Wpre_Op is the product of fruit set and the mean number of seeds per plant in the open pollination treatment, and Wpre_Ps is the equivalent calculated with pollen supplementation treatment results. We subsequently calculated the mean value of each L index for each population (Table 2).

In two cases we changed the formula for calculating the L index for an individual to L = (Ps/Op) – 1; this was done to correct the following errors: a) when dividing a non-zero value by zero, which would yield an undefined value or b) when the numerator is higher than the denominator, which would lead an overestimation of negative values. When the numerator and the denominator were both zero, we treated this as a missing value. The values of pollen limitation index ranged from −1 to +1, where negative and zero values mean non-pollen limitation while positive values mean pollen limitation.

2.3.2. Pollination biology of *S. rostratum*

2.3.2.1. Diversity and relative abundance of floral visitors

In order to describe the composition of visitors to *S. rostratum*, we conducted 115 hours of pollinator observations in three populations (CU, LP and TP; see Table 1). We chose three populations that represent different environmental conditions in Mexico. Population CU was from a perturbed area inside the Pedregal de San Ángel nature reserve. This urban nature reserve has a temperate climate (mean temperature of 15.6°C) and it is located inside one of the largest metropolises in the world (Mexico City; Castillo *et al.* 2007). Population TP was located in an abandoned field at the indigenous town located inside the Tehuacán-Cuicatlán nature reserve. This nature reserve has probably the highest biological diversity for an arid zone in North America (Casas *et al.* 2001). Finally, population LP was located in a rural area with relatively few human inhabitants (12 249 persons) and contains regions with a temperate and semiarid climate (INEGI 2003).

In these three populations, we recorded the visitation rate during between five and eight 30-minute periods throughout the day (07:00–19:00). The observation intervals were established by conducting preliminary observations during 12-hour periods to determine the period of activity of floral visitors to *S. rostratum*. For populations LP and TP we recorded at five time points between 09:30 and 16:00 during five days (from 21th July to 5th August 2011) and seven days (from 9th to 15th October 2010), respectively. In population CU, visitation started earlier (from 07:30) and finished around 18:30. Thus, we conducted eight observation periods per day during seven days (from 11th August to 09th September 2011). Observations were made of a 5 × 5 m quadrat by between one and three observers (during the peak visitation there was more than one observer). First, we located a big patch of *S. rostratum* plants and sampling quadrats were distributed haphazardly in this patch. The vegetation that surrounded each patch depended on the environmental condition and flora composition of each studied site. We conducted 35, 37.5, and 43 hours of observations in populations TP, LP, and CU, respectively (Table 3). To evaluate the diversity of visitors, we calculated Shannon's index of diversity for each population.

2.3.2.2. Morphological characteristics of floral visitors

To describe the morphological characteristics of floral visitors, we captured and measured at least ten individuals per bee species (from one to four in rare species) at the end of the visitation bout (i.e. when the visitor finished foraging in a sampling quadrat). All visitors captured were cooled on ice, measured and then released. The following measurements were taken with digital callipers: **L** = length of the visitor (from the top of the head to the tip of the abdomen); **TL** = thorax length; **TW** = thorax width (across the centre of the thorax); **AL =** abdomen length; and **AW** = abdomen width (across the centre of the abdomen). Four individuals of each species were identified and deposited as voucher specimens in the entomology collection of the Universidad de las Américas, Puebla (UDLA-P), Mexico.

2.3.2.3. Behaviour of floral visitors

For each visitor that arrived at a quadrat we recorded the following data: **A)** the morphospecies of the visitor; **B)** whether the visitor produced an audible buzz; **C)** whether the visitor touched the sexual organs of the plant, grouped in the following categories: **NC** = no contact with any sexual organs, petals only; **FA** = contact with the feeding anthers; **PA** = contact with the pollinating anthers; and **ST** = contact with the stigma/style. These categories were combined to describe different types of floral visits, e.g. when a visitor made contact with the pollinating anther and the stigma (PA, ST). In addition, we recorded: **D)** the number of flowers visited per bout and **E)** the length of each floral visit recorded with a chronometer. We defined a visitor as any insect that visited a flower and visit length as the period from the visitor landing to it leaving the flower.

To explore whether bees have specialized in collecting only *S. rostratum* pollen or if they collect pollen from different species, we removed the pollen load from the corbiculae (pollen baskets) and from the body of visitors in three of the populations (CU, LP and TP). Pollen was removed using a pair of forceps and placed in an eppendorf tube with 70% ethanol. In the laboratory we mounted a drop for each pollen samples in glycerine jelly on microscope slides (Kearns & Inouye 1993). We counted 100 pollen grains by light microscopy (Dialux 20EB, Leitz) and we calculated the proportion of pollen grains belonging to *S. rostratum* vs. non-*S. rostratum* pollen (Table 3). In the case that a preparation contained fewer than 100 pollen grains, we centrifuged the remaining sample for 2 min at 1800 rpm and used the sediment to prepare a new preparation with the same method described above, until we had counted 100 pollen grains.

2.3.2.4. Classification of visitors into legitimate and illegitimate categories

Firstly, we classified each floral visit depending on whether the visitor made contact with the sexual organs during the visit, into one of two categories: legitimate or illegitimate. We considered a floral visit as legitimate if the visitor touched the stigma and as illegitimate if the visitor did not touch the stigma (Table 3). We excluded from the statistical analysis those cases where visitors did not contact (NC) any sexual organs. We then characterized each species of visitor as legitimate or illegitimate according to the proportion of all floral visits (legitimate + illegitimate visits) that were legitimate. For the majority of species, less than 20% of visitors conducted a legitimate visit, and for only a few species were more than half of the visitors legitimate (Table 4). According to this information, we decided to classify a species as a legitimate visitor if at least 50% of individuals made contact with the stigma.

Secondly, we calculated the proportion of pollinators to thieves in each population and the number of visits conducted by each group. In order to obtain this information, we classified a visitor as a pollinator or thief according to the results of the pollination efficiency experiment (described below) and the proportion of legitimate visits. On the one hand, we considered a species to be a pollinator or legitimate visitor if the visitor usually touched the stigma and was thus capable of producing fruit. On the other hand, we classified the remaining species, which were pollen thieves (illegitimate visitors), into two groups: conditional thieves (poor pollinators) where the visitor occasionally touched the stigma or produced few fruits (such as *A. mellifera*), and habitual thieves where it rarely touched the stigma and was unable to induce fruit production. We test the hypothesis that pollinators of *S. rostratum* are large buzzing bees that conduct many brief visits per bout (see section 2.3.3 for details of this analysis).

2.3.2.5. Pollination efficiency experiment

To test the efficiency of the legitimate and illegitimate visitors in triggering fruit set, we conducted a pollination efficiency experiment using population CU. We enclosed inflorescences with only floral buds using big bags of fine-mesh (30 × 20 cm) and we used at least ten inflorescences per sampling quadrat. Later opened flowers of bagged inflorescences were exposed to visitors during the peak hours of observed visits for 30-minute periods (from 10:00 to 13:30). The flowers visited were labelled with paper tags with the visitor's identity and then isolated again, in smaller bags (10 × 10 cm), to avoid contamination by other visitors. At the end of the period, we labelled the unvisited flowers on the same inflorescence as a control and isolated them with the small bags until senescence. Next we enclosed the whole inflorescence again with a big bag. We repeated the exposure of the inflorescence to visitors for several days (from 22th August to 9th September 2011) and then recorded the extent of fruit set six weeks later.

2.3.3. Statistical analyses

All analyses were conducted using the statistical program *R* version 3.0.1 (R Core Development Team 2013). First, we calculated 95% confidence intervals by bootstrapping with 1000 permutations in order to validate the pollen limitation index (L) (Gomez *et al.* 2010) using the *boot* package in *R* (Canty & Ripley 2014). We subsequently analysed the morphological measurements of visitors using principal component analysis (PCA) based on correlations matrix, to determine which measurement best characterized visitor size. We used the first principal component (PC1) as an estimator of visitor size because this summarized most of the variation in the visitor's morphology and effectively characterized the size of the bees. To graph we used the thorax width as an estimator of the visitor's size because this measurement was strongly correlated (0.94) with PC1 and it is commonly used for comparing among bee sizes (Bullock, Del Rio & Ayala 1989; Goulson & Sparrow 2009; Goulson 2010; Peat

& Goulson 2005; Peat, Tucker & Goulson 2005). Finally, to compare the size of visitors among species we used an analysis of variance (ANOVA) of the PC1 scores and a Tukey as a post hoc test.

We next tested the hypothesis that legitimate visitors of *S. rostratum* are large buzzing bees that conducting brief and many visits per bout. All data from pollination observations were analysed using separate linear models (LM) or generalized linear models (GLM). Each model used a separate response variable as follows: the length of visit (the mean length of visits per individual visitor), the bout length (the number of flowers visited per individual visitor) and the size of the visitor (mean of the PC1 scores per species per population). The length of visit and the bout length were log-transformed prior to GLM analysis and fitted to a Gaussian error term. To evaluate visitor size, we used a LM because PCA assumes normality. All models used the type of visitor (legitimate and illegitimate) as the explanatory variable; these categories were determined per species according to the number of legitimate visits as a proportion of the total number of visits. Next, we conducted a chi-squared test on the GLMs and ANOVA on the LM to determine if visitor categories differed with respect the response variables. Outliers were excluded from each model in order to improve them. We excluded four records from the GLMs where the length of visit (1260 sec and 1140 sec from *A. mellifera,* and 83 sec from *Thygater analis*) or an unusual record of the bout length (143 visits by an *A. mellifera*).

Finally, we used chi-squared tests to compare fruit production between legitimate and illegitimate visitors of the pollen efficiency experiment, with buzzing and non-buzzing visitors considered separately. Also, we used a chi-squared test to compare the preference of pollen thieves for stealing pollen from feeding anthers, pollinating anthers or both types of anther.

2.4. RESULTS

2.4.1. Reproductive biology of *S. rostratum*

Solanum rostratum strongly depended on being visited by pollinators to produce seeds, because it did not produce seed autonomously (non-fruit production in Pe treatment, n = 153 flowers, populations CU in 2009 & 2011, LP in 2011 and TP in 2010) and it also lacked the capacity to set seed through agamospermy (non-fruit production in E treatment, n = 61 flowers, populations CU in 2009 and TP in 2010).

Furthermore, all native populations studied in Mexico were pollen limited in their fruit production (L_FS = 0.23 ± 0.06, CI = 0.1620–0.3815) and seed production (L_SS = 0.24 ± 0.06, CI = 0.2052–0.4458), except for population DHG, which was not pollen limited for seed set, although this was not statistically significant (Table 2). All sampled populations were pollen limited at the predispersal fitness level (L_Wpre = 0.33 ± 0.07, CI = 0.2334–0.5273), where we considered both the fruit and seed set to calculate the L index. The magnitude of pollen limitation was high and statistically significant in populations CU, LP and TEM, where seed set contributed heavily to the L_Wpre index (Table 2).

The main visitors of *S. rostratum* were bees (Hymenoptera: Apoidea) in the studied populations in the centre of Mexico. We rarely found flies (Diptera) visiting *S. rostratum* (Table 3). Other orders such as Hemiptera, Coleoptera and Lepidoptera were recorded in population TP, but were excluded from the analyses. These orders were also excluded from the pollination observations because they were scarce in the rest of the populations, never touched the sexual organs and did not buzz. In the particular case of the Coleoptera, beetles were observed eating the corolla and/or the anthers.

The diversity of visitors differed among the studied populations of *S. rostratum.* Population TP had the highest diversity index (Shannon index = 1.85), followed by population CU (Shannon index = 1.57), and population LP had the lowest diversity index (Shannon index = 0.66). The variation in these index values was probably related to the different environmental conditions and/or temporal variations in the pollinator fauna at the study sites. Population TP was located inside a nature reserve, which was probably the most conserved site studied, followed by population CU, which was in an urban nature reserve. The most perturbed site was probably population LP, which was located in a rural area. The genera *Apis*, *Lasioglossum* and *Xylocopa* were found at all sites sampled (Table 3).

The visitor's abundance differed among populations: at CU *A. mellifera* and *Exomalopsis mellipes* represented more than half of the visitors; at LP *A. mellifera* was the most abundant visitor (84% of the total); and at TP the most abundant visitors were *Augochlorella neglectula*, *Exomalopsis pueblana, Augocholora sp.* and *Pseudaugochlora graminea* (Table 3). The most abundant species also conducted the majority of visits in their corresponding populations. However, at CU we found that rare species (such as *Thygater analis* and *Xylocopa sp.*) conducted the same number of visits as common species (Table 3). At these three populations, rare species such as *T. analis, Xylocopa sp., Centris zacateca* and *C. mexicana* visited more flowers per bout (i.e. bout length) than the other species (Table 3).

The duration of visits differed among species. Some species (*T. analis*, *Xylocopa sp.* and *Centris spp.*) spent just a few seconds (1–2 sec) in each visit, compared with other species that spent more time (4–52 sec) per visit (Table 3, $F_{15,8725} = 616.55$, $P < 0.00001$). We found that almost all visitors of *Centris*, *Thygather* and *Xylocopa* buzzed when extracting pollen from flowers of *S. rostratum*. For other genera such as *Lasioglossum* (subgenera *Lasioglossum* and *Dialictus*), only some individuals buzzed when visiting. In contrast, *A. mellifera*, *Augocholora sp.* and species of Syrphidae did not buzz when collecting the reward (Table 3).

2.4.2. Morphological and behavioural characteristics of legitimate and illegitimate visitors

2.4.2.1. Contact with the sexual organs

Xylocopa sp., *Centris spp.* and *T. analis* (Fig. 1) were considered as legitimate visitors because the majority of individuals touched the stigma and the anthers of *S. rostratum* flowers (Table 4). In the specific case of the genus *Centris*, we only recorded two individuals and therefore need greater sampling effort of this genus to substantiate this finding. Previous studies have reported that *Centris* is a pollinator of other buzz-pollinated plants (Moco & Pinheiro 1999; Snow & Roubik 1987). The remaining bee species were considered to be illegitimate visitors because the majority of individuals did not touch the stigma and may thus be considered as pollen thieves (Table 4).

Most of the illegitimate visitors stole mostly from the feeding anthers (55% of the total of visitors), rather than the pollinating anthers (5% of the total of visitors) or from both sort of anthers (40% of the total of visitors) [comparison among all types of anther $\chi^2 = 38.52$, df = 2, n = 686, $P < 0.0001$; comparison between FA vs. FA&PA $\chi^2 =$

16.34, df = 1, n = 649, $P < 0.0001$]. In addition, all legitimate and illegitimate visitors had a high percentage of *S. rostratum* pollen (73-100%) in the corbiculae or on their bodies (Table 3).

2.4.2.2. Visitor morphology

In the PCA of the morphological measurements of visitors, PC1 explained 89% of the variance. It was interpreted as the visitor's size because all eigenvectors had similar values (TL = 0.4483, TW = 0.4505, AL = 0.4493, AW= 0.4373, L = 0.4503). Similar sizes of visitors (small, medium and large-sized visitors) were present in all populations. The range of sizes differed among populations, for example the largest visitor of population TP was bigger than the largest visitor of population CU ($F_{17,100} = 143.11$ $P < 0.0001$).

The legitimate visitors of *S. rostratum* were large-sized bees ($F_{1,906} = 714$, $P < 0.0001$) which were large enough to touch the sexual organs of the visited flower in one visit, spent only a few seconds per flower on each visit ($\chi^2 = 401.90$, df = 1, $P < 0.0001$) and visited many flowers per bout ($\chi^2 = 157.75$, df = 1, $P < 0.0001$; Fig. 2). In contrast, the illegitimate visitors were small bees that usually did not touch the sexual organs when visiting a flower, spent considerable time stealing the reward pollen and visited few flowers per bout

Table 2. Fruit set—the percentage of flowers maturing into fruits—, and seed set—mean number of seeds ± standard error (SE)— in six populations of *Solanum rostratum* in central Mexico. Flowers were exposed to natural pollination conditions either with (pollen supplementation treatment) or without (open pollination) addition of supplemental outcross-pollen. The mean value of the pollen limitation index (L) was calculated for each population for fruit set (L_FS), seed set (L_SS) and predispersal fitness (L_Wpre) using the formulas provided by Larson & Barrett (2000); the means are reported ± S.E. Values of L in bold are statistically significant when the confidence interval (CI) did not cross zero (the 95% CI were generated by bootstrapping analysis with 1000 permutations). The values in parentheses are: the number of flowers per treatment for the fruit set and the number of fruits counted in each population for the seed set.

	Population												
	CU			**DHG**		**LP**		**SLG**		**TEM**		**TP**	
	Fruit set 2009	Fruit set 2011	Seed set 2011	Fruit set 2011	Seed set	Fruit set 2011	Seed set	Fruit set 2011	Seed set	Fruit set 2011	Seed set	Fruit set 2010	Seed set
Pollen supplementation	54.8 (42)	67.9 (28)	65.1 ± 3.5 (17)	70.4 (27)	55.6 ± 3.9 (15)	92.9 (28)	57.6 ± 3.1 (24)	50.6 (28)	65.4 ± 6.2 (8)	72.7 (44)	50.1 ± 3 (27)	67.9 (28)	58.2 ± 6.7 (16)
Open pollination	42.2 (71)	50 (30)	63.5 ± 3.8 (15)	51.2 (43)	62.7 ± 3.1 (18)	62.1 (29)	46.1 ± 3.9 (17)	41.4 (29)	57.4 ± 6.2 (8)	47.4 (38)	39.1 ± 4.1 (14)	56.7 (30)	52.6 ± 4.8 (15)
L_FS/L_SS	0.19 ±0.17	**0.29** ±0.13	**0.34** ±0.12	**0.19** ±0.17	-0.41 ±0.13	**0.33** ±0.12	**0.39** ±0.12	0.17 ±0.19	0.43 ±0.17	**0.34** ±0.12	**0.48** ±0.12	0.11 ±0.14	0.15 ±0.18
L_Wpre	**0.45** ±0.13			0.07 ±0.22		**0.43** ±0.13		0.31 ±0.25		**0.51** ±0.13		0.19 ±0.19	

Table 3. Identity and characteristics of floral visitors of *Solanum rostratum* in three populations in central Mexico.

Visitor Code	No. individuals	Number of floral visits	% Total visits	Bout length ± S.E.	Length of visit (sec) ± S.E.	% individuals buzzing	% *S. rostratum* pollen ± S.E.	Thorax width (mm) ± S.E.
Pedregal, Mexico City (CU)								
Apis	139	1553	31.7	11.2 ± 1.1	13.3 ± 1.2	0	98.5 ± 0.5	3.9±0.1
Em	133	813	16.6	6.1 ± 0.5	16.4 ± 0.7	71	93.2 ± 2.1	3.1±0.1
LDsp	29	79	1.6	2.7 ± 0.4	22.2 ± 5	11	NA	1.5±0.2
Lj	37	139	2.8	3.8 ± 0.6	14.7 ± 1.5	19	89.2 ± 4.9	2.6±0.3
Ta	62	1622	33.1	26.2 ± 2.8	1.2 ± 0.05	98	83.3 ± 11.1	5.1±0.2
Xsp	25	697	14.2	27.9 ± 3.4	1.3 ± 0.03	100	92.5 ± 1.5	7.4±0.9
Total	**425 individuals**	**4903 visits**	**100%**	**425 visitors**	**4903 visits**	**403 visitors**	**26 visitors**	**37 visitors**
Libres, Puebla (LP)								
Apis	97	1810	95.8	18.7 ± 2.3	8 ± 0.2	0	98.7 ± 0.7	3.2±0.1
Asp	3	13	0.7	4.3 ± 2.0	4.1 ± 0.9	0	73.0	1.8
LLsp	8	19	1.0	2.4 ± 0.9	25.2 ± 7.3	37	100.0	3.4
Syr	4	20	1.0	5.0 ± 2.5	52.4 ± 10.3	0	NA	1.7±0.04
Xsp	4	28	1.5	7.0 ± 4.3	1.1 ± 0.14	100	NA	NA
Total	**116 individuals**	**1890 visits**	**100%**	**116 visitors**	**1890 visits**	**84 visitors**	**11 visitors**	**20 visitors**
Zapotitlán Salinas, Puebla (TP)								
Am	16	132	6.8	8.2 ± 2.0	10.6 ± 0.8	100	NA	2.9 ±0.1
An	87	342	17.6	3.9 ± 0.4	18.2 ± 1	99	78.9 ± 15.7	1.8±0.3
Apis	2	3	0.1	1.5 ± 0.5	2 ± 0	0	NA	NA
Asp/Pg	76	558	28.7	7.3 ± 0.8	12 ± 0.5	98	93.3 ± 3.0	2.7±0.1
Cm/Cz	2	43	2.2	21.5 ± 8.5	2.1 ± 0.04	100	97.0	5.3±0.2
Em	26	128	6.6	4.9 ± 0.8	12.2 ± 1	100	88.4 ± 6.3	3.7±0.2
Ep	102	459	23.6	4.5 ± 0.4	13.2 ±0.5	100	93.0 ± 2.8	3.3±0.1
LDsp	33	90	4.6	2.7 ± 0.4	22.2 ± 2.7	54	NA	1.0±0.2
LLsp	28	102	5.2	3.6 ± 0.6	13.9 ± 1.2	96	91.0 ± 8.0	2.3±0.3
Xc	4	90	4.6	22.5 ± 19.9	1.5 ± 0.06	100	NA	10.10
Total	**376 individuals**	**1947 visits**	**100%**	**376 visitors**	**1947 visits**	**325 visitors**	**24 visitors**	**61 visitors**

No. individuals = number of insects visiting the flowers during the observation period. A single insect was followed since it entered the flower patch until it left (a visitation bout). **Number of floral visits** = total number of flowers visited per species; a visit was recorded if the insect landed on the flower regardless of the amount of time it spent there. **% Total visits** = percentage of visits relative to the total visits recorded in each population. **Bout length** = mean number of flowers visited ± standard error, per species**;** mean was calculated as individuals per species, where we considered each visitor one visitation bout. **Length of visit in seconds** = mean length of visit ± standard error, per species; the time that visitors spend in each visit from when they land on a flower until they leave it. **% Individuals buzzing** = percentage of individuals that produced vibrations while stationary in the flower, per species. **% *S. rostratum* pollen** = mean percentage of pollen grains from *S. rostratum* ± standard error, per species**;** samples were collected from the pollen carried by the insect at the end of the visitation bout. **Thorax width** = mean thorax width of visitors in mm ± standard error, per species; measured below the point of insertion of the wings this was measured in a subsample of visitors per population. The observation hours for each population are as follows: CU: 43 hours; LP: 37.5 hours; TP: 35 hours. Visitor codes are provided in Table 4

Table 4. Classification of the legitimate and illegitimate visitors in three populations (CU, LP and TP) in central Mexico. Proportion of legitimate visits where visitor only contacted the stigma, both types of anther and the stigma (FA, PA, ST), or one sort of anther (feeding or pollinating anther) and the stigma (FA/PA, ST) out of the total number of visits including legitimate and illegitimate visits [if the visitor only contacted the feeding anthers (FA), the pollinating anther (PA) or both anthers (FA, PA)]. *In population TP we pooled observations of visits by *Augloclora sp.* with *P. graminea* because these species were difficult to distinguish in field.

		Sexual organ contacted during visit								
		Anthers and stigma		Only anthers			Only stigma			
Bee species		FA/PA, ST	FA,PA, ST	FA	PA	FA, PA		Total visits contacting stigma and anthers	Total visits observed	Proportion of legitimate visits
Legitimate visitors	CODE									
Xylocopa cyanea	Xc	0	3	0	0	0	0	3	3	1
Xylocopa sp.	Xsp	0	26	0	0	0	0	26	26	1
Thygater analis	Ta	0	49	1	3	4	0	49	57	0.86
Centris mexicana/ Centris zacateca	Cm/Cz	0	1	0	0	1	0	1	2	0.5
Sub-Total		**0**	**79**	**1**	**3**	**5**	**0**	**79**	**88**	**0.9**
Illegitimate visitors										
Apis mellifera	Apis	5	29	108	2	53	1	35	198	0.18
Exomalopsis mellipes	Em	0	17	23	15	95	0	17	150	0.11
Lasioglossum (Dialictus)sp.	LDsp	1	3	30	5	19	0	4	58	0.07
Lasioglossum jubatum	Lj	0	1	25	4	6	0	1	36	0.03
Augochlorella neglectula	An	1	0	57	3	22	0	1	83	0.01
Augochlora sp.	Asp	0	0	3	0	0	0	0	3	0
Augochloropsis metallica	Am	0	0	12	0	2	0	0	14	0
Exomalopsis pueblana	Ep	0	0	33	5	56	0	0	94	0
Lasioglossum (Lasioglossum)sp.	LLsp	0	0	26	0	7	0	0	33	0
*Pseudaugochlora graminea**	Pg	0	0	58	0	6	0	0	64	0
Sub-Total		**7**	**50**	**375**	**34**	**266**	**1**	**58**	**733**	**0.08**
Grand Total		**7**	**129**	**376**	**37**	**271**	**1**	**137**	**821**	**0.17**

2.4.2.3. Proportion of legitimate versus illegitimate visitors in natural populations

Population CU had proportionally more legitimate visitors (20% of the total number of visitors at CU) than the rest of the populations. These visitors accounted for 47% of the total of visits observed in this population. By comparison, populations LP and TP had a smaller proportion of legitimate visitors (3% and 2% of the total number of visitors at LP and TP, respectively), and which accounted for only 1.5% and 7% of the total of visits observed in these populations, respectively. *Apis mellifera* was the most abundant visitor in population LP and almost all recorded visits (97%) in this population were accounted by this species. While *A. mellifera* was abundant at site CU, it accounted for only 32% of floral visits there. Furthermore, *A. mellifera* was also abundant at site TP, it was rarely observed visiting *S. rostratum* flowers (only two honeybees). At this study site honeybees were observed visiting other plant species instead, such as *Leucaena sp.*, *Physalis sp.* and some species of Asteraceae (L. S. M. personal observation). Other illegitimate visitors conducted 93% of the visits recorded in this population (Fig. 3).

2.4.3. Pollination efficiency experiment

The pollen efficiency experiment suggested that the legitimate visitors (*Xylocopa sp.* and *Thygather analis*) mainly function as pollinators, because 33% of visited flowers produced fruit (n = 12 flowers). In the particular case of *A. mellifera,* a non-buzzing visitor, 18% of visited flowers produced fruit (n = 28 flowers). This result is similar to the visitor’s contact result, where *A. mellifera* occasionally (18% of visits) touched the

Fig. 1. The legitimate (Left: *Thygater analis*) and illegitimate visitors (Right: *Augocloropsis metallica*) of *S. rostratum* in Ciudad Universitaria, Reserva del Pedregal, Mexico City (CU) and Zapotitlan de Salinas, Puebla (TP). Photographs by LSM and CSM.

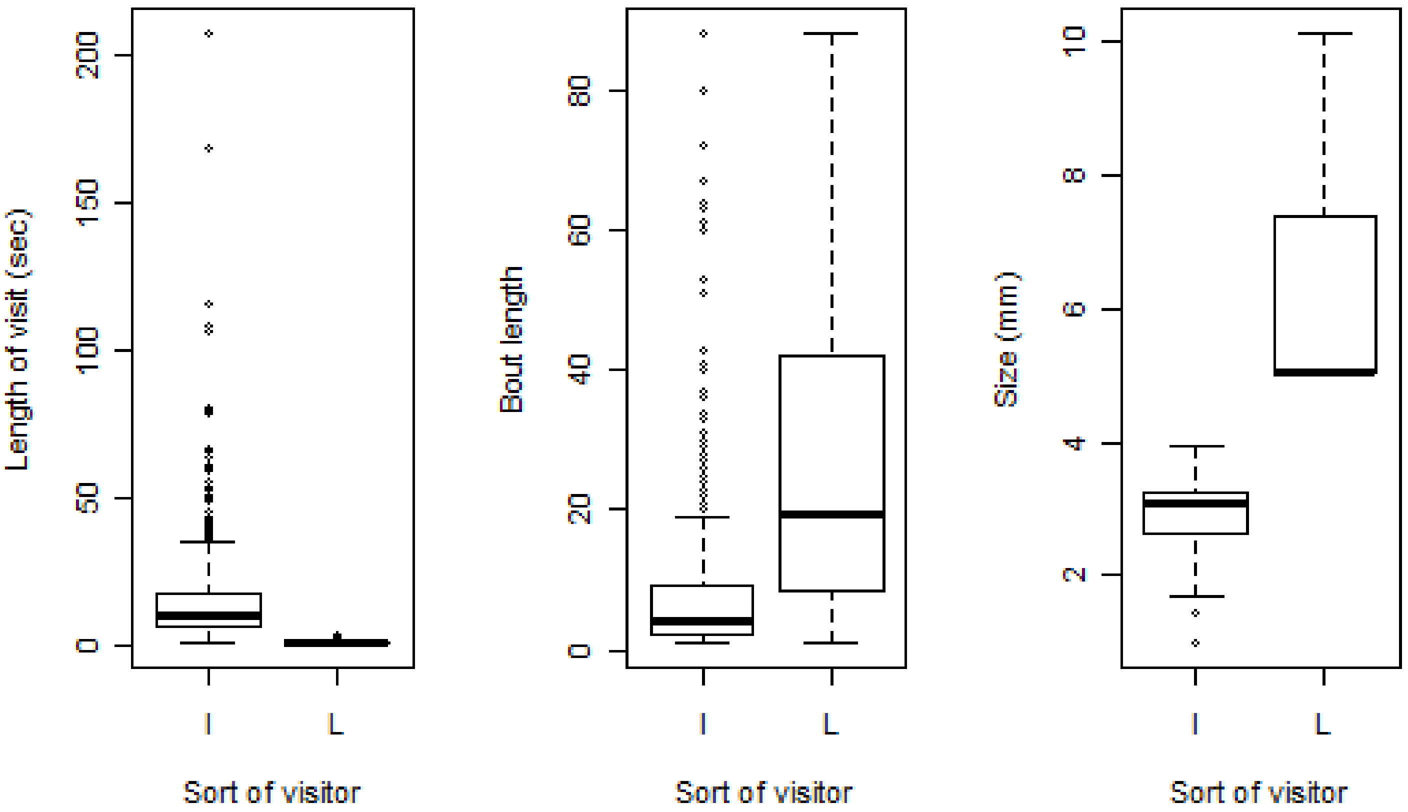
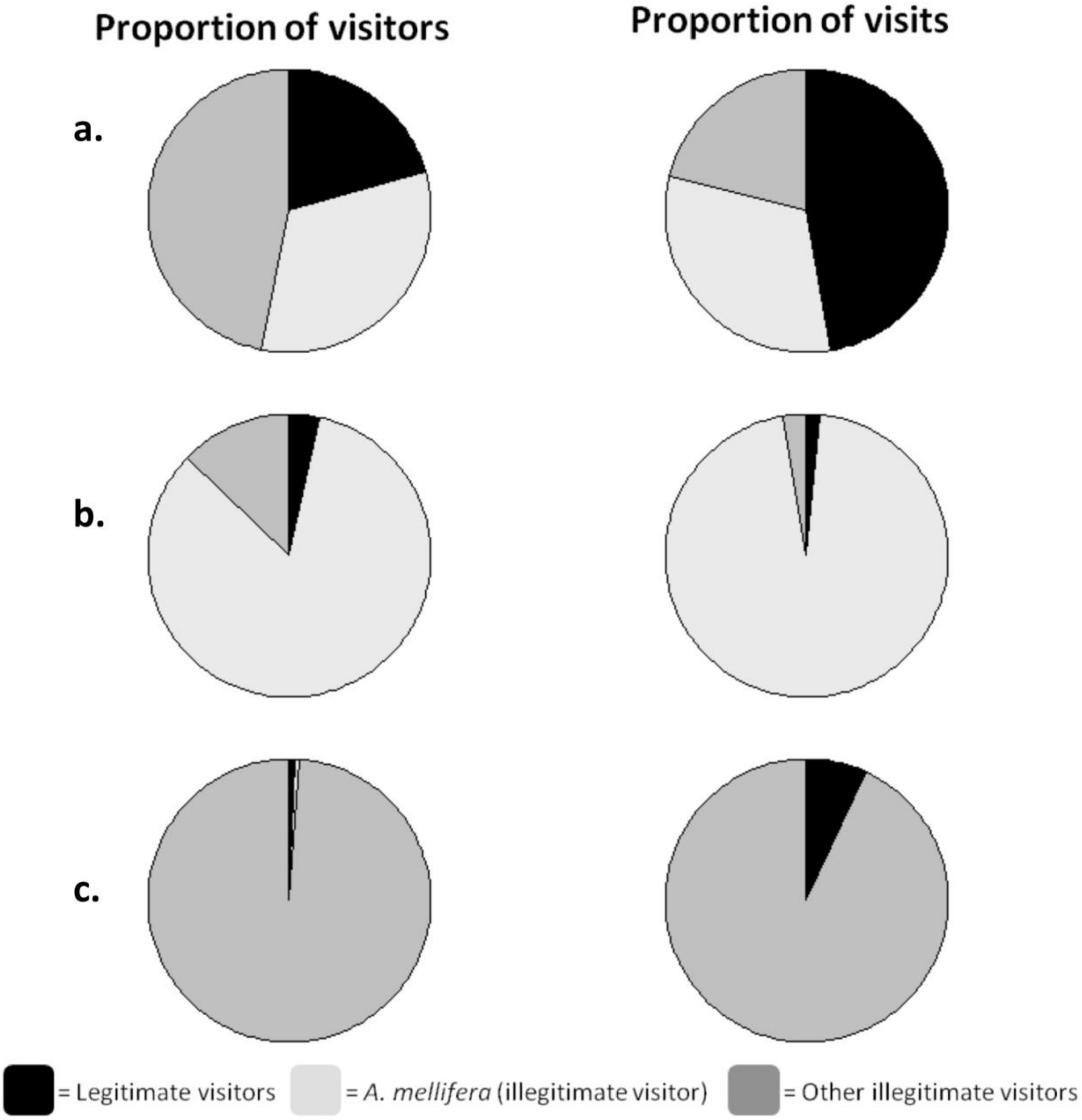


Fig.2. Box plots of the duration of visit in seconds (left), the number of flower visited in a bout (bout length) (centre) and the size of the visitor measured as thorax width in mm (right) between the legitimate (L) and illegitimate (I) visitors in three populations (the CU, LP and TP populations) of *S. rostratum* in Mexico.

Fig.3. Proportion of legitimate and illegitimate visitors (n = 425, 116, and 376 visitors in populations CU, LP and TP) and the proportion of floral visits (n = 4903, 1890 and 1947 visits, respectively) that visitors conducted at three sites: (a) Ciudad Universitaria, Pedregal, Mexico City (CU), (b) Libres, Puebla (LP) and (c) Zapotitlán de Salinas, Puebla (TP).



stigmas of flowers. Both results suggest that *A. mellifera* may function as a poor pollinator. We did not find any statistically significant difference in fruit production between the legitimate and non-buzzing illegitimate visitors in this experiment ($\chi^2 = 0.43$, df = 1, $P = 0.5$). Finally, the illegitimate buzzing visitors (*E. mellipes*, *L. jubatum* and *L. Dialictus sp.*) mainly function as pollen thieves because visited flowers did not produce fruits (n = 12). The control validated our results because unvisited flowers did not produce fruits (n = 10).

2.5. DISCUSSION

2.5.1. Reproductive biology and pollen limitation

Solanum rostratum strongly depends on pollinators to reproduce in natural conditions because it is unable to set seed autonomously or by endogenous mechanisms. The complex floral morphology (anther dimorphism and mirrored floral morphs) interacts with the size and behaviour of visitors promoting the high outcrossing rate previously reported in native populations of *S. rostratum* (Vallejo-Marín *et al.* 2013). However, pollen limitation is common in plants in which ovule fertilization depends on an adequate pollinator service (Burd 1994).

The present study shows that the native populations of *S. rostratum* in Mexico are pollen limited. The pollen limitation in this species is recorded at two levels; the level of fruit and of seed production, but it is higher for the latter. At the level of fruit production, *S. rostratum* has, on average, a lower score (L_FS = 0.23 ± 0.06) than other self-compatible (0.31 ± 0.03), herbaceous (0.32 ± 0.03), open-habitat (0.33 ± 0.03), non-autogamous (0.42 ± 0.03) or nectarless (0.47 ± 0.05) species (Larson & Barrett 2000).

In the particular case of the three populations for which pollinator observations were made, the pollen limitation experiments were conducted at the same time. Populations CU and LP had a higher score on the L_Wpre index than population TP. We suggest that the difference in the scores among the populations is attributable to an inequality of visitation rate by conditional thieves. *Apis mellifera* is considered to be a conditional thief because of its low pollination efficiency (Gross & Mackay 1998; Hargreaves, Harder & Johnson 2009), as our results corroborated. The honeybees are common visitors to populations LP and CU but not to population TP. We suggest two possible scenarios: (i) conditional thieves are common visitors and conduct the majority of visits while there are scarcely any visits by the legitimate visitor (e.g. population LP). In this case, when the honeybee is responsible for ensuring the reproduction of a population, it could be pollen limited because it is a poor pollinator. (ii) Conditional thieves are common but the population receives a high percentage of visits by the legitimate pollinators (e.g. population CU). Under this circumstance, *A. mellifera* would decrease the pollination success of a population if it removes pollen directly from the stigmas where pollen was previously deposited by legitimate pollinators (Gross & Mackay 1998), or indirectly if it reduces the attractiveness of flowers, so affecting the behaviour of legitimate pollinators arriving later (Hargreaves, Harder & Johnson 2009).

Even though the TP population was visited by many illegitimate visitors (except honeybees) at least 7% of the visitors were legitimate and so could assure reproduction in the population. Although *A. mellifera* was abundant at this site, it rarely visited *S. rostratum*, but visited other species of plants instead. It is possible that *A. mellifera* prefers to visit other plants that offer nectar as a reward (for example *Leucaena* has nectarless flowers but has extrafloral nectaries on its leaves; Zarate-Pedroche 1994) or that offer easier access to pollen (for example the tiny flowers of Asteraceae are inserted in a wide and flat receptacle).

2.5.2. Visitation of *S. rostratum* includes both buzzing and non-buzzing bees

Solanum rostratum is a buzz-pollinated plant which is visited by both buzzing and non-buzzing bees in natural populations. In the Mexican populations we found some genera of Halictidae (*Augochlora, Augochlorella, Augochlopsis, Lasioglossum,* and *Pseudoaugochlora*) and others of Anthophoridae (*Centris, Exomalopsis, Thygater* and *Xylocopa*) that have previously been reported as buzzing genera (Buchmann 1983). However, in this study not all visitors of *Lasioglossum* buzzed (as few as half of them) when they visited flowers of *S. rostratum*.

Even though *A. mellifera* uses vibration of its indirect flight muscles for communication or thermoregulation, this species has never been observed vibrating flowers to release pollen (Buchmann 1983). However, *A. mellifera* was a common visitor in some of the populations studied, but we did not observe honeybees buzzing the anthers of *S. rostratum* to obtain the pollen. To release the pollen from the porcidal anthers it is necessary that bees vibrate the anthers at a frequency in the range of 100 to 400 Hz (De Luca & Vallejo-Marín 2013). Therefore, how does a non-buzzing bee access pollen in a buzz-pollinated plant? Buchmann (1983) reported that *A. mellifera* gleans pollen grains from the corolla that the legitimate buzzing pollinators have left. In *Melastoma affine*, honeybees do not gather pollen from the anthers but instead glean pollen previously deposited by the legitimate pollinators from the stigmas, which decreases the fruit and seed production (Gross & Mackay 1998). We suggest that in *S. rostratum* honeybees may access the pollen by biting anthers or gathering pollen remaining on the anthers that was previously extracted by buzzing visitors. Occasionally (in 18% of total of visits) honeybees accidentally touched the stigma with their legs or bodies when manipulating the anthers. We rarely observed honeybees gathering pollen directly from the stigmas when extra pollen was deposited there during the pollen supplementation treatment (L. S. M. personal observation).

2.5.3. Behaviour and morphology of the legitimate and illegitimate visitors

What determines whether a visitor becomes a legitimate or illegitimate visitor? The illegitimate visitors mainly act as pollen thieves, i.e. they collect pollen without providing pollination services. These were small bees that stole pollen, visiting flowers for a long time and only a few flowers per bout. Conversely, the legitimate pollinators differed from pollen thieves in some morphological and behavioural characteristics, such as they were large-sized buzzing bees that spent only a few seconds in each visit, but which visited many flowers per bout. However, a medium-sized non-buzzing bee, such as *A. mellifera*, could act as poor pollinator.

The visitor’s size was an important difference between the legitimate and illegitimate visitors of *S. rostratum*. In buzz-pollinated plants the small bees have a minimal likelihood of pollination because they usually do not make contact with the stigmas when curling their bodies over an anther in order to vibrate it (Bowers 1975; Liu & Pemberton 2009). The common pollinators of *S. rostratum* in North America are large-sized species of the genus *Bombus* (Harris & Kuchs 1902; Linsley & Cazier 1963). However, we did not record *Bombus* as a visitor of this plant in central Mexico. Instead we found other large-sized legitimate visitors such as *Xylocopa spp., T. analis* and *Centris spp.*

Another important difference between the pollinators and the thieves is the time that they spend visiting a flower. For example, the large-sized buzzing bees (i.e. *Xylocopa micans* and *Euglossa viridissima*) move quickly among the flowers because they grasp the whole anther cone while buzzing and spend only a few seconds on each visit (Liu & Pemberton 2009). By comparison, small non-buzzing bees (e.g. *Trigona sp.*) or buzzing-bees (e.g. *Augochlrorella striata*, *Augochloropsis spp*. and *Lasioglossum sp.*) hold one anther at a time and so remain longer in each flower (Liu & Pemberton 2009; Snow & Roubik 1987).

Finally, another difference that we found was that the number of flowers visited differed between the pollinators and the thieves. However, we did not measure if consecutive visits were conducted within-individual or between-individuals. *Solanum rostratum* is a self-compatible species (Bower 1975) that is unable to reject its own pollen. In self-compatible plants self-fertilization can occur through pollen transfer within a flower (Jesson & Barrett 2005) or between flowers of the same individual (geitonomamy; Barrett, Jesson & Baker 2000). Dupont *et al.* (2004) reported that *A. mellifera* promotes self-pollination because it often forages in more flowers on the same inflorescence in comparison with native bees in Tenerife, Canary Islands. However, Sun *et al.* (2013) reported that *A. mellifera* potentially reduces geitonogamous pollination when visiting a bumblebee-pollinated plant (*Pedicularis densispica*) because it conducts fewer consecutive visits within-individual in comparison with *Bombus*. However, we need to further explore the role of *A. mellifera* as a poor pollinator of *S. rostratum.* In natural populations this plant registers 30% self-pollination, probably caused by multiple flowers opening at the same time within an individual (geitonogamy) or as a result of self-pollen transfer by illegitimate visitors (Vallejo-Marín *et al.* 2013) such as *A. mellifera.*

2.5.4. Pollinators versus pollen thieves

The pollination efficiency is measured through different parameters, such as the percentage of pollen removed from the anthers that will be deposited on the stigma (Conner, Davis & Rush 1995), and the ability of a pollinator to effect fruit-set and seed-set (Schemske & Horvitz 1984). In this research, we measured the ability of floral visitors to produce fruits. According to these results, we can infer the functionality of a visitor as a pollinator, if it can produce fruits, or as a pollen thief, when it does not produce fruits. Pollen thieves were common visitors of *S. rostratum*, a buzz-pollinated plant.

Since many buzz-pollinated plants offer pollen mainly as a reward, there is a dilemma that pollen is not only used as a gamete for fertilization but also as a food source to attract pollinators. Some studies have suggested that the “division of labour” among stamens resolves this conflict (Luo, Zhang & Renner 2008; Vallejo-Marin *et al.* 2009). The differentiation of anthers, or heteranthery, which is the presence of two or more types of anther in the same flower, reduces the trade-off between using the pollen as a reward and as a gamete (Vallejo-Marín *et al.* 2009). The feeding anthers offer pollen for reward and the pollinating anthers contain the fertilizing pollen (Buchmann 1983). The feeding anthers are more attractive to pollinator for foraging due to their bright colours and accessible placement (Luo, Zhang & Renner 2008). Recently studies (Luo, Zhang & Renner 2008; Vallejo-Marín *et al.* 2009) corroborate Müller's hypothesis (1883), who postulated that pollen collecting bees focus on feeding anthers to gather pollen rather than on pollinating anthers.

A precondition of the evolution of heteranthery is that pollinators act as a pollen thieves. In buzz-pollinated plants, the evolution of heteranthery represents a male strategy that influences pollen dispersal and reduces pollen consumption (Vallejo-Marín *et al.* 2010). However, pollen theft is still very prevalent in *S. rostratum* and is mainly focused on the feeding anthers. In this species the four feeding anthers do not differ in colour from the corolla (Bowers 1975) and contain 49% of the total pollen produced by the flower, but which are slightly larger (~0.1 μm) than the pollen from the pollinating anthers (Vallejo-Marín *et al.* 2009). We suggest that pollen thieves focus on the feeding anthers because it is easier to steal pollen from the four central feeding anthers. These anthers contain almost the same quantity of pollen as the pollinating anther and have slightly larger pollen grains. Furthermore, the pollinating anther is reflected in the flower being more difficult to manipulate it.

Pollen consumption without providing pollination service is common in *S. rostratum*. The consequences of pollen theft on reproduction and evolution of this species should depend on the relative abundance of thieves and pollinators. It could also depend on the intensity of the pollen theft and its consequences on plant fitness. Although we did not directly measure the effect on reproductive success of the pollen theft in *S. rostratum*, we found that some populations suffered a strong pollen theft (e.g. almost all visits in the LP population were conducted by pollen thieves). Gross and Mackay (1998) reported that honeybees reduced the fitness of a buzz-pollinated plant when removing pollen from the stigmas that had been previously deposited by a legitimate pollinator. Halictids are also common pollen thieves in our study and are known to negatively affect the fitness of plants that they visit, and their floral preferences play an important role in the evolution of floral traits (Lau & Galloway 2004). Future work should focus on the magnitude and impact of pollen theft on the reproductive success of *S. rostratum* in order to understand the ecological and evolutionary implications of pollen theft for this buzz-pollinated plant.

2.6. CONCLUSION

This is one of the first studies to quantify the rates of visitation by both buzzing and non-buzzing bees of a buzz-pollinated species of plant. *Solanum rostratum* is a buzz-pollinated plant that is mainly visited by bees capable of producing vibrations to extract pollen. Legitimate pollinators of *S. rostratum* are relatively large-sized buzzing-bees, which conduct multiple short visits. However, most visitors of *S. rostratum* act as pollen thieves. Our results show that high rates of visitation by pollen thieves (60-70% of visitors) mainly focus on the feeding anthers. Insect size, relative to the flower that is visited, is the main determinant of whether a visitor acts as a pollinator or a pollen thief. In addition, pollen limitation implies that pollen theft can act as selective forces through both the male and female fitness components. Finally, buzz-pollinated plants can be pollinated by non-buzzing bees when the legitimate pollinators are scarce or absent. However, these non-buzzing bees could increase pollen limitation when legitimate pollinators are abundant.

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CHAPTER 3:

The morphological fit between flower and pollinator influences pollen transfer dynamics in *Solanum rostratum*

This chapter is in preparation to be submitted as:

Solís-Montero, L. & M. Vallejo-Marín. Does morphological fit between flowers and pollinators affect pollen transfer? An experimental test in a buzz-pollinated species with anther dimorphism. *Functional Ecology*.

3.1. ABSTRACT

Some specialized pollination systems, such as pollination by vibration, are associated with complex floral morphologies that require a close physical interaction between floral sexual organs and insect visitors. In these systems, a pollinator's size relative to the flower may be an important feature determining whether the visitor touches both male and female sexual organs and thus transfers pollen between plants efficiently. To date, few studies have addressed whether in fact the 'fit' between flower and pollinator influences pollen transfer dynamics. Here we use *Solanum rostratum,* a buzz-pollinated plant with a relatively complex floral morphology (dimorphic anthers and mirror-image flowers) to investigate whether the morphological fit between the pollinator's body and floral morphology influences pollen deposition. Specifically, we hypothesized that when the size of the pollinator matches the separation between the sexual organs in a flower, more pollen should be transferred to the stigma than when the visitor is either too small or too big relative to the flower. To test this hypothesis, we exposed flowers of *S. rostratum* to bumblebees (*Bombus terrestris*) of different sizes and recorded the number of visits received, pollen deposition, and fruit and seed production. We found higher pollen deposition when bees were the same size or bigger than the separation between anther and stigma within a flower, compared to smaller bees. Fruit and seed production were recorded only in specific range of matching when the bee's abdomen was between approximately three-quarters to twice as big as the sexual organ separation. Our results suggest that the fit between flower and pollinator significantly influences pollen transfer in this buzz-pollinated species, with smaller bees functioning primarily as pollen thieves that do not deposit pollen onto stigmas. We speculate that in buzz-pollinated species where floral morphology and pollinators interact closely, variation in the visitor's size may determine whether it acts mainly as a pollinator or a pollen thief.

Keywords: *Bombus terrestris,* herkogamy, pollen deposition, pollinator size, *Solanum rostratum.*

3.2. INTRODUCTION

Most flowering plants are hermaphrodites, possessing both female and male organs in the same flower (Barrett 2002). The presence of both sexual organs in the same flower allows for self-pollination to occur and may result in sexual interference (Fetscher 2001). A mechanism for avoiding both self-pollination and sexual interference in hermaphroditic flowers is the spatial separation of the sites of pollen presentation and pollen receipt, known as herkogamy. This separation is traditionally interpreted as a mechanism for reducing selfing, but recently it has also been interpreted as a mechanism for avoiding physical interference between sexual functions (Fetscher 2001; Webb & Lloyd 1986).

When the sexual organs are spatially separated, visitors only contact one sexual organ at a time or touch both male and female organs but in different parts of the pollinator's body, which should, in principle, decrease pollen transfer efficiency. This problem is resolved in various ways in the different classes of herkogamy (Webb & Lloyd 1986). In the case of reciprocal herkogamy, the reciprocal positions of the sexual organs between two or three floral morphs in the same or different plants (Barrett 2002; Webb & Lloyd 1986) ensures that precise pollen transfer occurs between floral morphs, while self-pollination is reduced within morphs (Barrett 2002).

The evolution and function of reciprocal herkogamy have been well documented in heterostylous systems (style-length polymorphism). In contrast enantiostyly, an asymmetric polymorphism is not yet well understood (Jesson & Barrett 2003). This asymmetric polymorphism is characterised by the reciprocal deflection of the style either left or right side of the floral axis resulting in mirror-image flowers (Jesson & Barrett 2002; Jesson & Barrett 2003; Webb & Lloyd 1986). Enantiostyly is usually associated with a lack of nectaries, and therefore pollen is usually the main reward. It is also associated with heteranthery, where anthers are divided into two functions: pollination in the “pollinating anthers” and pollinator nutrition in the “feeding anthers” (Jesson & Barrett 2003). Furthermore, small apical pores on the tips of the anthers (poricidal anthers) are strongly associated with heterantherous plants that exhibit buzz-pollination (Vallejo-Marín *et al.* 2010). Buzz-pollination requires pollinators, usually bees, to release pollen from poricidal anthers through the vibration of indirect flight muscles (Buchmann 1983). When a pollinator approaches enantiostylous and heterantherous flowers, it grasps the feeding anthers and vibrates to extract the pollen while the pollinating anther deposits pollen on the side of the pollinator’s body, which will then be deposited on the opposite floral morph (Whalen 1979).

The complex floral morphology (enantiostylous and heterantherous flowers) associated with buzz-pollination system requires a close physical interaction between floral sexual organs and insect visitors. For example, when heteranthery evolves, it requires that the edible and fertilizing pollen grains are deposited on different parts of the pollinator’s body which must be large enough in relation to the flower to allow for the specialization of the anther function (Vallejo-Marín *et al.* 2010). The size of pollinators will also influence whether a pollinator makes contact with the sexual organs of heterantherous and enantiostylous flowers (Whalen 1979).

Studies to explore the size matching between pollinators and floral traits have focused on the proboscis length of insects and its fit with the depth of the structures that hold nectar (Stang *et al.* 2009). However, few studies have focused on size matching between pollinators and floral traits in relation to the offer of pollen as a reward (Bowers 1975; Duncan, Nicotra & Cunningham 2004; Gao *et al.* 2006; Kawai & Kudo 2009; Liu & Pemberton 2009). Furthermore, as far as is known about pollen transfer efficiency relates to the closeness of the fit between the pollinator and the floral sexual organs. We suggest that the degree of size matching between the pollinator body size and the floral sexual organ separation (herkogamy) is a fundamental issue for successful reproduction in buzz-pollinated plants. When the pollinator is sufficiently large, pollen is deposited on its body and is subsequently transferred to the stigmas of conspecific plants.

In this study, we experimentally test how reproductive success relates to pollinator-flower size matching in *Solanum rostratum* Dunal, a buzz-pollinated plant species. *Solanum rostratum* is a self-compatible, bee-pollinated, annual herb that is partially outcrossing (outcrossing rate: $t = 0.70 \pm 0.03$; Vallejo-Marín *et al.* 2013) and that inhabits open and disturbed habitats (Whalen 1979; Bowers 1975). *Solanum rostratum* has nectarless, heterantherous and enantiostylous flowers (Whalen 1979). This species strongly depends on pollinators for it to reproduce, and is unable to produce fruit autonomously or asexually (Chapter 2). In North America, it is distributed from central Mexico to the Great Plains of the USA and Canada (Whalen 1979). Pollinator observations conducted in its native range reveal that *S. rostratum* is mainly visited by bees of different sizes (Bowers 1975; Harris & Kuchs 1902; Jesson & Barrett 2005; Linsley & Cazier 1963). While larger bees usually make contact with the stigma, smaller bees are precluded from making contact (Bowers 1975). In central Mexico, populations are visited by 15 species of bees that range from 1 to 10 mm in thorax width. Legitimate pollinators of this species are large-sized bees (from 5 to 10 mm), and illegitimate pollinators are small and medium-sized bees (from 1 to 4 mm), which mainly act as pollen thieves or as poor pollinators (Chapter 2).

The main goal of this study is to determine how pollination efficiency varies in relation to the size matching between the pollinator and the plant's sexual organs. We addressed two specific questions: 1) Is more pollen deposited on stigmas when the difference between the size of the pollinator and the separation of the floral sexual organs is at a minimum? 2) Is fruit and seed production greater when the pollinator size closely matches the separation of the sexual organs? We expect that if a pollinator fits closely with the floral sexual organs, this will increase the extent of pollen deposition on stigmas and, consequently, increase the fruit and seed production.

3.3. MATERIALS AND METHODS

3.3.1. Floral morphology in native populations

In order to characterize the variation in floral morphology among natural populations, we collected samples from six populations of *S. rostratum* across a latitudinal gradient in Mexico (Fig. 1) during October and November of 2010 (Table 1). In each population, we measured between two and four flowers from 16 to 30 individuals (Table 1). For each flower, we measured the following ten traits with digital callipers: corolla length (1) and width (2); the length and width of the base of the anther, for both the feeding (3, 4) and pollinating anther (5, 6); the length of the style (7); the distances between: the stigma and the pollinating anther (8), the stigma and the nearest feeding anther (9) and the pollinating anther and the nearest feeding anther (10; Fig. 2).

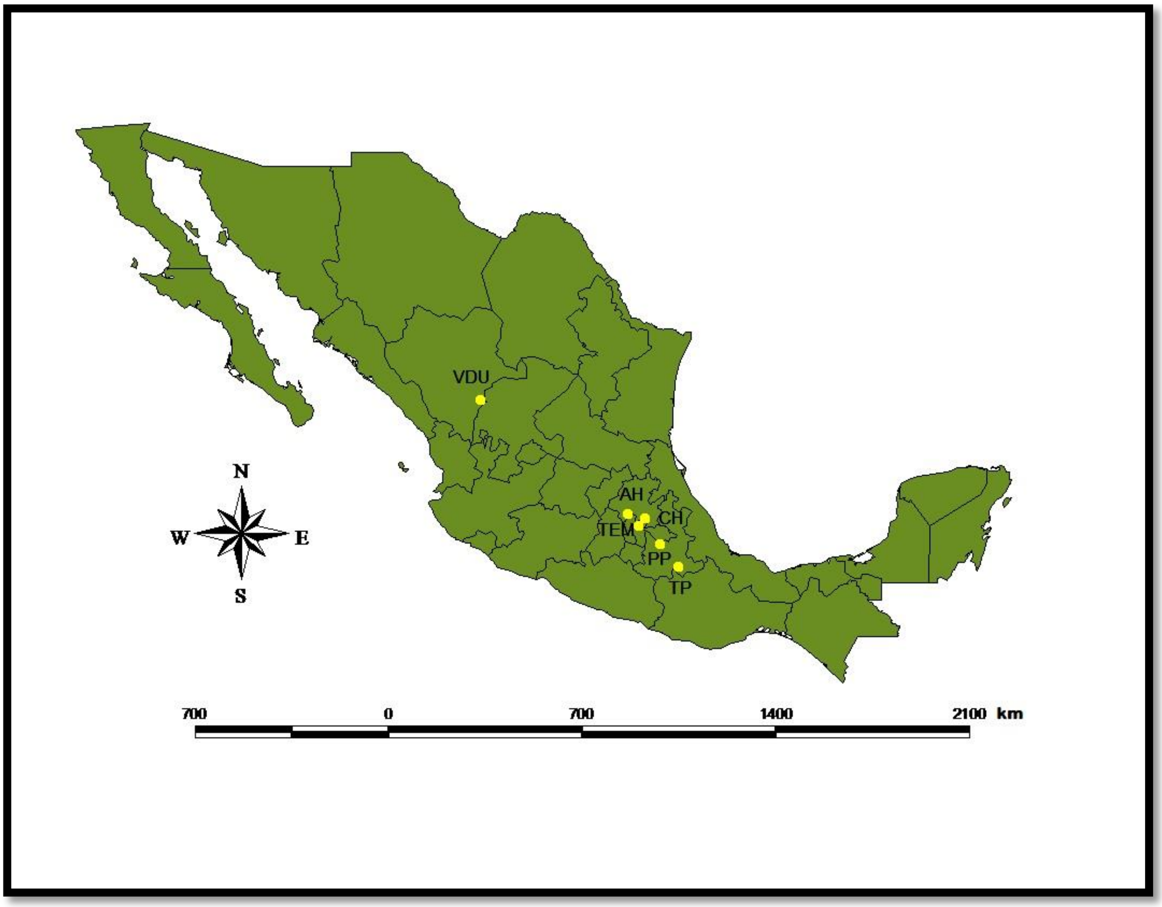


Fig. 1. Map of the six populations studied, located across a latitudinal gradient in Mexico. Each yellow point represents a population (Population codes are described with detail in Table 1).

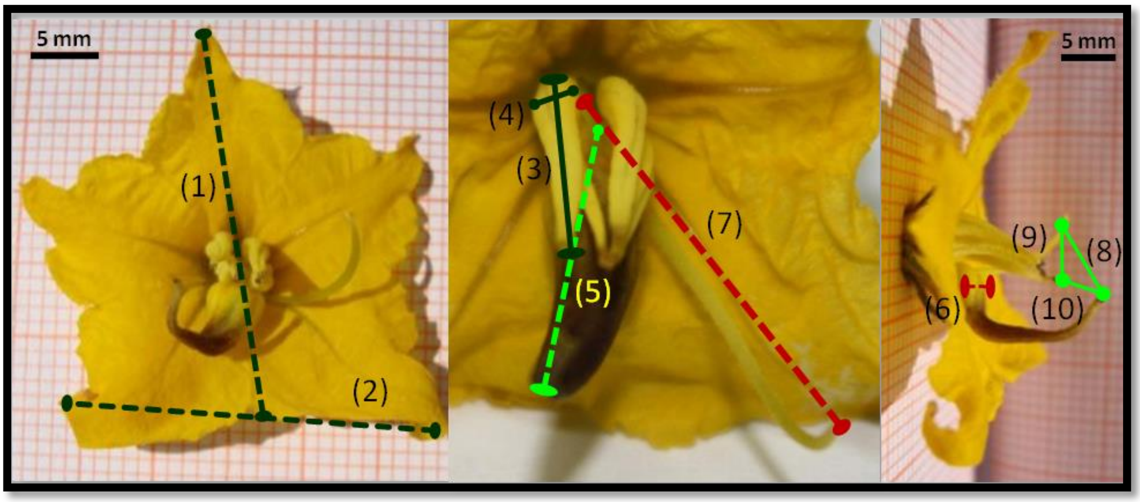


Fig. 2. The ten floral traits measured in flowers of *Solanum rostratum*. (1) Corolla length and (2) width; (3) the length of one feeding anther and (4) the width of the base of this anther; (5) the length of a pollinating anther and (6) the width of the base of this anther; (7) the length of the style; the distances between: (8) the stigma and the pollinating anther, (9) the stigma and the nearest feeding anther and (10) the pollinating anther and the nearest feeding anther.

Table 1. Floral morphology measured in six Mexican populations of *S. rostratum* sampled during October and November 2010. The values in parentheses are the number of individuals measured.

Pop. Code	Population	Latitude (N)	Longitude (W)	Elevation (m)	Number flowers measured (individuals)
AH	Atitalaquia, Hidalgo	20.07°	99.22°	2090	60 (30)
CH	Cempoala, Hidalgo	19.91°	98.65°	2467	32 (16)
PP	Puebla, Puebla	19.06°	98.16°	2198	60 (30)
TEM	Teotihuacán, Estado de México	19.68°	98.86°	2277	32 (16)
TP	Zapotitlán de Salinas, Puebla	18.33°	97.57°	1670	120 (30)
VDU	Vicente Guerrero, Durango	23.74°	104°	1926	60 (30)

3.3.2. Plant growth for pollination experiment

In order to generate plants for the pollination experiment, we collected seeds from two of the six populations measured in the field (PP and VDU; Table 1). We selected these two populations because they exhibited the extreme values for the separation between the sexual organs within a flower (Fig. 4). Seeds from 20 plants (hereafter maternal families) per population were extracted from the fruits and stored in paper bags at 5–7°C until planting. Five seeds per maternal family (5 x 20 = 100 plants per population) were planted in glasshouses at the University of Stirling. To induce the seed germination, seeds were pre-treated with a 1000 ppm aqueous solution of gibberellic acid (GA_3; Sigma-Aldrich, Dorset, UK) for 24 hours and then incubated overnight in a growth chamber at 20°C and 12:12 hours light:dark regimen. Seeds were sown in plastic trays with Modular Seed Growing Medium compost (William Sinclair

Horticulture PLC, Lincoln, UK), and kept in a glasshouse at 14–22°C with natural daylight supplemented with compact-fluorescent lamps for 14 hours per day. After 2-3 weeks, seedlings were transplanted into individual 0.37 L pots with All Purpose Growing Medium and perlite (William Sinclair Horticulture) in a 3:1 ratio, and fertilised with slow-release fertiliser (Osmocote16:9:12; Scotts Miracle-Gro Co, Marysville Ohio). After four weeks, plants were transplanted to 1.5 L pots with the same growth medium described above.

3.3.3. Pollination experiment according to bumblebee fit with sexual organs

In order to investigate patterns of pollen transfer and both fruit and seed set, experimental plant arrays were exposed to visits by captive bumblebees (*Bombus terrestris* L.). We chose this species of bumblebee for our experiment because individuals showed considerable size variation (thorax width: 2.3–8.8 mm; Goulson 2010), and colonies are readily available from commercial providers as they are used in the pollination of crops, including other buzz-pollinated species such as tomatoes (*Solanum lycopersicum*). Moreover, bumblebees are pollinators of *S. rostratum* in North America (Bowers 1975 see Table 1 in Chapter 1), and *B. terrestris* has been previously used in pollination experiments with this species (De Luca *et al.* 2013; De Luca & Vallejo-Marín 2013).

Experimental arrays (35 blocks) consisting of 10 potted plants were placed in a flight cage (dimensions: 4 x 3 x 2 m) and exposed to visitation by captive bumblebees. Plants were arranged in two parallel rows of five plants, each placed 0.5 m apart and with 1 m of separation between each row. Each array contained five individuals from each of the two experimental populations (PP and VDU). The floral display of each plant in the array was standardized to four flowers (two for each enantiostylous morph); the remaining flowers were either removed or bagged with fine mesh to exclude bees. Each flower was individually labelled and the following floral traits measured; the distance between (8) the stigma and the pollinating anther, (9) the stigma and the nearest feeding anther, and (10) the pollinating anther and the nearest feeding anther (Fig. 2).

Each array (40 flowers from 10 plants per array) was exposed for 20 min to a single bumblebee, and the number of visits to each flower was recorded. A bee landing on a flower and making contact with the sexual organs was scored as a visit. After 20 minutes, the bee was captured and the following five measurements were taken using digital callipers: the thorax width (1) and length (2), the abdomen width (3) and length (4) and the overall length of the bumblebee (5). In order to count the number of pollen grains deposited on the stigma, the terminal end of the style was collected from all the flowers of plants that received at least one visit. The top third of the style, including the diminutive stigma, was harvested after 24 hours and placed on a slide with fuchsine-stained glycerol jelly (Kearns & Inouye 1993). The 24-hour delay between pollination and style collection was done to allow pollen tubes to grow and reach the ovary, as we were also interested in recording fruit and seed set in the experimental flowers. The total number of pollen grains deposited on each stigma was counted at 400× magnification under a light microscope (Dialux 20EB, Leitz). Six weeks later, we recorded whether fruits had formed and counted the number of seeds produced.

3.3.4. Statistical analyses

3.3.4.1. Floral morphology in native populations

We analysed the floral measurements with principal component analysis (PCA) over correlations and differences among populations were calculated with an analysis of variances (ANOVA) of the scores and a Tukey post-hoc test.

3.3.4.2. Size-matching index

We predicted that the degree of size-matching between the spatial separation of the floral sexual organs and the body size of the visiting bumblebee would influence the probability of it making contact with the anthers and stigmas, and thus affect the number of pollen grains transferred between flowers. To investigate this hypothesis, we calculated the difference between the distance from the pollinating anther to stigma (DPAST), and the bumblebee's abdomen width (BAW) as shown in Fig. 3. Hereafter we refer to this index as the size-matching index or SMI (SMI = DPAST − BAW). The size-matching index has a straightforward interpretation: when SMI = 0 the abdomen of the bumblebee fits exactly into the space between the pollinating anther and stigma. Positive values of SMI indicate that the space between the sexual organs is larger than the size of the bumblebee's abdomen, and thus the bee cannot simultaneously touch both pollinating anther and stigma. Finally, negative values of SMI indicate that the separation between sexual organs is smaller than the abdomen's width of the visiting bumblebee, allowing for simultaneous contact of the pollinating anther and stigma during a visit.

Fig. 3. The size-matching index (SMI) was defined as the difference between the distance from the pollinating anther to stigma (DPAST) and the bumblebee's abdomen width (BAW). Photograph by L. Bernstein.

3.3.5. Pollination efficiency according to bumblebee fit with sexual organs

The number of pollen grains deposited, and the number of seeds and fruit set in the experimental arrays were analysed using separate generalized linear mixed models (GLMM). For all analyses, we used the statistical package *R* ver. 3.0.3. (R Core Development Team 2014). Mixed models were fitted with the *lme4* package (Bates, Maechler & Bolker 2013) and *P*-values were calculated with the *lmerTest* package (Zeileis & Hothorn 2002). The mixed models were visualized using the *plotLMER.fnc* function of the *languageR* package (Baayen 2008), which plots the predictors of each model. In addition to plotting a quadratic term, we used the *optimix* package, which optimizes the fitting of a smooth function to a model in *R* (Nash & Varadhan 2011).

The models used the number of visits and the size-matching index as fixed effects, and the individual plants per array and the array-block as random effects. Models included both linear and quadratic coefficients for the fixed effects. The best model was selected by backward elimination by comparing the log-likelihood and Akaike Information Criterion of the nested models. In the pollen deposition value including zeros, where no pollen grains were deposited, this variable was modified adding one to allow that zero values to be log-transformed when this model was fitted with a Poisson error term. In the case of fruit set, which can take individual values of either zero or one, and seed production (seed counting), the models were fitted with a binomial error distribution (logit link) and a Poisson error distribution (log link), respectively. For all models to estimate the variance and covariance of the random effects were using the *ranef* function (package *lme4*). Random effects that were not significant were eliminated from the model.

3.4. RESULTS

3.4.1. Floral morphology of *S. rostratum* in native populations

Throughout its distribution of *S. rostratum* in Mexico, this plant species differed in flower size and in the separation between the sexual organs within its flowers. The principal components summarized the observed variation in the floral morphology in the native populations. The first two components explained a total of 55% of the variance in floral morphology. The first principal component (PC1) explained 39% of this variance and was interpreted as reflecting flower size since almost all eigenvectors were positive and of similar magnitude (Table 2). As derived from PC1, population PP had the smallest flowers and population TP had the largest ($F_{5,358} = 56.86$ $P < 0.0001$; Fig. 4). For example, population PP has smaller size of corolla (corolla length = 18.55 ± 0.32 mm and corolla width = 19.94 ± 0.34 mm) than population TP (corolla length = 24.56 ± 0.26 mm and corolla width = 25.61 ± 0.26 mm). The second principal component (PC2) explained 16% of the variance, for which the highest eigenvector scores were for variables that defined the space separating the sexual organs (Table 2). The southern populations (PP and TP) had more widely separated sexual organs than the northern populations ($F_{5,358} = 9.42$, $P < 0.0001$; Fig. 4). For example, the distance between the pollinating anther and the stigma is wider in the populations PP and TP (DPAST = 7.65 ± 0.27 mm and 8.28 ± 0.14 mm, respectively) than in the populations in AH and VDU (DPAST = 6.95 ± 0.19 mm and 6.74 ± 0.20 mm, respectively).

Table 2. Eigenvectors of the first two principal components (PC1 and PC2) of the Principal Component Analysis of floral morphology traits in *S. rostratum*. Abbreviations used: L (length), W (width), FAnther (feeding anther), PAnther (pollinating anther), DPAST (the distance between the stigma and the pollinating anther), ST (the length of the style), DFAPA (the distance between the pollinating and the closest feeding anther) and DFAST (the distance between the stigma and the closest feeding anther).

Floral traits	PC1	PC2
Corolla L	0.40244	-0.15767
Corolla W	0.39609	-0.14222
FAnther L	0.36753	-0.0503
FAnther W	0.3756	0.08514
PAnther L	0.38226	0.01058
PAnther W	0.35442	0.21053
DPAST	0.12533	0.63123
ST	0.33034	0.02859
DFAPA	-0.01954	0.38381
DFAST	-0.08977	0.59508

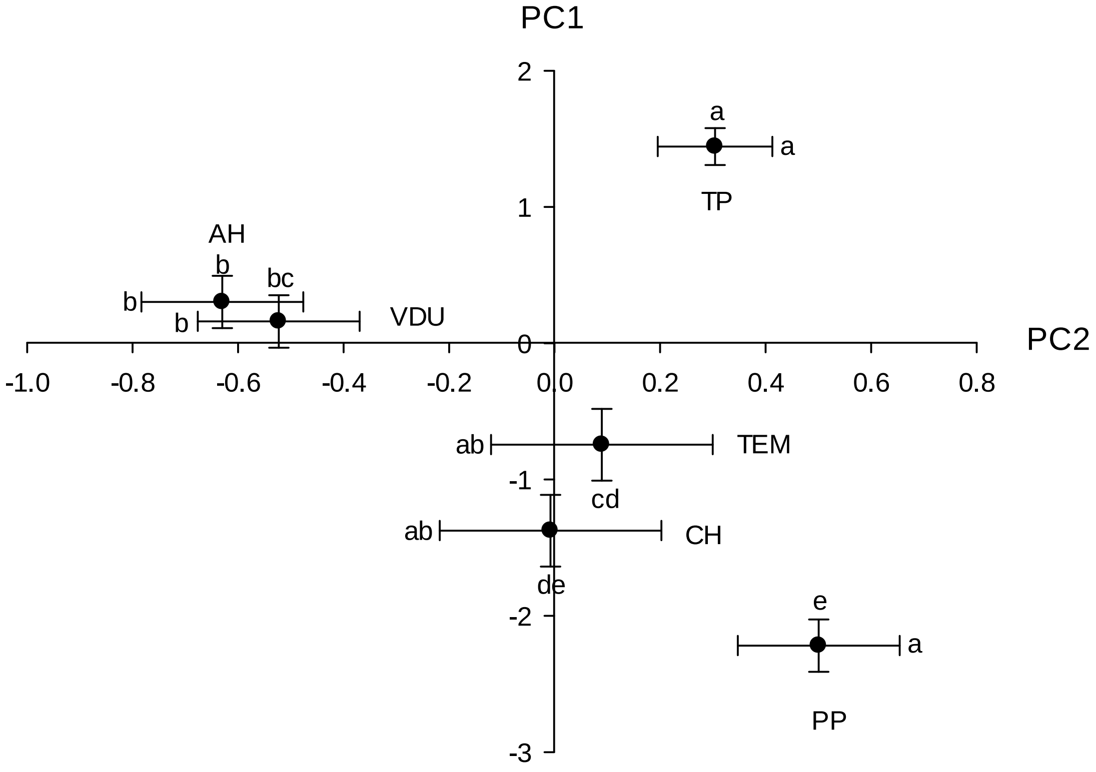


Fig. 4. Mean values and standard errors of principal component scores derived by principal component analysis. The mean scores for six native populations (AH, CH, PP, TEM, TP and VDU) of *S. rostratum* are plotted, with principal component one (PC1) on the Y axis and principal component two (PC2) on the X axis. The lowercase letters indicate statistically significant differences among populations after Tukey as a post-hoc test. Population codes (uppercase letters) are given, with further details, in Table 1.

In the pollination experiment, to maximize the variation in the measures of sexual organ separation, plants from populations PP and VDU were used. During the pollination of *S. rostratum*, while a pollinator is collecting pollen from the feeding anthers, the pollinating anther touches one side of the pollinator’s body and the stigma touches the corresponding position on the opposite side (Bowers 1975). We focused on the distance between the pollinating anther and the stigma because this should play an important role in pollen transfer due to the direct interaction between pollinator and this floral trait.

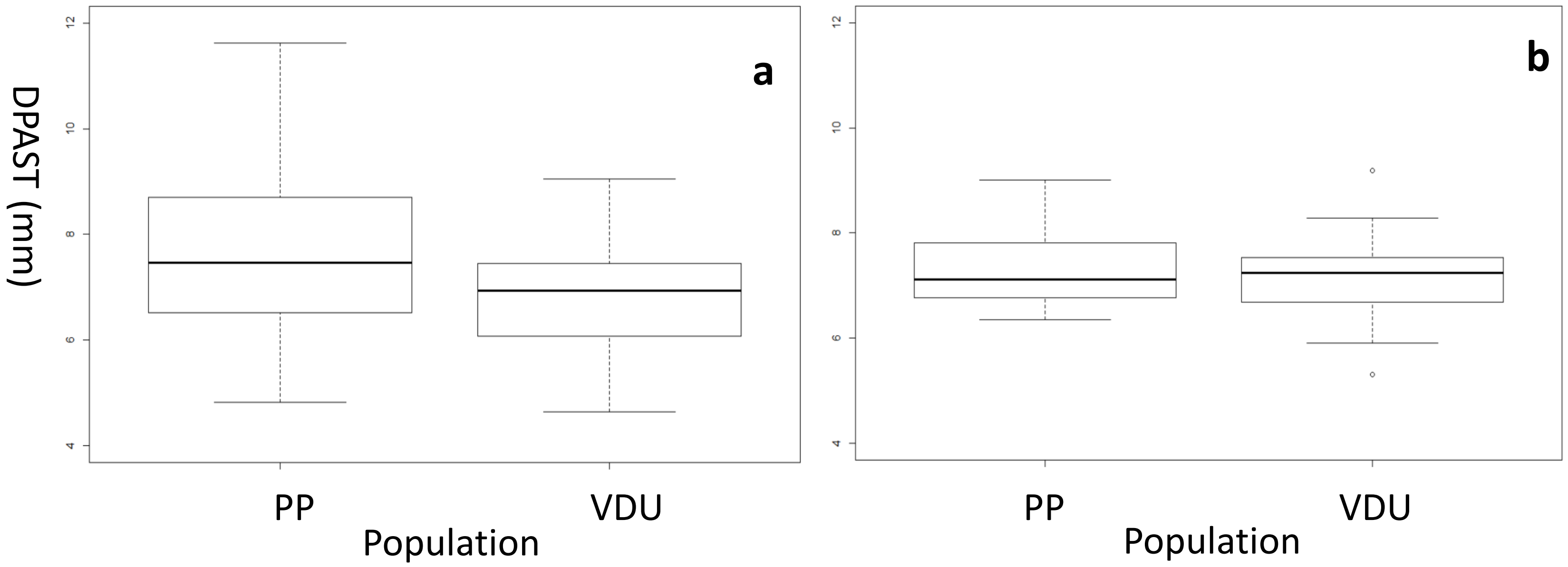
Both of the populations that were selected for the pollination experiment (PP and VDU) differed in the distance between the pollinating anther and the stigma ($F_{1,58} = 5.50$, $P = 0.02$; Fig. 5a) in the field, but this difference was not statistically significant in the progeny grown in the glasshouse in Scotland ($F_{1,35} = 0.28$, $P = 0.60$; Fig. 5b). However, we found enough variation in the distance between the pollinating anther and the stigma in the progeny of both populations (1.31–17.94 mm) to conduct the pollination experiment.

3.4.2. Pollination efficiency according to bumblebee fit with sexual organs

In the pollination experiment where flowers were exposed to bumblebees of different sizes (5.3–9.3 mm of abdomen width), the variation in the floral sexual organ separation resulted in sufficient variation in the SMI to test the hypothesis proposed (Fig. 6). For bumblebees that differed in terms of their SMI, the measurement of pollination efficiency was composed of two components: a) the number of pollen grains deposited by the bumblebee onto stigmas and b) the production of fruits and seeds.

(a) Number of pollen grains deposited by bumblebees on the stigma. The number of pollen grains deposited on a stigma rises with an increased number of visits, but is lower for flowers that receive many visits (Table 3; Fig. 7a,b). Pollen grains were found on unvisited flowers. However, our results suggest a pattern of pollen deposition according to the SMI (Fig. 7c). There was a negative relationship between the number of pollen grains deposited on stigmas and the SMI, for negative values of SMI was high the number of pollen grains deposited while for positive values of SMI this number was low (Table 3; Fig. 7d). This means that when the abdominal width of a bumblebee is

Fig. 5. Box plots showing the comparison of the mean distance separating the pollinating anther from the stigma (DPAST) in mm, between (**a**) two native populations in Mexico (n = 30 plants) and (**b**) the progeny of these populations grown in the glasshouse at the University of Stirling, UK (n = 20 families). Population codes are given with further details in Table 1.



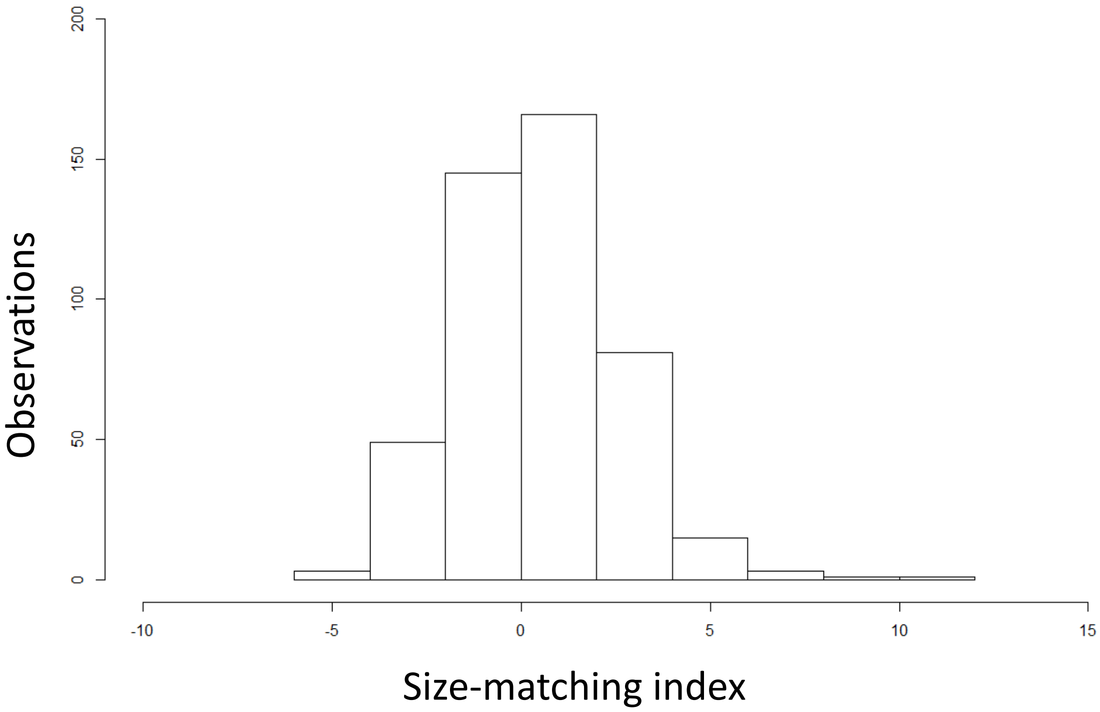


Fig. 6. The frequency distribution of the size-matching index (SMI) recorded in the experimental array blocks. The SMI is the difference between the distance from the pollinating anther to stigma and the width of the bumblebee's abdomen.

larger than the separation between the pollinating anther and stigma (negative values of SMI), more pollen grains are deposited on the stigma. Conversely, when the abdominal width of the bumblebee is smaller than this separation (positive values of SIM) it is difficult for the bumblebee to make contact with the sexual organs, and thus fewer pollen grains are deposited onto the stigma.

Table 3. Summary statistics of the three generalized linear mixed models (GLMM). The values in parentheses are the standard error of the estimate for fixed effects and the standard deviation of the variance for random effects.

Variable	Estimate (SE)	Test statistic (z)	*P* value
Pollen grain deposition on stigmas			
Fixed effect			
Number of visits	0.555405 (0.030850)	18.004	<0.001
Number of visits^2	-0.069730 (0.005001)	-13.943	<0.001
Size-matching index	-0.068846 (0.008761)	-7.858	<0.001
Random effect	**Variance (SD)**		
Individual per array	0.3238 (0.5691)		
Array-block	0.2734 (0.5229)		
Fruit production			
Fixed effect			
Size-matching index	-0.1864 (0.1113)	-1.675	0.094
Random effect	**Variance (SD)**		
Array-block	0.79987 (0.89436)		
Seed production			
Fixed effect			
Size-matching index	0.21440 (0.03759)	5.704	<0.001
Random effect	**Variance (SD)**		
Individual per array	0.19087 (0.4369)		
Array-block	0.08216 (0.2866)		

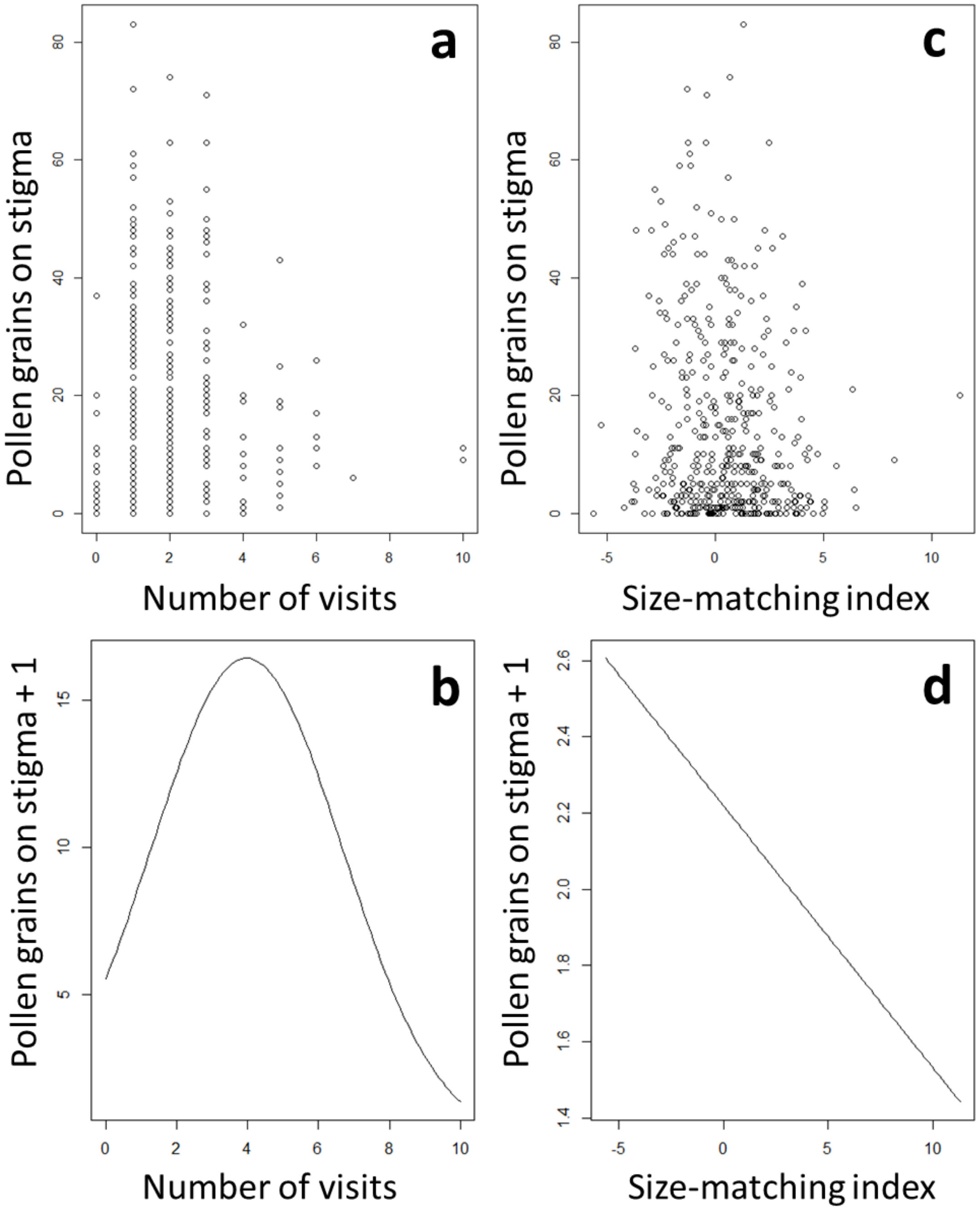


Fig. 7. Pollen grains deposited on stigmas of *S. rostratum* according to (**a**) the number of visits conducted by *B. terrestris* these data were fitted with a quadratic curve (**b**) and according to (**c**) the size-matching index these data were fitted with a linear regression (**d**), which has a negative slope.

(b) Fruit and seed production in relation to a pollinator's fit with the floral sexual organs. Fruits and seeds were only produced over a limited range of the SMI (-3.71–2.6). The bumblebees that produced fruit and seed the width of their abdomen were between approximately twice as big to three-quarters the size of the separation between the floral sexual organs. However, there was no significant effect of the SMI on whether or not pollinator-flower pairs produced fruit (Table 3). Nevertheless, there was a positive relationship (Fig. 8b) between the number of seeds and the SMI when this index was positive (Fig. 8a), which means that more seeds were produced when the bumblebee's abdomen was smaller than sexual organ separation.

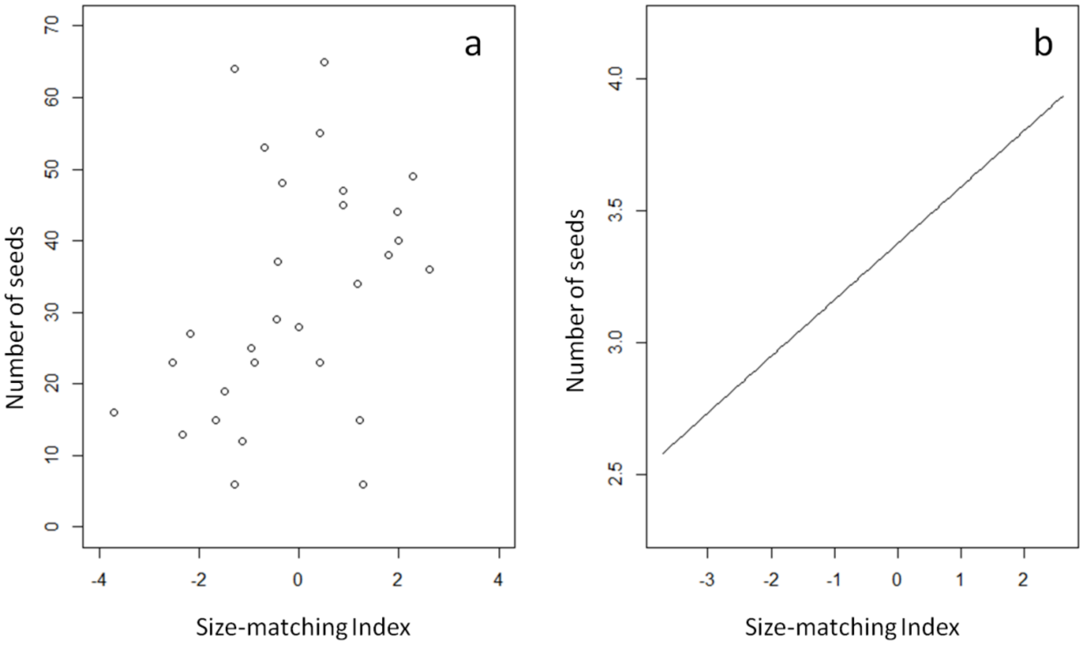


Fig. 8. (**a**) Number of seeds produced by plants arranged in arrays according to the size-matching index of the *B. terrestris*, these data were fitted with a linear regression (**b**) which has a positive slope.

3.5. DISCUSSION

The size of a flower is considered to be an important trait for pollinator attraction. For example, large flowers can cause an increase in pollinator visitation (Conner & Rush 1996; Galen & Newport 1987; Young & Stanton 1990). In natural populations of *S. rostratum* in Mexico, Whalen (1978) previously observed floral size variation and attributed this to “character displacement”. This occurs when this species coexists in sympatry with another *Solanum* species of section *Androceras* as a mechanism of reproductive isolation. The specialized floral morphology of section *Androceras* restricts the size range of efficient pollinators and coexisting species with distinct floral sizes may exploit different parts of pollinator body. For example, *S. rostratum* and *S. citrullifolium*, which typically have large-sized flowers except where both species coexist near the city of Chihuahua (northern Mexico). Here *S. rostratum* has smaller flowers (a mean pollinating anther length of 6 mm). Conversely, where two populations of *S. rostratum* grow alone a few miles away of the city of Chihuahua (5 miles south and 15 miles north), their flowers usually have larger size (a mean pollinating anther length of 11 mm).

In the present study, we corroborated that *S. rostratum* does have different flower sizes among native populations. Unlike Whalen (1978), who characterised floral size as the length of the pollinating anther, in this study we characterized floral size using the first principal component (PC1) that summarized the variance of all floral traits measured. According to PC1, the populations with the largest and the smallest flowers were both located in the south of the native range. Furthermore, we did not observe any *Solanum* species coexisting in these populations during the sampling period. Only two populations of *S. rostratum* (CH and AH) were recorded as coexisting with other species of *Solanum*: two small-flowered species of section *Androceras* (*S. heterodoxum* and *S. fructo-tecto*, respectively). However, more sampling effort is necessary, particularly of populations where *S. rostratum* coexists with larger-flowered species, to test the hypothesis that floral character displacement has driven the differentiation in floral size recorded in *S. rostratum* populations.

Therefore, flower size is important for pollinator attraction. However, once the pollinator has been attracted it is the distance separating the sexual organs (herkogamy) that determines the extent of pollen transfer and deposition by a pollinator (Webb & Lloyd 1986). In the analysis of *S. rostratum* floral morphology, PC2 was interpreted as reflecting the distance among the sexual organs which was widest in the southern populations and narrowest in the northern populations. Moreover, the highest value of PC2 corresponded to the separation between the pollinating anther and the stigma (Table 2). The present chapter shows that the relationship between the separation of the sexual organs and pollinator size determines the pattern of pollen deposition and the extent of fruit production in experimental arrays. It is possible that natural populations elsewhere along the latitudinal range of this species, they may be different in organ separation and they may be exposed to different sizes of pollinators; this variation in size matching may affect the efficiency of pollination among populations.

The variation in floral traits found in *S. rostratum* provides the opportunity to test if pollen transfer efficiency increases with the fit of the pollinator to the floral sexual organs. Pollination efficiency was estimate in this study through the female fitness of the plant, by assessing the extent of pollen deposition onto the stigmas of the flowers and by the extent of fruit and seed production. The stigmas of buzz-pollinated plants are not saturated with deposited pollen grains during the first visit of a pollinator, and receive more pollen with additional visits (Kawai & Kudo 2009). In fact, more than one visit is required to achieve the maximum seed set (Snow & Roubik 1987). However, in this study we found that the cumulative pollen deposition decreased after flowers received more than three visits. A possible explanation for this is that when bumblebees visit the same flower many times in an experimental array they could remove pollen previously deposited on the stigma due to the quantity of available pollen being limited (only 40 flowers were open at the same time).

Pollen deposition in *S. rostratum* increased when the bumblebee was larger than the sexual organ separation, compared to when the bumblebee was smaller. A possible explanation for this observation is that when the bee is larger than the herkogamy, it is also more likely to touch the stigma (Armbruster *et al.* 1989), and so would deposit pollen more often. Conversely, when the bee is smaller than the herkogamy it may touch the stigma more rarely and pollen grains would be deposited less often. Fruit and seed were produced only with a specific range of size matching between the floral visitor and the sexual organs (Fig. 8). Nevertheless, flower-pollinator combinations that produced fruit did not differ in their size-matching index from those that did not produce fruit, possibly because fruit and seed production depend on other factors, such as the allocation of resources for sexual reproduction (Obeso 2004).

The difference between bee size and the herkogamy determines that the visitor functions as a pollinator when the difference is small or as pollen-thief when this difference is large (Armbruster *et al.* 1989). For example, *S. rostratum* is visited by bees of a wide size range, of which the larger bees are considered to be pollinators because they usually make contact with the sexual organs and induce the production of fruits, while the smaller bees mainly act as pollen thieves because they regularly fail to contact the stigma (Chapter 2). Furthermore, when the bee is too large it can damage the stigma with the energy of its vibration (Dulberger 1981); the amplitude, which reflects the energy of the buzzes, increases with bee mass (De Luca *et al.* 2013). In such cases, style deflection along the median plane of symmetry could protect diminutive stigmas from injuries caused by these high intensity vibrations (Dulberger 1981).

We found that pollen was deposited on the stigmas of unvisited flowers in experimental arrays; 66% of unvisited flowers contained from 1 to 37 pollen grains. In the field the native populations of *S. rostratum* did not produce fruits through autonomous fertilization (Chapter 2). Although pollen deposition on unvisited flowers was recorded in this experiment, we still observed a markedly pattern of pollen deposition according to the fit of the pollinator with the floral sexual organs. It is possible that pollen deposition on unvisited flowers may have occurred cause by artificial vibration of the anthers when the plants were transported from the glasshouse to the flight cage. An alternative explanation is that there is automatic pollen transfer within the flowers of *S. rostratum*, but that this is insufficient to produce fruit under natural conditions.

In summary, when bumblebees were larger than the distance between the sexual organs, they deposited more pollen grains. Conversely, we found that when the bumblebee was smaller than the herkogamy it induced the flower to produce a larger seed set. A possible explanation of these contradictory results is that when many pollen grains were deposited on diminutive stigmas, there was a high competition among pollen grains to gain a space in the tiny stigmas and fertilize ovules (from 40 to 80 seeds; Whalen 1979). Another way of explaining this, which is not exclusive of the first explanation, would be to consider the effect of inbreeding on seed set. In this experimental design each individual plant in an experimental array had four flowers (two per floral morph) open at the same time. Therefore, pollinators could have transferred either self- or outcross-pollen to the plant's stigma. The transfer of self-pollen could occur between flowers of the opposite morph on the same plant (geitonogamy). In *Aquilegia caerulea*, for example, self-pollination results in fewer seed being set because of a higher rate of seed abortion than with outcross-pollination, which results from inbreeding depression during seed development (Montalvo 1992). *Solanum rostratum* did not differ in fruit or seed production whether self- or cross-pollinated in an experiment that Bowers (1975) conducted on a few populations in the USA. However, inbreeding effects vary among environments and populations (Keller & Waller 2002). Since the pollination experiment conducted in this study only registered the total amount of pollen deposited on the stigma and did not quantify the proportions of self- and cross-pollen, further work would be needed to explore the extent of pollen saturation on unexpanded *S. rostratum* stigmas. Furthermore, a properly experiment to quantify the inbreeding depression needs to be conduct using samples from a greater number of populations.

This study determined the pollen transfer efficiency through female fitness, without giving consideration to male fitness in the estimation. However, other studies have comprehensively documented pollen removal in buzz-pollinated species (De Luca *et al.* 2013; De Luca & Vallejo-Marín 2013; Harder & Barclay 1994; Kawai & Kudo 2009). Some buzzing properties (i.e. duration, frequency and amplitude) vary among bee species and have been shown to determine the release of pollen from poricidal anthers (De Luca & Vallejo-Marín 2013). For example, De Luca *et al.* (2013) measured the variability of buzzing properties in a commercial colony of *B. terrestris* to explore the effect of pollen removal in *S. rostratum*. They generated "synthetic buzzes" and found that buzzes with longer duration and greater amplitude caused greater pollen removal, while variation in frequency had no effect on the amount of pollen removed. Moreover, these authors found that heavier workers produced greater amplitude buzzes and that these resulted in larger pollen collection loads. We can infer from these findings that in our experiment the largest bumblebees deposited more pollen grains, not only because they fitted with the sexual organs but also because they probably released and transported more pollen grains on their bodies. Another manner by which male success can be quantified is by determining the paternity of the progeny using the 13 microsatellites newly developed for *S. rostratum* (Vallejo-Marin *et al.* 2011; Vallejo-Marin *et al.* 2013). Future studies could make use of these genetic markers to determine the pollination efficiency through male fitness relate to size matching.

Finally, this experiment shows that size matching between the pollinator and the floral sexual organ separation determines the extent of pollen deposition and consequently the level of fruit and seed production in *S. rostratum* pollinated by captive bumblebees. However, does size matching between the pollinator and the floral sexual organ separation explain the efficiency of pollen transfer under natural conditions? This experiment provided evidence that size matching between the pollinator and the herkogamy influences the pollination efficiency within a bumblebee species. In previous pollination observations conducted in Mexico, *S. rostratum* was visited by many bee species, which ranged widely in abdomen diameter (from 1.11 to 19.51 mm). Moreover, natural populations of *S. rostratum* exhibit a huge variation in the separation between the pollinating anther and stigma (from 3.45 to 14.25 mm). Knowing both the size of visiting bees and the herkogamy in different natural populations of *S. rostratum* would be helpful for predicting which bee species are likely to function as pollinators or as thieves, at different locations within this plant's distribution.

3.6. CONCLUSIONS

Due to the complex floral morphology (heteranthery and enantiostyly) of *S. rostratum* that is associated with its specialized reproductive system (buzz-pollination), it is crucial that pollinators fit closely with the sexual organs during the pollination process. The size matching between a pollinator and the sexual organ separation determines the pattern of pollen deposition in *S. rostratum*. When the pollinator was bigger than the separation of the sexual organs, more pollen grains were deposited on stigmas. However, it seems that seed production not only depends on the quantity of pollen deposited but also on other factors (e.g. pollen competition and inbreeding effects). The fruit set is only produced in a specific range of size matching between the pollinator and the sexual organ separation; fruit were set when the bee was three-quarters to twice the size of the herkogamy. We suggest that where visitor-flower combinations are far from this optimal range of size matching, the visitor would ineffective at depositing pollen and would act as a pollen-thief.

3.7. ACKNOWLEGMENTS

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CHAPTER 4:

Thirteen microsatellites developed for *Solanum rostratum* and related species

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4.1. ABSTRACT

Microsatellite markers were developed using second-generation sequencing in *Solanum rostratum* as a tool to study the reproductive biology and genetic structure of this invasive species. Thirteen microsatellites were successfully discovered and amplified in a single multiplexed PCR. All loci showed genetic variation in *S. rostratum*. Cross – amplification in five closely related taxa was successful for a subset of loci. The set of 13 microsatellite markers developed here provides a time-effective and cost-effective genetic tool to study the reproductive biology of *S. rostratum*. The demonstrated transferability of the PCR multiplex to related taxa also highlights its usefulness for evolutionary studies across *Solanum* sect. *Androceras*.

Key words: invasive species; population genetics; reproductive biology; *Solanum rostratum; Solanum* sect. *Androceras*.

4.2. INTRODUCTION

Solanum rostratum Dunal (Solanaceae) is a diploid, annual, self-compatible herb with weakly zygomorphic bee-pollinated nectarless yellow flowers (Whalen 1979). It forms part of a clade of 12 species of *Solanum* Section *Androceras*, a group that has been used as a model to investigate the relationship between flower form and reproductive isolation and mating patterns (e.g. Vallejo-Marín *et al.* 2009, Whalen 1979). The native range of *S. rostratum* extends from Central Mexico to the United States (Whalen 1979). However, it is now found in China, Russia, Australia, and Europe (Lin & Tan 2007; Vallejo-Marín unpublished; Whalen 1979). The limited availability of genetic markers in *S. rostratum* currently thwarts studies on the reproductive biology and genetic structure of both native and invasive populations.

In this study, we describe 13 new microsatellite markers for *S. rostratum* to enable further studies on its phylogeography and reproductive biology. We used second-generation sequencing and bioinformatic tools to optimize a single microsatellite PCR multiplex (Guichoux *et al.* 2011) for cost and time-effective amplification of these markers in *S. rostratum* and related taxa.

4.3. METHODS AND RESULTS

Seven *S. rostratum* individuals were sampled from two Mexican populations (Tehuacán and Mexico City, Table 1). Genomic DNA was isolated from silica-dried leaf tissue with DNeasy Plant Mini kit (QIAGEN, Crawley, West Sussex, UK) and sent to Genoscreen (Lille, France) for microsatellite-enriched library preparation and sequencing by 454 GS FLX Titanium (Roche Applied Science, Indianapolis, Indiana, USA) according to Malausa *et al.* (2011). Briefly, the pooled sample of seven individuals was subject to genomic DNA fragmentation, ligated to standard adapters, and enriched with eight microsatellite probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC). The enriched DNA was then amplified using adapter-specific primers as described in Malausa *et al.* (2011). The resulting library was tagged with a specific multiplex identifier (MID) tag sequence and pooled together with eight other samples in a quarter of a 454 GS FLX Titanium run for sequencing. The resulting 33 491 reads (average length = 254 ±107 bp; mean ± SD) were analyzed with QDD version 1.3 (Meglécz *et al*. 2010) to design microsatellite primers using selection criteria detailed in Lepais and Bacles (2011). These criteria were chosen to optimize potential for single

PCR multiplexing of the designed primers, and included limiting the length of the expected PCR product to between 90 and 400 bp, optimal primer length of 24 bp (range 21-30 bp), optimal annealing temperature of 63°C (range 60-66°C), and 50% GC content (range 40-60%). Five hundred fifty-seven microsatellites were identified, from which 355 had designed primers.

Table 1. Voucher information for taxa used in this study. All vouchers deposited at the University of Stirling (STIU).

Species—Country and Locality, Accession number, (Latitude, Longitude).
***Solanum rostratum* Dunal** — Mexico, Tehuacán, Puebla, 08s104, (18.48° N, 97.41° W).
***Solanum rostratum* Dunal** — Mexico, Mexico City, Distrito Federal, 10s110, (19.313° N, 99.178° W).
***Solanum rostratum* Dunal** — Mexico, Plan de Fierro, Puebla, TP-8, (18.33° N, 97.57° W).
***Solanum rostratum* Dunal** — Mexico, Teotihuacán, Estado de México, TEM-19, (19.68° N, 98.86° W).
***Solanum fructu-tecto* Cav.** — Mexico, Atitalaquia, Hidalgo, AH-9, (20.07° N, 99.22° W).
***Solanum heterodoxum* Dunal** — Mexico, Fresnillo, Zacatecas, FZ-24, (23.10° N, 102.80° W).
***Solanum grayi var.grandiflorum* Whalen**—Mexico, Los Zapotes, Sinaloa,07s197,(23.45°N,100.13°W)
***Solanum grayi var. grayi* Whalen** — Mexico, Los Álamos, Sonora, 07s189, (27.00° N, 108.93° W).
***Solanum lumholtzianum* Bartlett** — Mexico, El Progreso, Sinaloa, 07s41.

Two screenings of 24 primer pairs were performed following the selection strategy of Lepais and Bacles (2011). In brief, microsatellite loci containing dinucleotide (AG and AC) and trinucleotide (AAC, AAG and AGG) repeat motifs were categorized in one of six expected PCR product size classes and ranked based on the number of motif repeats. In the first screening, a selection of 24 primer pairs representing all six size classes was chosen for testing in simplex PCR format on a panel of 19 *S. rostratum* individuals. Based on the results of this first screening, a new set of 24 primer pairs was then selected to try to obtain successfully amplifying loci across all size classes, and screened in the same 19 individuals. Simplex PCR cycles consisted of a denaturing step of 5 min at 94°C; followed by 30 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, and then eight cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 45 s; and a final elongation step of 10 min at 72°C (Lepais & Bacles 2011). Fragment analysis was performed on an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, California, USA) at DNA Sequencing & Services (Dundee, UK) and subsequently analyzed using STRAND (VGL, University of California, Davis, California, USA). Out of 48 tested primer pairs, 29 successfully amplified, and 15 were polymorphic with repeatable profiles.

Thirteen loci were found to be compatible for simultaneous PCR multiplexing using Multiplex Manager (Holleley & Geerts 2009) and were evaluated using a panel of 38 *S. rostratum* individuals from two populations (Teotihuacán and Plan de Fierro; Table 1). In addition, marker transferability and multiplex applicability were tested on two individuals from each of five taxa in *Solanum* Sect. *Androceras*: *S. fructu-tecto* Cav., *S. heterodoxum* Dunal, *S. grayi* Rose var. *grandiflorum* Whalen, *S. grayi* var. *grayi* Whalen, and *S. lumholtzianum* Bartlett (Table 1). The multiplex PCR reaction was performed using 1X QIAGEN Type-it Microsatellite PCR Kit (QIAGEN), various concentrations (Table 2) of each of the 13 fluorescent forward primers labeled with one of 6-FAM (Eurofins MWG Operon, Ebersberg, Germany), VIC, PET or NED (Applied Biosystems) dyes and reverse primer and approximately 5 ng of template DNA. PCR cycles consisted of a denaturing step of 5 min at 95˚C, followed by 30 cycles of 95˚C for 30 s, 58˚C for 180 s, and 72˚C for 30 s, and a final elongation step of 30 min at 60˚C. Products were analyzed in an ABI3730xl capillary sequencer (Applied Biosystems). Fluorescence profiles were analyzed using STRAND and exported to MSATALLELE (Alberto 2009) in *R* version 2.12.0 (R Development Core Team 2010) to determine suitable allele bin range.

Fig. 1: Example of a typical electropherogram profile obtained for one individual with the multiplex PCR genotyping protocol presented here (a), and diagram showing the allele size range and fluorescent dyes of each of the 13 loci (b). In (a), down-turned triangles indicate alleles at each locus; fragments sizes (bp) of the 500 LIZ size standards are indicated by numbers above each corresponding peaks. In (b), dark rectangles represent the observed allele range in 34 *S. rostratum* individuals; light rectangles represent an arbitrary potential allele range used during the multiplex design to avoid overlap of loci with the same fluorescent dye.

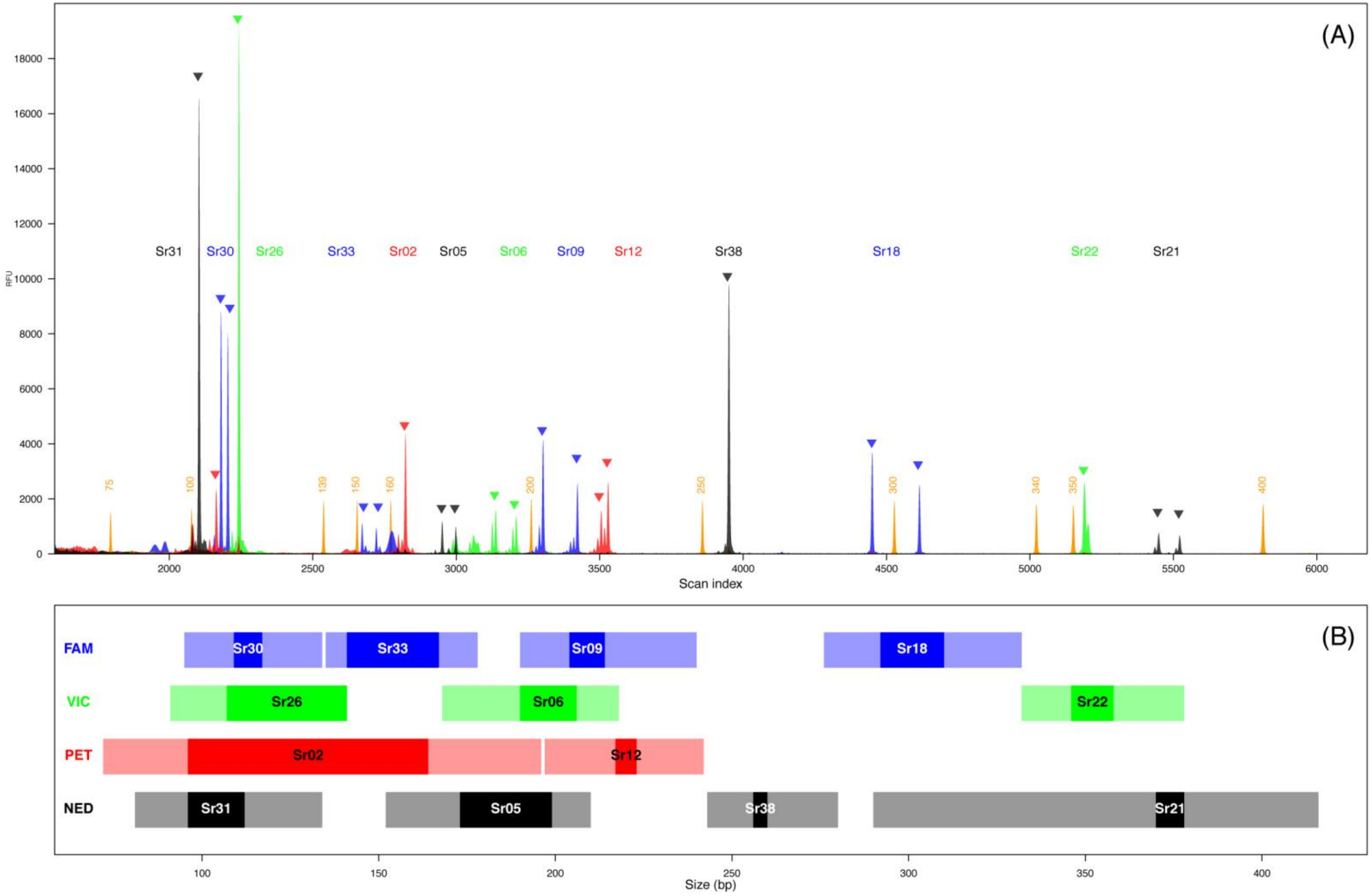

Table 2. Characteristics of 13 microsatellite primers developed in *Solanum rostratum* and optimized to coamplify in a single multiplex PCR.

Locus	Repeat type	EMBL accession	Primer sequences (5' -> 3')		Dye	[Primer] (nM)	Size range (bp)
Sr09	$(AC)_8$	FR846150	F:	TCACTTTGAGACCCCTAACACCTC	FAM	170	204-214
			R:	TAAGAGGAACAGGAAGAAGAGGGC			
Sr18	$(CA)_6$	FR846159	F:	AATCACCCACCTACTGTGACGTTT	FAM	170	292-310
			R:	ATCCAGTGCTTGTGTTGATAGGCT			
Sr30	$(TC)_8$	FR846171	F:	ATGCTCCCCATTTTCCATTTTC	FAM	120	109-117
			R:	ATCTGCTGAGAAGTTGAATTTCCG			
Sr33	$(GT)_6$	FR846174	F:	ATACTTCATTTGTTGCAGGAGCTG	FAM	340	141-167
			R:	CAAAAGCTAAAACCCAAGACAGGA			
Sr06	$(AG)_8$	FR846147	F:	ATGAGGACCCAGTTGAGTTTCTTG	VIC	340	190-206
			R:	CTTTAAATTCCTCCCATCCAGCTC			
Sr22	$(AAC)_6$	FR846163	F:	CTAACAATTTCTCCAACAACCTTGG	VIC	170	346-358
			R:	CCAAAACTTTCACCAGAAAACTCAC			
Sr26	$(CT)_9$	FR846167	F:	GCTATTTCCCCTACTCCGGTTCTT	VIC	120	107-141
			R:	GTAGGTGCCCAAATATTGATCCAG			
Sr05	$(TC)_9$	FR846146	F:	CTGAATGTTGTAATTGGGTGTCCA	NED	340	173-199
			R:	ACAAGAACCGAAAACGAAGAACAG			
Sr21	$(AAC)_8$	FR846162	F:	GGTCGATTGCCTCTATCTACTGTTG	NED	200	370-378
			R:	TGGTAGTGGTAAGGTCTGCGTACA			
Sr31	$(TC)_7$	FR846172	F:	AACTCAGCCATAGTTCCAGACACC	NED	170	96-112
			R:	AGAGGTGCTGGAGTTGAGAAAAGA			
Sr38	$(GAA)_6$	FR846179	F:	GATCTCAAAGAAGGGTCTCCCCTA	NED	170	256-260
			R:	AGTGCAGAAAATGAAGTGCTCTGG			
Sr02	$(CT)_{13}$	FR846143	F:	GGAATAGAGGGAGTTATACAGAATACACGA	PET	200	96-164
			R:	GGCGAGACCAGTTCTTGTCATATT			
Sr12	$(TC)_7$	FR846153	F:	GGTTAGGCCCAAACGTTGAAATAA	PET	170	217-223
			R:	ACCAGAGATGGATCAAACTTCAGC			

Notes: Shown for each primer pair are the repeated motif type, the accession number at the European Molecular Biology Laboratory—Nucleotide Sequence Database (EMBL; http://www.ebi.ac.uk/embl/), the forward and the reverse primer sequences, the fluorescent dye added to the 5’ end of the forward primer, the final primer concentration ([Primer]) in the PCR mixture (nM), and the allele size range (bp).

All 13 loci were polymorphic in at least one population with two to 13 alleles detected (Fig. 1; Table 3), and showed moderate genetic diversity with expected heterozygosity ranging from 0.00 to 0.86 (Table 3). All loci amplified in *S. fructu-tecto*; Sr21, Sr06 and Sr02 failed to amplify in S. *heterodoxum*; Sr21 and Sr06 did not amplify in *S. grayi* var. *grayi*; Sr21, Sr06 and Sr02 did not amplify in *S. grayi* var. *grandiflorum*; and Sr21, Sr06 and Sr26 failed to amplify in *S. lumholtzianum*. It is important to note that loci that amplified in these taxa did so within the expected size range, thus demonstrating the transferability of the multiplex protocol.

Table 3. Results of initial loci screening in two populations of *Solanum rostratum*.

	Population 1 (N=15)		Population 2 (N=23)		Total
Loci	N_a	H_e	N_a	H_e	N_a
Sr09	2	0.238	3	0.343	4
Sr18	2	0.186	6	0.783	6
Sr30	3	0.476	3	0.573	5
Sr33	4	0.612	4	0.489	5
Sr06	4	0.667	5	0.612	6
Sr22	4	0.352	3	0.606	4
Sr26	4	0.531	5	0.501	6
Sr05	8	0.852	6	0.754	12
Sr21	2	0.457	3	0.625	3
Sr31	3	0.440	6	0.792	8
Sr38	1	0.00	2	0.417	2
Sr02	7	0.660	9	0.862	13
Sr12	3	0.676	5	0.543	5

Notes: N = Number of genotyped individuals, N_a = number of alleles;
H_e = expected heterozygosity.
Population 1 = Teotihuacán, Estado de México;
Population 2 = Plan de Fierro, Puebla.

4.4. CONCLUSIONS

Second-generation sequencing and novel bioinformatic approaches are very effective tools to isolate microsatellite markers in nonmodel organisms. This allows discovery of numerous microsatellites that can be combined in one or few PCR reactions, reducing both time and cost of genotyping (Lepais & Bacles 2011). Here we developed a set of 13 polymorphic microsatellite markers for *S. rostratum* that can be amplified in a single multiplexed PCR and demonstrated its potential use in related taxa, thus enabling future investigation of numerous ecological and evolutionary questions.

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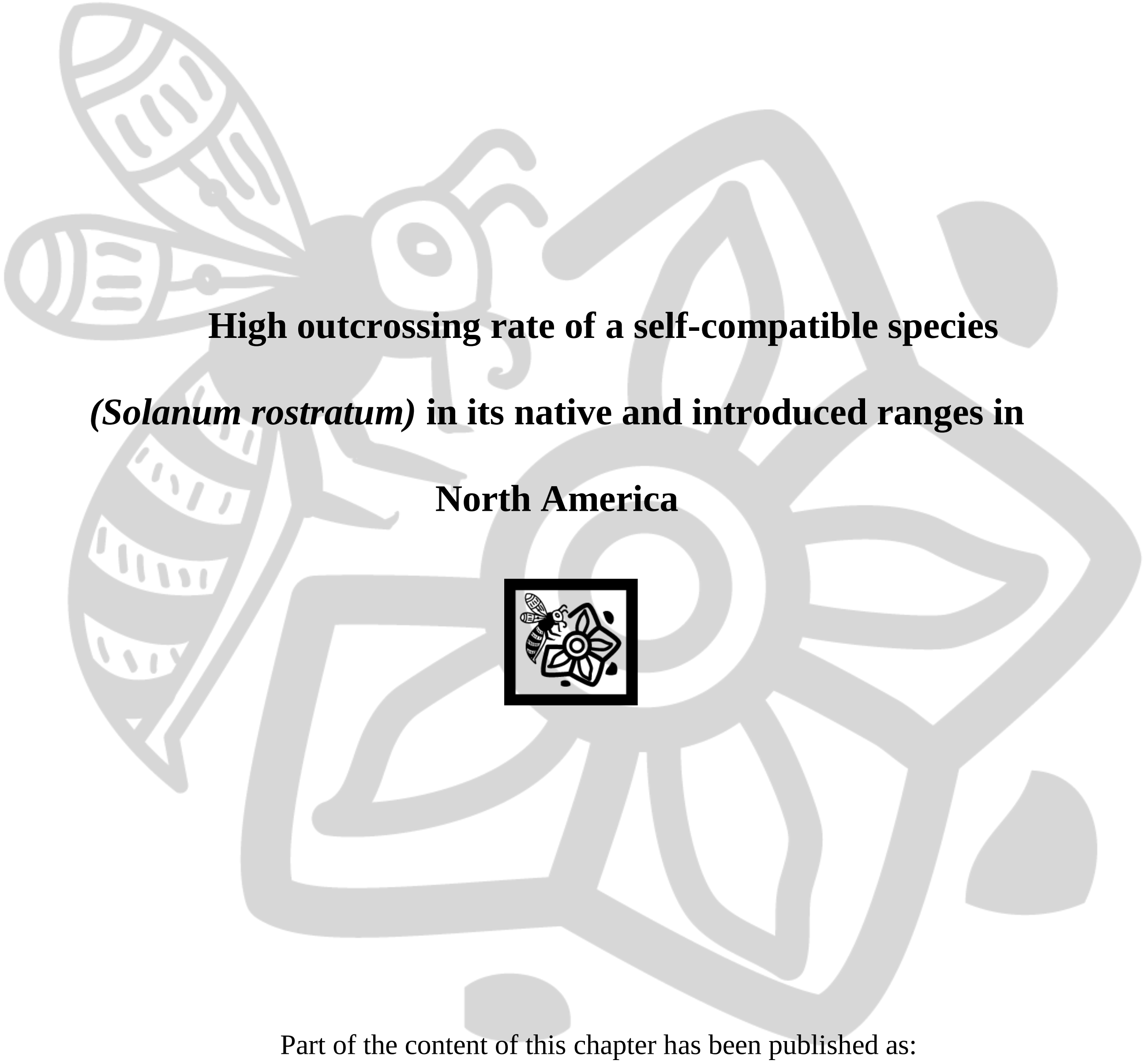
Whalen, M. (1979). Taxonomy of *Solanum* section *Androceras*. *Gentes Herbarum*, **11**, 359-426.

CHAPTER 5:



High outcrossing rate of a self-compatible species *(Solanum rostratum)* in its native and introduced ranges in North America

Part of the content of this chapter has been published as:

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5.1. ABSTRACT

Enantiostyly, a floral polymorphism where the style is deflected to either the right or left of the axis of symmetry, occurs in some self-compatible species that lack the physiological mechanisms for rejecting self-pollen. Most enantiostylous species produce both floral morphs (left- and right-styled) in the same plant (monomorphic enantiostyly), and self-pollination can occur among flowers of different morphs on the same plant (geitonogamy). Nevertheless, monomorphic enantiostyly can still promote cross-fertilization by reducing geitonogamy between flowers of the same morph. To date, little has been discovered about whether enantiostylous species are able to maintain high rates of cross-fertilization in introduced populations when they are exposed to different pollination services. Here, I studied *Solanum rostratum,* a self-compatible, bee-pollinated and enantiostylous species that depends on pollinators to reproduce. The main goal of this research was to compare the outcrossing rates between native and introduced areas throughout its distribution of *S. rostratum* in North America. In order to achieve this goal, I determined the mating system (the contributions of self- and cross-fertilization to seed set) of two populations that were introduced into Kansas (USA) 130 years ago, and compared these calculations with those previously performed on four native Mexican populations. To estimate the mating system in each population, I genotyped 10–12 sibs of 20 families for each population using 13 microsatellites loci. I found that, on average, *S. rostratum* maintained a high outcrossing rate (t_m = 0.75 ± 0.03) across the six populations. There was no significant difference between the Mexican (average t_m = 0.71 ± 0.02) and USA populations (average t_m = 0.82 ± 0.001). I conclude that *S. rostratum* maintains high outcrossing rates between native and introduced populations of *S. rostratum* in North America,

probably facilitated by pollinators in the introduced range that function as equivalents to pollinators native to the plant's centre of distribution in Mexico (i.e. large buzzing-bees).

Keywords: Buzz-pollination, mating system, *Solanum rostratum*, Mexico, USA.

5.2. INTRODUCTION

Flowering plants have morphological and physiological mechanisms that influence their mating patterns (Eckert & Barrett 1994), which can act during and after pollination (Harder & Barrett 1996). While floral morphology and phenology influence the quantity and quality of pollen dispersed during pollination, post-pollination mechanisms act at the pistil as physiological level (i.e. self-incompatibility systems), by recognition and rejection of self-pollen or pollen from relatives (Barrett 1998). Self-compatible species lack these physiological mechanisms for rejecting self-pollen and the variation in floral traits (i.e. floral size and the extent of stigma exsertion) plays an important role in determining the mating pattern (Rick, Holle & Thorp 1978).

Self-compatible plants that exhibit floral differences could record extensive variation in the degree of outcrossing (Rick, Holle & Thorp 1978). For example, in *Eichhornia paniculata*, a self-compatible plant with style length polymorphism, the outcrossing rate is found to vary widely (t = 0.002–0.960 among the 32 populations studied) and this rate is higher where three style morphs are present (tristylous populations; Barrett & Husband 1990). In contrast, self-incompatible species are usually associated with high outcrossing rates (0.08–1; Goodwillie, Kalisz & Eckert 2005).

Self-fertilization occurs in self-compatible plants through intrafloral self-pollination (Jesson & Barrett 2005) or as geitonogamous self-pollination when pollen is transferred among flowers on the same plant (Barrett, Jesson & Baker 2000). On the one hand, the spatial separation of male and female organs within a flower (herkogamy) reduces intrafloral self-fertilization; this is also recently interpreted as a mechanism for avoiding physical interference between the sexual functions (Fetscher 2001; Webb & Lloyd 1986). On the other hand, floral polymorphisms with reciprocal herkogamy in the style deflection as mirror-image flowers (enantiostyly) reduce the level of geitonogamy through right- and left-styled morphs, on the same (monomorphic enantiostyly) or different plants (dimorphic enantiostyly; Jesson & Barrett 2005). Monomorphic enantiostyly is the most common type of enantiostyly found in angiosperms (Barrett, Jesson & Baker 2000). This type of enantiostyly has been recorded less geitonogamous self-pollination than in non-enantiostylous plants in experimental arrays (Jesson & Barrett 2005). However, it is unknown if this asymmetrical polymorphism maintains cross-fertilization under natural conditions across populations in self-compatible species.

It is not known whether this polymorphism is able to maintain cross-fertilization in novel environments with different pollinator services. The ability of an alien plant to colonize a new environment depends significantly on its ability to reproduce in a novel pollination fauna. For this reason, the reproductive system is considered to be a trait that greatly influences the life-history of the plant, because it can determine the demographic and genetic structure of invasive populations. Whether offspring arise from cross- or self-fertilization is relevant, because mates and pollinators may be limited during the establishment of a new population or during subsequent colonizing episodes (Barrett 2011).

I used *Solanum rostratum* as a model of a self-compatible and monomorphic enantiostylous plant (Bowers 1975). This species is native to central Mexico (Whalen 1979) and it has spread not only northwards in the Americas (USA and Canada), but also around the world (Asia, Europe and Australia; The Global Biodiversity Information Facility 2013; Tropicos 2013; Whalen 1979), having the potential to grow aggressively in newly colonized habitats (Zhong *et al.* 2009).

Previous studies have suggested that *S. rostratum* promotes cross-fertilization in both experimental arrays (t = 0.74 ± 0.06; Jesson & Barrett 2005) and in natural populations (t = 0.70 ± 0.03; Vallejo-Marín *et al.* 2013). This species strongly depends on specific size of buzzing bees for reproduction which must closely match the floral morphology of the plant to effectively transfer pollen (Chapter 2 and 3). When a pollinator approaches *S. rostratum* flowers, it vibrates the feeding anthers (specialized for rewarding pollinators) to extract pollen, at the same time the pollinating anther (specialized for fertilization) will usually make contact with the pollinator on the side of its abdomen, while the stigma touches the visitor on the opposite side (Bowers 1975). The side of the abdomen on which pollen is deposited and the side from which pollen is collected, alternate in the two enantiostylous floral morphs, promoting pollen transfer between flowers of different morphs. This asymmetric floral polymorphism has been interpreted as a mechanism by which the precision of cross-pollination is increased in bee-pollinated plants, by reducing the incidence of geitonogamy (Jesson & Barrett 2005). But does this polymorphism maintain cross-fertilization in invasive populations under different pollinator services?

The main goal of this research was to compare the outcrossing rates between native and introduced populations throughout the range of *S. rostratum* in North

America. I compared populations from the centre of its distribution (Mexico), where it has high morphological (Whalen 1979) and genetic diversity (Zhao *et al.* 2013), with populations from the USA, where this species was introduced approximately 130 years ago (according to herbarium records) and it has low genetic diversity (Zhao *et al.* 2013). This species was probably accidentally transported by Spanish caravans during the sixteenth or seventeenth century from locations in southern Mexico, northward into the USA through Texas, New Mexico and Arizona. It later spread into the Great Plains in association with the activity of people or animals (Tower 1906).

In order to compare the native and introduced populations of *S. rostratum* in North America, I used previously published data from four Mexican populations (Vallejo-Marín *et al.* 2013) and I characterized the mating system of two populations from Kansas, USA. To estimate the mating system in each population, 10–12 sibs were genotyped for each of 20 maternal families from each population, using 13 microsatellites that were previously developed for *S. rostratum* (Vallejo-Marín *et al.* 2011). I used the expectation maximization method to estimate the outcrossing rates for each family and I calculated a population mean for each geographic region. I hypothesize that *S. rostratum* maintains a relatively high outcrossing rate in introduced populations, probably promoted by the complex floral structure (enantiostylous and anther dimorphic flowers with both style-deflected morphs in the same plant) and facilitated by pollinators that function as equivalents to the legitimate native pollinators (i.e. large buzzing-bees) of its centre of distribution.

5.3. MATERIALS AND METHODS

5.3.1. Sampling design

To compare the mating systems of native and introduced populations of *S. rostratum* in North America, I used the genetic information previously generated by Vallejo-Marín *et al.* (2013) of four Mexican populations. I also generated genetic information from two populations in Kansas, USA (Table 1). The closest populations in Mexico were separated by a distance of 40 km, and the northernmost and southernmost Mexican populations were separated by 690 km (Fig.1; Vallejo-Marín *et al.* 2013). The populations from Kansas, USA were separate by 12 km (Table 1). The distance between the northernmost Mexican population and the southernmost USA population was 1828 km (Fig.1).

All populations were sampled between 20 October 2010 and 12 October 2011. We collected seeds from between 19 and 20 randomly selected individuals per population, with 2-6 mature fruits sampled per plant, depending on fruit availability. To dry the material and to prevent fungal attacks, we placed the fruits in paper bags and kept them at room temperature. Where fruit was collected before it had opened, as was the case for some of the Mexican population, it was briefly placed in a drying oven at 40°C. We then extracted the seeds from the fruits and kept them in waxed paper bags. These were transported to the University of Stirling, where they were stored at 5°C until they were planted for cultivation.

In order to obtain material for genetic analysis, we collected leaf tissue from young seedlings (2–3 weeks after germination). To induce germination, seeds were pre-treated with a 1000 ppm aqueous solution of gibberellic acid (GA_3) for 24 hours and

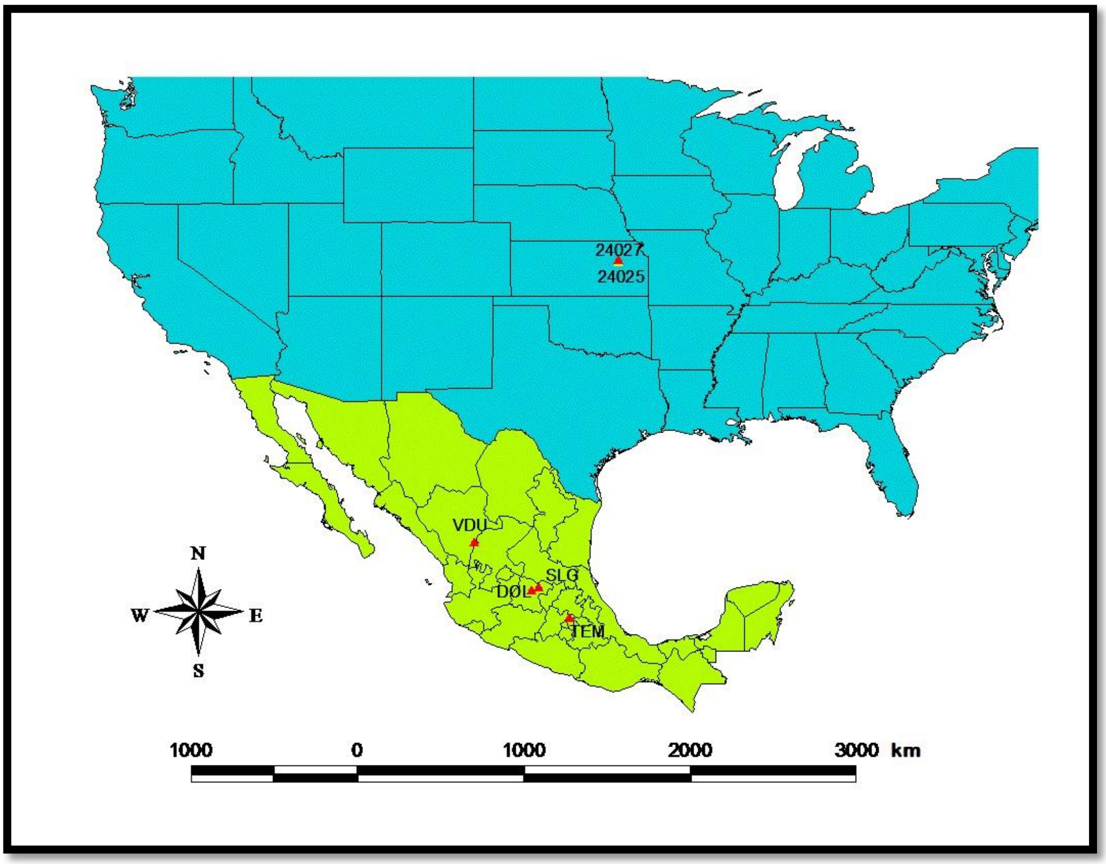


Fig. 1. Map of the four Mexican populations (DOL, SLG, TEM and VDU) and the two USA populations (24025 and 24027). Each red triangle represents a population. The colours represent the countries, with Mexico in green and the USA in blue. In the case of population 24025 is represent by yellow triangle to distinguish from 24027. Population codes are given with further details in Table 1.

then incubated overnight in a growth chamber at 20°C and 12:12 hours light:dark regimen. Between 5 and 22 seeds per family were planted, depending on the seed availability. Seeds were planted in plastic trays that contained Modular Seed Growing Medium compost (William Sinclair Horticulture PLC, Lincoln, UK), and kept in a glasshouse at 14–22°C with natural daylight supplemented with compact-fluorescent lamps ensuring at least 14 hours of light per day. Tissue samples were collected from 10–12 randomly selected seedlings from each family and were stored in silica gel (Fisher Scientific UK Ltd, Leicestershire, UK) until DNA extraction. We sampled a total of 1077 offspring belonging to 117 maternal families from the six populations, with between 156 and 193 seedlings per population (Table 1).

Table 1. Locations and sample sizes of six populations of *Solanum rostratum* in Mexico (MEX) and the USA used to assess the genetic diversity and mating system. Germination proportion represents the average across families in each population, for a total of 2017 seeds planted from the six populations. The data for the four Mexican populations were obtained from Vallejo-Marin *et al.* (2013). Population sizes are only approximate values estimated by one observer; for the USA populations, this was a qualitative description of the abundance.

Code	**Population**	**Latitude (N)**	**Longitude (W)**	**Elevation (m)**	**Population size** (number of plants)	**Maternal families sampled**	**Genotyped offspring** (Mean; median; range per family)	**Germination proportion** (S.E.)
DOL	Dolores, Guanajuato, MEX	21.161°	100.886°	1913	150	19	179 (9.42; 10; 2-12)	0.723 (0.06)
SLG	San Luis La Paz, Guanajuato, MEX	21.309°	100.514°	2050	50	19	156 (8.21; 8; 5-12)	0.956 (0.02)
TEM	Teotihuacán, Estado de México, MEX	19.683°	98.858°	2277	2000	19	187 (9.84; 10; 2-12)	0.763 (0.05)
VDU	Vicente Guerrero, Durango, MEX	23.744°	103.996°	1926	150	20	178 (8.90; 10; 3-12)	0.738 (0.06)
24025	Wabaunsee County, Kansas, USA	38.871°	96.207°	405	Locally abundant	20	184 (9.20; 9; 8-10)	0.903 (0.03)
24027	Wabaunsee County, Kansas, USA	38.974°	96.210°	427	Locally abundant	20	193 (9.65; 10; 8-10)	0.932 (0.02)
Total						**117**	**1077**	

5.3.2. DNA extraction and genotyping

We extracted DNA from leaves using a CTAB protocol (Doyle & Doyle 1990) and quantified the resulting DNA using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). We genotyped each individual at 13 microsatellite loci according to an existing protocol (Vallejo-Marin *et al.* 2011); these loci had previously been probed successfully in four of these populations (Vallejo-Marín *et al.* 2013). The 13 microsatellite loci were amplified in a single multiplex reaction which contained 1× Type-it Microsatellite PCR Kit (QIAGEN, Crawley, West Sussex, UK) and various concentrations of each of the 13 fluorescent forward primers, which were labelled with one of 6-FAM (Eurofins MWG Operon, Ebersberg, Germany), VIC, PET, or NED (Applied Biosystems, Foster City, California, USA), the reverse primers (Vallejo-Marin *et al.* 2011), and 2.5–30 ng of template DNA. PCR cycles were performed in a Veriti thermocycler (Applied Biosystems), which consisted of a denaturing step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 180 s, and 72°C for 30 s, and a final elongation step of 30 min at 60°C (Vallejo-Marín *et al.* 2013). Fragment analysis was performed on an ABI 3730xl capillary sequencer with a GeneScan 500 LIZ internal size standard (Applied Biosystems, Foster City, California, USA) at DNA Sequencing & Services (Dundee, UK).

5.3.3. Genetic and statistical analysis

In order to score the microsatellites, we first analysed the fluorescence profiles using STRAND version 2.4.59 (Toonen & Hughes 2001). We exported the data to MSATALLELE (Alberto 2009) in *R* version 3.0.1 (R Core Development Team 2013) to assign peaks to suitable allele bins range. For each population, I reanalysed all data and calculated the number of alleles, the average number of alleles per locus (N_a), the unbiased heterozygosity (H_e), observed heterozygosity (H_o) and Inbreeding coefficients (F_{is}) using GENALEX 6.5 (Peakall & Smouse 2012).

5.3.4. Mating system analysis

Parameters of the mating system were estimated from the genotypes of the progeny using the program MLTR 3.4 (Ritland 2002). Vallejo-Marín *et al.* (2013) excluded the loci that recorded null alleles when analysing the mating system of the Mexican populations. However, the authors mention that the estimates of outcrossing rate only changed slightly if these loci were excluded from the analysis. Accordingly, I decided to include all 13 loci in the estimation of the mating system parameters in the six populations. I calculated the multi-locus (t_m) and single-locus (t_s) outcrossing rate and the difference between these rates ($t_m - t_s$), which is used to estimate the level of biparental inbreeding that results from mating among relatives and that causes an increase in homozygosity (Ritland 2002). With biparental inbreeding, the difference between the rates is positive because single-locus estimates include mating among relatives, whereas multi-locus estimates exclude much of the selfing that is due to mating between relatives (Ritland 2002). These mating parameters were estimated using the expectation maximization (EM) method and standard errors were approximated as the standard deviation of 1000-resample bootstrap maternal families.

5.4. RESULTS

5.4.1. Genetic diversity

All 13 loci used in Mexican populations were polymorphic (Vallejo-Marín *et al.* 2013) in comparison with the USA populations, where at least one or two of these loci were monomorphic (Sr9 in both USA populations and Sr21 in population 24027). In general, the Mexican populations had a higher average number of alleles per locus (N_a = 4.67 ± 0.36 alleles; a maximum of 12 alleles per locus) than the USA populations (N_a = 3.19 ± 0.50 alleles; a maximum of 7 alleles per locus; Table 2). The expected average heterozygosity among the loci ranged between 0.43 and 0.57 (Table 2). The average expected heterozygosity in the Mexican populations was higher (H_e = 0.58 ± 0.05, Vallejo-Marin *et al.* 2013) than in the USA populations (H_e = 0.45 ± 0.05). The inbreeding coefficient (F_{is}) found to be higher in the Mexican populations than in the USA populations (Table 2). However, this coefficient did not differ among the six populations when the 95% confidence intervals were considered (graph not shown).

5.4.2. Outcrossing rates

Populations of *S. rostratum* analysed in this study presented intermediate to high outcrossing rates (Table 3). The average multi-locus outcrossing rate across the six populations was 0.75 ± 0.03, ranging from 0.690 ± 0.054 in population TEM to 0.821 ± 0.057 in population 24025. The USA populations presented slightly higher outcrossing rates (average t_m = 0.82 ± 0.001) than the Mexican populations (average t_m = 0.71 ± 0.02), although this difference was not significant (Fig. 2). Positive differences between t_m and t_s suggest biparental inbreeding, which was higher in the USA populations than in the Mexican populations (Fig. 3).

Table 2. Summary of the genetic diversity of the six populations of *Solanum rostratum*, obtained by high-throughput genotyping at 13 microsatellite loci. The number of individuals genotyped (N_{ind}), the number of polymorphic loci (P), the average number of alleles per locus (N_a), the unbiased heterozygosity (H_e), the observed heterozygosity (H_o) and the inbreeding coefficient (F_{is}) were calculated using GenAlEx (Peakall & Smouse, 2006). Total values were calculated on a combined data set of all populations.

Population	N_{ind}	P	N_a (range)	H_e(S.E.)	H_o(S.E.)	F_{is}(S.E.)
DOL	179	13	4.92 (2–12)	0.505 (0.061)	0.358 (0.042)	0.283 (0.034)
SLG	156	13	4.54 (2–9)	0.481 (0.070)	0.342 (0.056)	0.254 (0.051)
TEM	187	13	4.23 (2–8)	0.429 (0.062)	0.319 (0.050)	0.212 (0.055)
VDU	178	13	5.00 (2–10)	0.566 (0.039)	0.451 (0.039)	0.208 (0.030)
24025	184	12	3.385 (1–7)	0.450 (0.067)	0.396 (0.073)	0.092 (0.102)
24027	193	11	3.000 (1–7)	0.443 (0.062)	0.409 (0.070)	0.077 (0.096)
Total	1077	13	4.179 (3–17)	0.479 (0.025)	0.379 (0.023)	0.192 (0.027)

Table 3. The mating system parameters in the six populations of *Solanum rostratum*. The parameters were calculated by expectation maximization using MLTR (Ritland 2002), and where the pollen and ovule frequencies were assumed to be equal. The multi-locus outcrossing rate (t_m), single-locus outcrossing rate (t_s) and the difference between these rates (biparental inbreeding; $t_m - t_s$) are given, with the standard error shown in parentheses. Each standard error was calculated from 1000 bootstrap replicates by resampling the maternal families.

Population	N_{ind}	t_m	t_s	$t_m - t_s$
DOL	179	0.693 (0.062)	0.349 (0.042)	0.344 (0.045)
SLG	156	0.692 (0.050)	0.366 (0.031)	0.327 (0.034)
TEM	187	0.690 (0.054)	0.307 (0.034)	0.383 (0.032)
VDU	178	0.770 (0.048)	0.474 (0.044)	0.296 (0.034)
24025	174	0.821 (0.057)	0.308 (0.050)	0.513 (0.027)
24027	189	0.819 (0.048)	0.314 (0.041)	0.505 (0.027)

Fig. 2. Multi-locus outcrossing rate (t_m) with 95% confidence intervals in the six populations of *Solanum rostratum.* The dashed line shows the average outcrossing rate across all six populations ($t_m = 0.75 \pm 0.03$). Populations are grouped by country and names are given in Table 1.

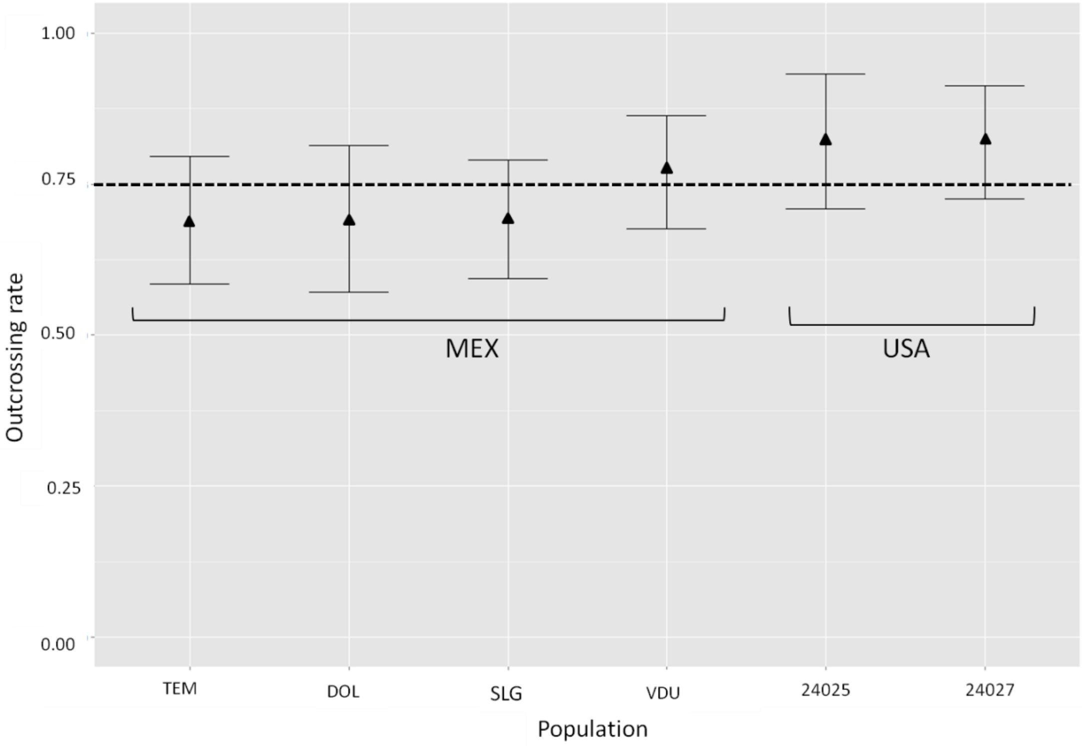
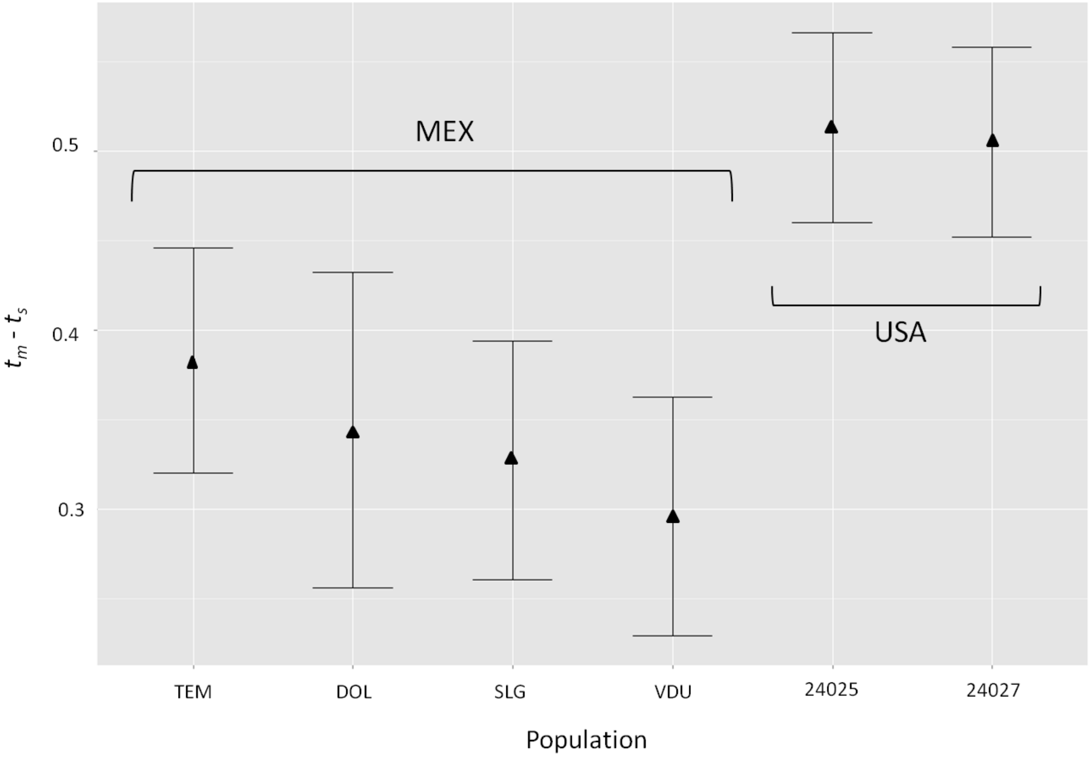


Fig. 3. Biparental inbreeding ($t_m - t_s$) with the 95% confidence intervals in the six populations of *Solanum rostratum.* Populations are grouped by country and names are given in Table 1.



5.5. DISCUSSION

We found similar levels of genetic diversity in this study to those reported by Zhao *et al.* (2013), who investigated other Mexican and USA populations using 10 of the 13 microsatellites. The Mexican populations had on average a higher number of alleles per locus (N_a = 4.67 ± 0.36 alleles; N_a = 4.60 ± 0.16 alleles, Zhao *et al.* 2013) and a higher average of expected heterozygosity (H_e = 0.58 ± 0.05, Vallejo-Marín *et al.* 2013; H_e = 0.53 ± 0.02, Zhao *et al*. 2013) than the USA populations (N_a = 3.19 ± 0.50 alleles; 3.4 ± 0.09 alleles, Zhao *et al.* 2013 and H_e = 0.45 ± 0.05; H_e = 0.45 ± 0.02, Zhao *et al*. 2013). Unlike in this study, where all 13 loci were found to be polymorphic in the Mexican populations (Vallejo-Marín *et al.* 2013), Zhao *et al.* found two monomorphic loci (Sr9 and Sr12) in Mexican populations (SLP and QSJ, respectively). Also both Mexican and USA populations had a deficit of heterozygotes shown by the significant values of the inbreeding coefficients reported (F_{is} = 0.256, Vallejo-Marín *et al.* 2013; F_{is} = 0.104–0.415, Zhao *et al.* 2013). In neither of the previous studies did the inbreeding coefficients differ between native and introduced populations. However, in this study the two USA populations that we sampled were close together in Kansas, we found similar levels of genetic diversity to those of Zhao and colleagues (2013), who sampled five populations across Oklahoma and Kansas. More sampling effort in the plant's introduced range is necessary to clearly determine its invasion history in the USA. Furthermore, sampling in the north of Mexico will be relevant to understanding this invasion history, because this region separates the known Mexican populations, which have a higher level of genetic diversity, from these USA populations.

Despite this limitation, our results are a good beginning that demonstrates that outcrossing rates are similar between native and invasive populations. How is this self-compatible species able to maintain similar outcrossing rates between native and introduced populations? The complex floral morphology and inflorescence architecture of *S. rostratum* probably maintain the relatively high outcrossing rates ($t_m = 0.75 \pm 0.03$) across the native and introduced range in North America. The outcrossing rate found in *S. rostratum* is at the high end of the range for mixed mating systems ($0.2 < t > 0.8$; Schemske & Lande 1985). This high cross-fertilization rate is probably facilitated by bees that function as equivalents to the native pollinators at the centre of the plant's distribution.

The complex floral morphology of *S. rostratum* requires a close physical interaction between insect visitors and the floral sexual organs. The size of a pollinator relative to the flower is an important feature that determines whether contact with the sexual organs is made and determines the dynamics of pollen transfer between plants (Chapter 3). In Mexico, only a small set of visitors act as pollinators, which are mainly large buzzing bees, with the rest acting as pollen thieves (Chapter 2). When large bees forage in buzz-pollinated flowers, they collect largest quantity of pollen per flower visited (De Luca & Vallejo-Marín 2013). These large buzzing bees touch the pollinating anther with one side of their abdomen and the stigma with the opposite side when the pollen is released from the feeding anthers by vibration (Bowers 1975).

Firstly, I speculate that native large buzzing bees such as *Bombus spp.*, *Centris spp.* and *Xylocopa spp.* (see Chapter 1, Table 1), which were previously reported as being visitors of *S. rostratum* in the USA (Bowers 1975; Harris & Kuchs 1902; Jesson & Barrett 2005; Linsley & Cazier 1963), function as equivalents to the principal pollinators of this plant in Mexico. *Bombus* are the most frequent pollinators of *S. rostratum* in the USA (Bowers 1975), whereas in Mexico other large buzzing bees were observed (*Centris spp., Xylocopa spp.* and *Thygater sp.* see Chapter 2). In North America, the genus *Bombus* is common in regions of temperate climate; in the USA 42 species have been recorded, and in Mexico 19 species have been recorded (Ayala, Griswold & Bullock 1993). In central Mexico, we did not observe *Bombus* visiting *S. rostratum* flowers in the three populations that we sampled (Chapter 2). This was probably because the species of *Bombus* present in Mexico are found principally in mountainous areas forested by *Pinus, Quercus* and *Abies* (Ayala, Griswold & Bullock 1993), while *S. rostratum* usually occurs in disturbed habitats such as roadsides and abandoned fields (Vallejo-Marín *et al.* 2013).

Secondly, exotic bees (i.e. honeybees and bumblebees) are likely to pollinate *S. rostratum* in introduced range. These bees usually have a high demand for resources and prefer to visit exotic plants (Goulson 2003) because they are abundant and provide high quantities of pollen or/and nectar. This would subsequently give rise to a mutualistic relationship between both alien organisms (Morales, Traveset & Ramírez 2009). For example, *Solanum torvum*, a buzz-pollinated plant that reproduces successfully in regions outside its native range, is pollinated by both native bees (i.e. halictids and carpenter bees) and exotic bees (i.e. *Euglossa viridissima*) in southern Florida, where the most efficient pollinators are the exotic bees (Liu & Pemberton 2009). In the particular case of *S. rostratum*, the non-buzzing *A. mellifera* is considered a poor pollinator. Honeybees are widely distributed around the world and can play an important role in the establishment of *S. rostratum* in newly invaded habitats. Finally, an alternative explanation, to ensure the reproduction of *S. rostratum* in invasive populations, is probably attributed to modifications in the floral morphology that are associated with the evolution of self-fertilization or asexuality in this plant that ensure reproduction when outcrossing is unpredictable, such as when mating partners or pollinators are limited (Eckert *et al.* 2006). However, there is no evidence of autonomous self-fertilization or asexual reproduction in native populations of *S. rostratum* (Chapter 2).

However, *S. rostratum* maintains relatively high rates of outcrossing across its populations, while self-fertilization accounts for only 25 % of progeny, which may be facilitated by pollinators through intrafloral selfing or geitonogamy in individuals with large floral displays (Vallejo-Marín *et al.* 2013). The illegitimate manipulation of *S. rostratum* flowers by *Apis mellifera* (a non-buzzing bee) may contribute to self-fertilization, since it is a poor pollinator that does occasionally make contact with the stigma during a visit. The remaining instances of self-pollination may be due to legitimate pollinators (large buzzing-bees) that produce small clouds of pollen when vibrating the anthers. These pollen grains may land on the stigma of the same flower, thus promoting selfing by the flower (Larson & Barrett 1999; Wanigasekara & Karunaratne 2012). Another option is that these bees promote geitonogamous selfing by transferring pollen between the flowers of opposite morphs on a plant with a large floral display (Vallejo-Marín *et al.* 2013).

The inbreeding coefficient found in *S. rostratum* was low (F_{is} = 0.192) in the studied populations. Although an inbreeding depression experiment was not conducted, the rate of germination found was high (above of 70%) across populations and the outcrossing rate did not correlate with the proportion of seeds that germinated (Vallejo-Marín *et al.* 2013). It seems that if there is inbreeding depression, it would be at a low level in selfed seeds during germination. Another way that inbreeding occurs is through mating between related individuals (biparental inbreeding), which reduces the genetic cost of outcrossing (i.e. the meiotic cost) because it increases the relatedness of parents to their outcrossed progeny (Uyenoyama 1986). The biparental coefficient ($t_m - t_s$) was higher in the USA than in the Mexican populations (Fig. 3) without a significant difference in the outcrossing rates between the native and introduced populations (Fig. 2). This result suggests that cross-fertilization occurred more frequently between relatives in the USA populations than in the Mexican populations. It is likely that the high biparental coefficient is related to the mechanisms of seed dispersal being less efficient in the USA populations, which would increase the possibility that relatives grow close together and pollinate between them. However, the mechanisms or vectors of seed dispersal of this species are not well known at the centre of its distribution. In China, where this species is invasive, the water of irrigation canals is the primary seed dispersal agent, and the secondary, localised dispersal away from the canals is facilitated by sheep, wind and ants (Amanulla *et al.* 2013). Additional work is necessary to further explore these seed dispersal mechanisms and how they explain the differences between the native and introduced populations in the mating among relatives.

Finally, similar rates of outcrossing in *S. rostratum* across native and introduced populations with different levels of genetic diversity (Mexico > USA) suggest that the low genetic diversity found in the USA was not related to changes in the mating system. For example, in *Eichhornia paniculata* the proportion of polymorphic loci and the average heterozygosity of populations decline with an increase in the incidence of self-fertilization (Barrett & Husband 1990). In this case, the low genetic diversity in USA could be attributed to another factor, such as bottleneck acting on introduced populations (Zhao *et al.* 2013).

5.6. CONCLUSION

The rates of outcrossing determined in this study suggest that the complex floral morphology (heterantherous and enantiostylous flowers) of *S. rostratum* maintains a high cross-fertilization rate across natural populations ($t_m = 0.75 \pm 0.03$) in native and introduced range, which is probably facilitated by pollinators that function as equivalents to legitimate pollinators from the centre of its distribution (i.e. large buzzing-bees). Further studies of the reproductive biology of invasions of *S. rostratum* will be needed to inform strategies for the management of the spread of this weed around the world.

5.7. ACKNOWLEDGMENTS

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CHAPTER 6:

General Discussion

6.1. GENERAL DISCUSSION

This research shows that *Solanum rostraum*, a monomorphic enantiostylous species, exhibits a relatively high outcrossing rate (t_m = 0.75 ± 0.03) and a low inbreeding coefficient (F_{is} = 0.192) in natural populations. Furthermore, the biparental inbreeding coefficients suggest that outcrossing occurs between relatives. However, self-fertilization produces 25% of progeny, which may be facilitated by pollinators through intrafloral selfing or geitonogamous self-pollination in individuals with large floral displays. Although an inbreeding depression experiment was not conducted, the rate of germination was found to be very high (above of 70%). It seems that if there is inbreeding depression, it would be at a low level for selfed seeds during germination, although additional work is needed to further explore this topic.

Self-fertilization may occur in *S. rostratum*, an enantiostylous buzz-pollinated species, when either buzzing or non-buzzing visitors (e.g. small bees or other opportunistic visitors) illegitimately manipulate the flower (Vallejo-Marín *et al.* 2013). As shown in Chapter 2, small bees usually fail to contact the stigmas and, consequently, the probability of fruit set after visitation by smaller bees is relatively low. Intermediate rates of self-pollination (17-31%) could be largely attributed to the following reasons. Firstly, illegitimate manipulation of flowers by non-buzzing bees (*A. mellifera*) resulting in fruit production because of occasional contact with flower's stigma. Secondly, large buzzing-bees could promote intrafloral self-pollination when they produce small pollen clouds during vibration of the anthers. These pollen grains may land on the stigma of the same flower promoting selfing within-flower (Larson & Barrett 1999; Wanigasekara & Karunaratne 2012). Finally, large buzzing-bees may transfer pollen among flowers on a plant with a large floral display (Vallejo-Marín *et al.* 2013). Therefore, although floral morphology and pollinator visitation may promote outcrossing, visitation by either buzzing or non-buzzing pollinators to a flower or to individuals with large floral displays may result in selfing.

Enantiostyly has been interpreted as a polymorphism that reduces geitonogamy and increases the precision of cross-pollination in bee-pollinated plants (Jesson & Barrett 2005). In dimorphic enantiostyly, a rare polymorphism (Barrett, Jesson & Baker 2000), all flowers of an individual plant have the same stylar deflection (Jesson & Barrett 2003) and style-deflected morphs being separate in different individuals. This type of enantiostyly effectively reduces the level of geitonogamy (Jesson & Barrett 2005). In monomorphic enantiostyly, which is the most common style-deflected polymorphism, both the right and left-styled morphs are on the same plant (Barrett, Jesson & Baker 2000). However, monomorphic enantiostyly reduces the incidence of geitonogamous self-pollination in comparison with the incidence of self-pollination in non-enantiostylous plants. Significant level of geitonogamy occurs in experimental arrays of *S. rostratum* (Jesson & Barrett 2005). This research demonstrates that natural populations of *S. rostratum* have relatively high outcrossing rates facilitated by legitimate pollinator visitation. However, self-fertilization still occurs, probably also facilitated by legitimate pollinators, through pollen transfer within a flower or between the flowers of an individual with a large floral display.

6.1.1. Legitimate pollinators of buzz-pollinated plants

Traditionally, the physiological or behavioural ability of bees to buzz flowers has been described as an important characteristic for pollinators of buzz-pollinated species (Buchmann 1983). However, the size of the pollinator relative to the flower may also be important because it determines the probability of contact with the plant's sexual organs (Whalen 1979). This research demonstrates experimentally that pollen transfer in a buzz-pollinated plant depends on the matching between the pollinator and the plant's sexual organs. When bees are smaller than the separation between the sexual organs, pollen deposition onto stigmas is lower and these bees probably act mainly as pollen thieves. Moreover, fruit and seed are produced when the fit between pollinator size and sexual organ separation is closer. The effect of size-matching is more restrictive when the bee is smaller than when the bee is larger in relation to the distance between the sexual organs in the flower. This result suggests that pollen theft is frequently associated with small bees because they do not deposit pollen onto stigmas.

6.1.2. Pollen theft occurrence in buzz-pollinated plants

The majority of floral visitors (both buzzing and non-buzzing bees) of *S. rostratum* act as pollen thieves and only a subset of visitors act as pollinators. However, a precondition of the evolution of heteranthery is that a pollinator acts as a pollen thief (Vallejo-Marín *et al.* 2010). Even after heteranthery had evolved in buzz-pollinated plants, there is still a high cost associated with sustaining many parasites that only consume the reward without providing pollination services (pollen thieves). The high incidence of pollen theft in *S. rostratum* increases the magnitude of pollen limitation in some native populations (Table 2 and Fig. 3 in Chapter 2).

For this species, the majority of the pollen thieves are specialized bees that use vibrations to steal pollen primarily from the feeding anthers. There is a possibility that they are attracted to the feeding anthers for the same reasons that pollinators are attracted (i.e. bright colors and accessible placement; Luo, Zhang & Renner 2008). Pollen theft from feeding anthers does not diminish the quantity of pollen available for fertilization, which is mainly contained in the pollinating anther. However, the findings of this research give rise to more questions: Does pollen theft affect the attractiveness of robbed flowers? Do robbed flowers receive less pollinator visits after the theft? Does pollen theft have a negative effect on fruit and seed production? *Solanum rostratum* could be used as a model system for further examining the ecological and evolutionary implications of pollen theft in a buzz -pollinated plant.

6.1.3. Fertilization of non-buzzing visitors in buzz-pollinated plants

Buzz-pollinated plants are usually pollinated by large buzzing-bees. However, the mid-sized honeybee (*A. mellifera*), a non-buzzing bee, has been reported as a low efficiency pollinator of buzz-pollinated plants (Gross & Mackay 1998; Macias-Macias *et al.* 2009). Despite this, *A. mellifera* has never been observed using vibrations to extract pollen from poricidal anthers (Buchmann 1983). How does a non-buzzing bee obtain pollen from poricidal anthers? The mechanism behind this is still unclear and possible explanations include: a) *Apis mellifera* collects the pollen from the anthers and corolla which have fallen during previous buzzes by other visitors (Buchmann 1983) or b) *A. mellifera* gather the pollen that has been previously deposited by legitimate pollinators directly from the stigma (Gross & Mackay 1998). The first mechanism is more likely common in *S. rostratum* where *A. mellifera* was observed primarily gathering pollen from feeding anthers and less so from pollinating anthers. Honeybees were rarely observed picking up pollen directly from the stigma.

This research verifies that *A. mellifera* is a poor pollinator of *S. rostratum*, but it seems that its functionality depends on the abundance of legitimate pollinators. The conditional thieves (low efficient pollinators) act as pollen thieves if other visitors provide more efficient pollination, but serve as pollinators when they are the only visitor to a plant (Hargreaves, Harder & Johnson 2009; Thomson & Thomson 1992). For *S. rostratum*, honeybees can be an alternative pollinator when legitimate pollinators are scarce but act as pollen thieves when pollinators are abundant.

Apis mellifera is abundant at sites where buzz-pollinated plants occur, though this does not imply that *A. mellifera* is a common visitor to these plants (Duncan, Nicotra & Cunningham 2004; population TP in this study). This could be dependent on the resources available at the study site. *Apis mellifera* may prefer foraging on nectar-rewarding plants or plants which have pollen that is easily accessible for collection, rather than gathering pollen from a buzz-pollinated plant. However, future work needs to be conducted to validate this hypothesis.

Finally, the role of *A. mellifera* as a pollinator of native plants has been a topic of debate in the scientific community (Aebi *et al.* 2012; Ollerton J. 2012). *Apis mellifera* is native to Africa, western Asia and Europe (Michener 1974). However, it is presently widespread throughout the world (Butz 1997). Many studies reported that *A. mellifera* has a negative effect on the fruit and seed production of plants (Hargreaves, Harder & Johnson 2009) because of its limited ability to transfer pollen in comparison with native bees (Gross & Mackay 1998). Due to its high demands for pollen and nectar, *A. mellifera* competes with native visitors for floral resources (Goulson 2003). This intensive competition could suppress native pollinators, decreasing their abundance in natural populations (Sun, Huang & Guo 2013).

Under certain conditions, such as fragmentation of habitat or the low abundance of native pollinators, honeybee could be a potential pollinator since its high foraging activity could compensate its inefficiency at removing and depositing pollen compared to native pollinators; it may even increase fruit and seed set in wild plants under these conditions (Cayuela, Ruiz-Arriaga & Ozers 2011; Sun, Huang & Guo 2013). However, observations in agricultural crops suggest that honeybees do not substitute the contribution of wild pollinators to set fruit and do not maximize the fruit production in crops around the world (Garibaldi *et al.* 2013).

What are the consequences of non-buzzing bees, like *A. mellifera*, on the reproduction and evolution of buzz-pollinated plants, especially in cases where they are the primary pollinator? There will probably be effects on the mating system from a diminished outcrossing rate because of an increase in self-pollination resulting from honeybees visiting more flowers per inflorescence (Dupont *et al.* 2004). Another possible consequence is that strong pollen theft causes the breakdown of the buzz-pollination syndrome. Finally, honeybees could select traits that permit easy pollen theft (e.g. larger opening of pores), or traits that increase the probability of fruits being produced by mid-sized honeybees (e.g. a closer distance between a flower’s sexual organs). Additional work is necessary to understand the impact of poor non-buzzing pollinators on the reproduction and evolution of buzz-pollinated plants.

6.1.4. Potential invasiveness of *S. rostratum*

Solanum rostratum is considered to be a noxious weed outside of its centre of distribution (central Mexico; Whalen 1979) in several regions around the world (Bassett & Munro 1985; Whalen 1979; Zhao *et al.* 2013), and it has the potential to grow aggressively in newly colonized habitat (Zhong *et al.* 2009). How does a buzz-pollinated plant, with complex floral morphology (heterantherous and enantiostylous flowers) that depends on pollinator to transfer pollen among flowers, reproduce successfully in a new habitat? From the information generated in this research, I suggest three possible explanations which are not mutually exclusive: a) wild pollinators in the new habitat function as equivalents of native pollinators at the centre of the plants distribution, b) exotic bees successfully fertilize this weed in its introduced range, c) changes in floral morphology in invasive populations promote self-fertilization or cross-pollination by floral visitors similar to that by pollen thieves in the plant’s native range, such that these visitors can act as pollinators in the new habitat.

In the first explanation wild pollinators in a new habitat would be functional equivalents of native legitimate pollinators: large buzzing-bees that that fit better with location of the flower’s sexual organs and so effect transfer of pollen grains onto stigmas. These wild pollinators would be able to maintain the high outcrossing rate of *S. rostratum.* For example, when two geographical areas (Mexico and the USA) in the *S. rostratum* distribution were compared, the populations maintained a similar outcrossing rate, promoted by enantiostyly and facilitated by legitimate pollinators. In the USA, some wild pollinators, which were previously observed visiting *S. rostratum* (e.g. *Bombus sp*., *Centris sp*. and *Xylocopa sp*.; see Table 1 in Chapter 1), may perform the same function as legitimate pollinators in Mexico (*Centris sp*., *Thygather sp*. and

Xylocopa sp.). Furthermore, this study demonstrated that *Bombus terrestris* was an efficient pollinator of *S. rostratum* under greenhouse conditions (see Chapter 3). This European bee could be a potential pollinator for this noxious herb in places where both species are present.

In the second explanation, exotic bees (i.e. honeybees and bumblebees) usually have a high demand for resources and prefer to visit exotic plants (Goulson 2003) because they are abundant and provide high quantities of pollen or/and nectar. This subsequently gives rise to a mutualistic relationship between both alien organisms (Morales, Traveset & Ramírez 2009). For example, *Solanum torvum*, a buzz-plant that reproduces successfully in non-native conditions, is pollinated by both native bees (i.e. halicitids and carpenter bees) and exotic bees (i.e. *Euglossa viridissima*) in southern Florida, where the most efficient pollinators are the exotic bees (Liu & Pemberton 2009). In the particular case of *S. rostratum*, the non-buzzing *A. mellifera* is considered a poor pollinator. Honeybees are widely distributed around the world and can play an important role in the establishment of *S. rostratum* in newly invaded habitats.

In the third explanation, modifications in the floral morphology associated with the evolution of self-fertilization or asexuality in *S. rostratum* could ensure reproduction when outcrossing is unpredictable (i.e. when mating partners or pollinators vectors are limited; Eckert *et al.* 2006). However, there is no evidence of autonomous self-fertilization or asexual reproduction in native populations of *S. rostratum* (Chapter 2). Local adaptations involved in non-native populations would need to be caused by new mutations or by alleles already present in population that are exposed by the new selection pressures (Barrett & Schluter 2007). These new selective pressures may cause evolutionary modification of the floral characteristics that control pollen transfer in invasive populations of *S. rostratum*. For example, it is possible that a reduction in flower size may permit visitors that are functionally similar to small buzzing bees that mainly act as pollen thieves in the plants native distribution, to act as pollinators in a new habitat. Further studies of the reproductive biology of invasions of *S. rostratum* are needed to propose strategies to manage the spread of this weed around the world.

6.1.5. Relevance of buzz-pollination ecology studies

The study of buzz-pollination is relevant for the production of crop plants from the family Solaneceae, such as tomato, eggplant and red pepper (Raw 2000). Despite the importance of buzz-pollination for food crops, there have been relatively few studies which have described buzz-pollination biology compared with the huge number of angiosperm species that exhibit this pollination system. Studies of pollination biology are needed to successfully identify viable native pollinators for crops that exhibit this pollinator syndrome (Nunes-Silva, Hrncir & Imperatriz-Fonseca 2010).

Detecting commercialized native pollinators is important for reducing the introduction of non-native pollinators. For example, in North America non-native bumblebees were introduced for the purpose of commercial crop pollination. In Mexico, in 1995–1996, colonies of *Bombus terrestris* (a European-bumblebee) were introduced to greenhouses for tomato production (Kimberly *et al.* 2006). When the non-native *B. terrestris* dispersed into natural populations, they threatened native pollinators for several reasons. These included the transmission of new diseases and parasites, the displacement of native pollinators through competition for resources, and the hybridization with native bumblebee species (Kimberly *et al.* 2006). Since 1996, imports of *B. terrestris* into Mexico have been restricted (Kimberly *et al.* 2006) and in majority they have been replaced with *B. impatiens* (Vergara & Fonseca-Buendía 2012) another non-native bumblebee but from the western USA (The Global Biodiversity Information Facility 2013).

Bombus impatiens is now considered to be a potential risk for native Mexican bumblebees (Medina-Valdez 2010). A possible solution to this issue could be achieved through encouraging the utilization of indigenous species of bumblebee as commercial pollinators of agricultural crops (Medina-Valdez 2010; Kimberly *et al.* 2006). Little effort has been made to use Mexican native bees. *Bombus ephippiatus* (Vergara & Fonseca-Buendía 2012) and *Nannotrigona perilampoides* (Cauich *et al.* 2004) have been tested as potential pollinators to be used in greenhouse-based tomato plantations. Exploring the contribution of wild bees to open crop pollination may present an alternative solution. For example, native bees are efficient pollinators and contribute significantly to the pollination of tomato and habanero pepper crops in Yucatan, Mexico (Macias-Macias *et al.* 2009). However, efforts to use native bees as pollinators of agricultural crops have exposed two main problems: insufficient large scale production of bee colonies and a lack of knowledge of the pollination efficiency of different species of bee used in crop production (Nunes-Silva, Hrncir & Imperatriz-Fonseca 2010).

The present study uncovers some important aspects of the pollination ecology of a buzz-pollinated plant that can give insight into the ecology of other buzz-pollinated plant species. We have shown that the fit of the pollinator to floral morphology is important in determining dynamic pollen transfer and fruit production in a buzz-pollinated plant; knowing this is helpful for predicting potential pollinators for crops with known floral morphology. The characterization of visitors as pollinators versus pollen thieves is relevant due to the antagonistic interaction between plant and visitor since the resulting pollen theft could have critical implications for the reproduction and evolution of buzz-pollinated plants. Finally, understanding more about the reproductive strategies of buzz-pollinated plants would assist in the determination of adequate strategies for management of invasive weeds.

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