

**LIEDSON TAVARES DE SOUSA CARNEIRO**

**Estrutura geográfica da interação entre abelhas coletoras de óleo  
e *Krameria* Loefl. (Krameriaceae): funcionalidade e integração  
fenotípica de caracteres florais**

*Geographical structure of the interaction between oil-collecting bees and  
*Krameria* Loefl. (Krameriaceae): functional significance and phenotypic  
integration of floral traits*

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Orientador (a): Dra. Isabel Alves dos Santos

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## Resumo

Nesse estudo, explorei aspectos da ecologia evolutiva da interação planta-polinizador, ao avaliar o fenótipo floral sob pressões seletivas geograficamente divergentes. Para isso utilizei a interação entre abelhas coletoras de óleo e *Krameria* (Krameriaceae) como sistema modelo. A tese abrange a história natural do sistema e manipulações experimentais *in situ* que deram suporte à investigação sobre integração floral no contexto multipopulacional. No primeiro capítulo, estudei a biologia da polinização de *Krameria tomentosa*, listando as espécies de visitantes florais associadas a suas flores. Assim, mostrei que essa espécie depende de seus polinizadores para o sucesso na polinização e que a maioria das abelhas coletoras de óleo associada a suas flores pertence ao gênero *Centris* (Centridini). No entanto, observei uma alta frequência de abelhas coletoras de pólen nessa espécie que parecem contribuir com a manutenção da polinização na população. No segundo capítulo, mostrei que há uma variação geográfica na assembleia de polinizadores de *K. grandiflora* que inclui diferentes comportamentos e ajustes à morfologia floral da espécie. Desse modo, testei a significância funcional das três estruturas especializadas das flores de *Krameria* (cálice conspícuo, pétalas petaloides e elaióforos) em dois cenários contrastantes de ajuste entre a arquitetura floral e o polinizador. No entanto, diferenças não foram detectadas. O ajuste mecânico que os elaióforos provêm parece essencial para o sucesso na polinização no grupo. No terceiro capítulo, demonstrei que populações similares no padrão de correlação de seus caracteres florais, apresentam composição e diversidade funcional de polinizadores semelhantes, sugerindo que diferenças na morfologia combinadas à abundância de polinizadores influenciam o padrão de integração fenotípica. Portanto, esses resultados mostram uma situação em que a variação geográfica na assembleia de polinizadores parece promover a diferenciação da estrutura correlacional do fenótipo floral.

## Abstract

In this study, I investigate some aspects of the evolutionary ecology of a plant-pollinator interaction, by evaluating the floral phenotype under geographically divergent selective forces. For this, I used the interaction between oil-collecting bees and *Krameria* (Krameriaceae) as a system model. This thesis comprises the natural history of the studied system and manipulative experiments *in situ* to support the investigation on the phenotypic integration in a multipopulation context. In the first chapter, I studied the pollination biology of *Krameria tomentosa*, listing the flower visitor species associated to its flowers. Thus, I showed that this species depends on their pollinator for pollination success and that most oil-collecting bees recorded belongs to *Centris* genus (Centridini). However, I observed a high frequency of pollen-collecting bees on this species which might contribute to pollination maintenance in the population. In the second chapter, I showed that there is a geographic variation in the pollinator assemblage of *K. grandiflora* which includes different behavior and matching to *Krameria* floral architecture. Thus, I tested the functional significance of the three specialized structures of *Krameria* flowers (showy calyx, petaloid petals and elaiophores) in two contrasting pollinator-matching scenarios; however, no differences were detected. The mechanical fit provided by the elaiophores might be a keystone for the pollination success in *Krameria*. O ajuste mecânico que os elaióforos provêm parece essencial para o sucesso na polinização no grupo. In the third chapter, I demonstrated that populations with similar patterns of floral trait correlation comprise similar pollinator composition and functional diversity, suggesting that differences in pollinator morphology combined to abundance influences the pattern of phenotypic integration. Therefore, these results show a condition in which geographic variation in pollinator assemblage might provide divergence in the phenotypic correlation structure.

## Introdução geral

Nas interações ecológicas, as espécies exploram umas as outras em diferentes formas. Juntamente com determinantes abióticos, essas explorações podem refletir em mudanças de atributos ao longo de suas histórias evolutivas (Rausher, 1992; Harder e Johnson, 2009). Essas mudanças proporcionam um melhor ajuste entre as espécies e a diversificação de linhagens gerando biodiversidade (Thompson, 2005). Nessa dinâmica de ajuste, os atributos morfológicos, fisiológicos e comportamentais podem ser moldados como resultado de seleção natural (Ehrlich e Raven, 1964; Connell, 1980; Ridenhour, 2005). As percepções sobre a seleção mediada por espécies interagentes tiveram início com os estudos de polinização, com as observações da interação orquídea-esfingídeo por Charles Darwin, que previu que a existência de esporões longos na corola de *Angraecum sesquipedale* Thouars (Orchidaceae) se relacionaria com um animal polinizador que possuiria um aparelho bucal longo suficiente para a exploração do recurso oferecido (Nilsson et al., 1987; Arditti et al., 2012). Na mesma época, também surgiram as ideias sobre mimetismo, estabelecidas por Henry Bates, que sugeriu a convergência no padrão de coloração de espécies palatáveis e modelos impalatáveis, mediada por um predador (Pasteur, 1982).

O estudo da evolução floral se baseia nos valores adaptativos da interação planta-polinizador, pois atributos florais possuem um efeito direto sobre o fitness da planta. A partir do forrageio por recursos e da transferência de pólen promovida por polinizadores (*e.g.* abelhas, morcegos, lepidópteros e beija-flores), fenótipos florais são selecionados para que haja um melhor ajuste à morfologia, fisiologia e comportamento desses animais (Herrera e Pellmyr, 2009; Moré et al., 2012; Schiestl e Johnson, 2013), provavelmente refletindo na exploração mais eficiente de recursos. Desse modo, grande parte da diversidade de morfologias florais encontradas nas angiospermas evoluiu em resposta à seleção mediada pela interação com seus polinizadores (Fenster et al., 2004; Harder e Johnson, 2009). As interações entre plantas e seus vetores de pólen podem promover a evolução de atributos florais, tais como, tamanho, forma e cor das flores, guias de néctar, quantidade de recurso oferecido, entre outros (Medel et al., 2003; Gómez et al., 2006; Benitez-Vieyra et al., 2010; 2014; Moré et al., 2012). Portanto, interações planta-polinizador são modelos promissores para o entendimento da mudança evolutiva por meio de pressões entre espécies interagentes.

Apesar dos caracteres florais serem alvos de seleção, a seleção natural não opera apenas em caracteres únicos, mas sim em vários atributos inter-relacionados, uma vez que as flores são órgãos complexos formados por verticilos interconectados (Berg, 1960; Armbruster e Schwaegerle, 1996; Murren, 2002). A forma como os atributos de um organismo estão associados entre si é denominada de integração fenotípica, detectada pela magnitude e pelo padrão da correlação entre caracteres (Berg, 1960; Ordano et al., 2008; Armbruster et al., 2009). A procura por seleção natural agindo de forma correlacional em atributos tem sido incluída nos modelos multivariados de seleção (Lande e Arnold, 1983), mas por um lado sua detecção não tem sido obtida devido às limitações estatísticas desses métodos (Roff e Fairbairn, 2012). No entanto, métodos multivariados podem ser aplicados para testar hipóteses evolutivas e descrever padrões sobre integração floral (Ordano et al., 2008; Ancantara et al., 2013; Pérez-Barrales et al., 2014; González et al., 2015; Lázaro e Santamaría, 2016).

Modularidade é outro conceito relacionado à associação entre caracteres fenotípicos e junto com o conceito de integração fenotípica está envolvido na regulação de variância e covariância fenotípica (Armbruster et al., 2009; Baranzelli et al., 2014). Módulos, ou “plêiades de correlação”, são conjuntos ou subconjuntos de caracteres integrados que possuem uma função coletiva e, geralmente, covariam independentemente de outros módulos. Caracteres dentro de um módulo podem ser geneticamente (ou epigeneticamente) integrados sob o efeito de seleção natural (Ordano et al., 2008). Por exemplo, caracteres florais são mais correlacionados entre si do que com caracteres vegetativos e, portanto, evoluem de modo independente (Berg, 1960). Nesse contexto, as flores podem ser compostas por intra-módulos que possuem determinada função no processo de polinização. Assim, caracteres envolvidos na atração de polinizadores que compõem o perianto (cálice e corola), por exemplo, formariam um módulo, enquanto que caracteres envolvidos na transferência e deposição de pólen (androceu e pistilo) formariam outro. Considerando essas ideias de modularidade, Berg (1960) sugeriu que padrões de correlação fenotípica parecem refletir operação direta de seleção natural e, em plantas, esses padrões deveriam diferir entre espécies vegetais com relações especializadas e generalistas com seus polinizadores. A predição básica para essa hipótese é que polinizadores de sistemas especializados são mais ajustados às flores por meio de seleção natural favorecendo a integração fenotípica. Por sua vez, a associação entre os caracteres florais estaria relaxada em sistemas de polinização generalistas. No entanto, essa

hipótese tem sido pouco suportada e mais investigações são necessárias para compreender a variação em integração floral (Armbruster et al., 1999). Da mesma forma que a pesquisa em polinização no Brasil carece de estudos de seleção fenotípica (Benitez-Vieyra et al., 2014), a integração floral também tem sido negligenciada.

Seleção fenotípica e integração floral são duas abordagens interligadas no contexto da polinização e se complementam para o entendimento dos padrões e mecanismos inerentes à evolução floral. Enquanto que na perspectiva de seleção fenotípica se detecta a seleção natural atuando em um determinado momento, na abordagem de integração fenotípica se avalia o efeito acumulado ao longo do tempo de pressões seletivas sobre o fenótipo. No entanto, ambas são espacialmente variáveis à medida que os cenários seletivos mudam (Thompson, 1997; Herrera et al., 2002; 2006; Armbruster et al., 2009; Medel et al., 2007; Pérez-Barrales et al., 2007; Ferreiro et al., 2017). Esta variação espacial pode gerar linhagens diferenciadas e desencadear o processo de especiação. Porém, a maioria dos estudos sobre seleção mediada por polinizadores e integração floral tem sido conduzida em populações únicas ou entre espécies relacionadas (Medel e Nattero, 2009; Moré et al., 2012), excluindo a variação intraespecífica em que processos microevolutivos atuam na divergência genética e fenotípica.

De modo geral, as interações entre as espécies variam geograficamente, e provêm respostas evolutivas divergentes, dependendo da comunidade nas quais as populações estão inseridas (Thompson, 1999; 2005). Na Teoria do Mosaico Geográfico da Coevolução, Thompson (2005) sugere que muito das dinâmicas de evolução entre pares ou grupos de espécies frequentemente ocorre em uma escala geográfica, ligando processos ecológicos locais e padrões filogeográficos. Uma das hipóteses que permeiam a teoria contempla a existência de um mosaico de seleção entre populações, que favorece trajetórias evolutivas diferentes nas interações em diferentes populações. Nesse contexto, explorei aspectos da ecologia evolutiva da interação planta-polinizador, ao avaliar as respostas evolutivas do fenótipo floral em cenários intraespecíficos divergentes. Como sistema modelo, utilizei a interação entre abelhas coletoras de óleo e uma espécie produtora de óleo floral do gênero *Krameria* Loefl. (Krameriaceae) que ocorre em formações abertas ao longo da “diagonal seca” brasileira (Werneck et al., 2011). O estudo reúne, portanto, abordagens ainda não exploradas da integração fenotípica de caracteres florais em escala multipopulacional em uma região tropical. Além disso, o estudo também abrange a história natural dessa interação.

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## Capítulo 1

# ***Krameria tomentosa* oil flowers and their pollinators: bees specialized on trichome elaiophores exploit its epithelial oil glands**

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**Abstract:** Oil flowers are spread over eleven angiosperm families and exploited by bees specialized on oil gathering. This reward is produced by glandular surfaces of specific floral whorls called elaiophores. Unlike trichome elaiophores, such as those of Plantaginaceae, *Krameria* species bear lateral petals modified into epithelial elaiophore, convergently similar to oil-secreting Malpighiaceae calyx. Krameriaceae is an oil-offering monotypic group of eighteen species with little information on their pollination biology and interactions with oil-collecting bees. In this study, we evaluated the breeding system and described the floral biology of *Krameria tomentosa*, a widespread species that occurs mainly in open vegetation habitats of northeastern Brazil. We listed its flower visitors, surveyed throughout four years, and recorded their visits by season. The pollination treatments showed that *K. tomentosa* is self-compatible; however, its flowers depend on pollinators to set fruits. Twenty-one Apidae visited the flowers, including the oil-bee *Centris hyptidis* specialized on trichome elaiophores and stingless-bees which foraged for pollen. *Centris nitens* and *Trigona fulviventris* were the most frequent visitors. *Krameria tomentosa* flowers received relatively few oil visits throughout the day and on some days no flower visitors were seen on its flowers. Nevertheless, this did not reflect on a low natural fruit set. *Centris hyptidis* has been associated with *Angelonia* (Plantaginaceae) trichome elaiophores mainly due to its long forelegs and their specialized oil-collecting apparatus. Nonetheless, it exploited the *K. tomentosa* epithelial elaiophores in an area with no Plantaginaceae species. We presumed that the association between the morphology of the oil-collecting apparatus and the elaiophore type is not enough to predict relationships in oil-systems. Additionally, we concluded that the diversity of bee visitors suggests that pollen-collecting bees also play an important role in the maintenance of *K. tomentosa* pollination.

**Keywords:** floral oil / pollination / *Centris hyptidis* / rhatany / carrapicho-de-boi.

**Resumo:** As flores de óleo estão distribuídas em onze famílias de angiospermas e são exploradas por abelhas especializadas na coleta de óleo. Este recurso é produzido por superfícies glandulares de verticilos florais específicos chamadas de elaióforos. Diferentemente de elaióforos tricomáticos, tais como os de espécies de Plantaginaceae, as espécies de *Krameria* pétalas laterais modificadas em elaióforos epiteliais, convergentemente similares ao cálice secretor de óleo de espécies de Malpighiaceae. Krameriaceae é uma família monotípica composta por dezoito espécies pouco estudadas quanto a sua biologia da polinização e interações com abelhas coletoras de óleo. Neste estudo, nós avaliamos o sistema reprodutivo e descrevemos a biologia floral de *Krameria tomentosa*, uma espécie amplamente distribuída que ocorre principalmente em ambientes abertos do Nordeste brasileiro. Nós listamos seus visitantes florais amostrados ao longo de quatro anos, e registramos a visitação por estação (seca e chuvosa). Os tratamentos de polinização mostraram que *K. tomentosa* é autocompatível, porém suas flores dependem de polinizadores para a produção de frutos. Vinte e uma espécies de Apidae visitaram as flores, incluindo a espécie coletora de óleo *Centris hyptidis*, especializada em elaióforos tricomáticos, e espécies de abelha sem ferrão que forragearam por pólen. *Centris nitens* e *Trigona fulviventris* foram os visitantes florais mais frequentes. As flores de *Krameria tomentosa* receberam relativamente poucas visitas de óleo ao do dia e em alguns dias de amostragem nenhum visitante floral foi registrado em suas flores. Contudo, isso não refletiu em uma baixa frutificação na área. *Centris hyptidis* tem sido associada aos elaióforos tricomáticos de *Angelonia* (Plantaginaceae) principalmente devido a suas pernas anteriores longas e seu aparato coletor de óleo. Não obstante, essa espécie explorou os elaióforos epiteliais de *K. tomentosa* em uma área onde espécies de Plantaginaceae são ausentes. Nós presumimos que a associação entre a morfologia do aparato coletor de óleo e o tipo de elaióforo não é suficiente para predizer sobre relações em sistemas de óleo. Além disso, concluímos que a diversidade de abelhas visitantes sugere que abelhas coletoras de pólen empregam um papel importante na manutenção da polinização de *K. tomentosa*.

**Palavras-chave:** óleo floral / polinização / *Centris hyptidis* / rhatany / carrapicho-de-boi.

## 1. Introduction

The interactions between oil-foraging bees and oil flowers represent a highly specialized bee-plant mutualism in which fatty oils secreted by floral elaiophores are gathered by bees through a particular collecting apparatus composed of sets of specialized setae (Vogel, 1974; Neff and Simpson, 1981; Buchmann, 1987). Floral oil-offering plants are spread over 11 angiosperm families (Renner and Schaefer, 2010) and are exploited by oil-collecting female bees for brood cell provisioning and water-resistant lining (Vogel, 1974; Simpson and Neff, 1981) and by some male oil-bees for uncertain purposes (Aguiar and Melo, 2009; Cappellari et al., 2012).

Elaiophores comprise oil-secreting surfaces in the corolla, calyx, androecium or gynoecium where the oil is retained between the cuticle and the secretory epidermal cells (epithelial type) or actively secreted via patches of many glandular trichomes (trichome type) (Vogel, 1974; Buchmann, 1987). The oil-collecting apparatus is variable within oil-bee taxa and its variants (see Vogel, 1974; Neff and Simpson, 1981) have been thought to be associated with the elaiophore type exploited. *Centris* is a speciose group of oil-collecting bees, and the specialized sets of setae vary even within this group. This diversity promotes asymmetric specializations to some extent within oil-system networks. Several *Centris* (*Paracentris*, *Penthemisia*, *Wagenknechtia* and the *hyptidis* group) and Tapinotaspidini (*e.g.* *Caenonomada*, *Chalepogenus*, *Lanthanomelissa* and *Tapinotaspis*) species are usually associated with trichome elaiophores such as those characteristic of *Angelonia* and *Monttea* (Plantaginaceae) (Simpson et al., 1990; Vogel and Machado, 1991; Sersic and Cocucci, 1999; Machado et al., 2002; Martins et al., 2013). However, as suggested by several reports (Simpson, et al., 1990; Aguiar et al., 2003; Aguiar and Melo, 2009), some of these associations do not seem to be as close as generally believed.

In the Neotropics, epithelial elaiophores independently evolved in the Krameriaceae and Malpighiaceae flowers, while the trichome type is found in some species of Calceolariaceae, Iridaceae, Plantaginaceae and Solanaceae (Vogel, 1974; Buchmann, 1987; Machado, 2004). Orchidaceae species bear epithelial or trichome elaiophores or even both in the same flower (Pansarin et al., 2009). The interaction between oil-collecting bees and epithelial elaiophores has been extensively studied in the Malpighiaceae pollination system (*e.g.* Vogel, 1990; Anderson, 1982; Sigrist and Sazima, 2004; Bezerra et al., 2009b; Mello et

al., 2012); however little has been reported about the interactions between oil-bees and *Krameria* species as well as their pollination biology (Machado et al., 1997; Gimenes and Lobão, 2006; Zanella, 2010; Silva et al., 2012). The Krameriaceae flower comprises a complex morphology in which the sepals are petaloid, brightly colored, and the petals are reduced and dimorphic: two or three (sometimes four) petaloid petals and a pair of lateral fleshy ones, which bears the elaiophore. The anthers are didynamous and poricidal with the pollen pushed out of each in sticky masses when the anthers are touched or senesce (Simpson, 1989).

Although the New World Malpighiaceae and Krameriaceae independently converged to produce a histologically and functionally convergent oil-secreting structure (Vogel, 1974) in symmetrical arrangement on the flowers (Simpson, 1982), these groups are divergent in terms of species richness, diversification and age. The first is the oldest clade among oil-offering groups and comprises approximately 900 floral oil-producing species (Vogel, 1990) while the latter is a younger monotypic family (Renner and Schaefer, 2010; Martins, 2014) with only 18 species (Simpson, 1989; Simpson et al., 2004). Their distributional ranges often overlap throughout the open vegetation in Neotropical xeric regions, where *Krameria* usually occurs. Unlike the malpigh elaiophore borne on the calyx epidermis, *Krameria* elaiophores comprise the outer secreting surface of the lateral pair of fleshy petals which are exploited, as in malpighs, mainly by *Centris* bees (Simpson, 1982; 1989). According to Simpson (1989), *Centris* populations are dependent on *Krameria* floral oil in regions, where there is no overlap with the malpighs (*e.g.* northern Texas).

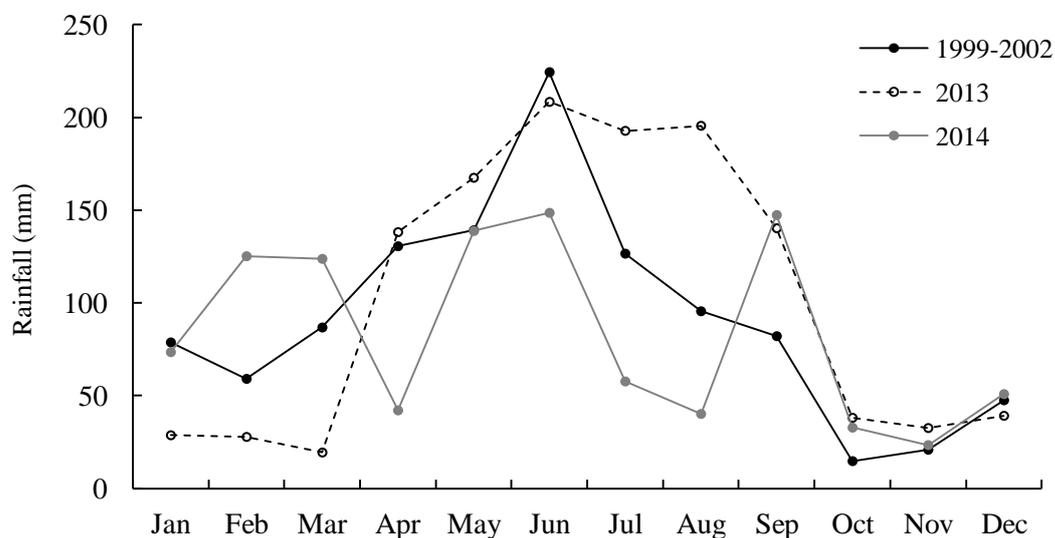
Among oil-offering plants, there are species that do not show dependence on oil-collecting bees or even secondary pollinators to set seeds. Self-fertilization and even agamospermy have been reported within *Angelonia*, *Banisteriopsis*, *Byrsonima*, *Peixotoa* and *Tetrapteryx* (Anderson, 1982; Vogel and Machado, 1991; Sigrist and Sazima, 2004; Benezar and Pessoni, 2006; Mendes et al., 2012); however, most oil plants require pollen transfer by their pollinators for seed set, even self-compatible ones (Rêgo and Albuquerque, 1989; Teixeira and Machado, 2000; Pereira and Freitas, 2002; Machado et al., 2002; Sigrist and Sazima, 2004; Oliveira et al., 2007; Bezerra et al., 2009a). Within *Krameria*, data on the mating system are scarce, and self-fertilization has not been reported (Simpson, 1989; Gimenes and Lobão, 2006).

In this study, we focused on the floral biology and the breeding system of *K. tomentosa* A. St.-Hill as well as the interaction between oil-collecting bees and its flowers. This species is the most widespread among the five *Krameria* species in Brazilian open habitats (Simpson, 1989) and occurs in the Atlantic rainforest (*tabuleiros* and coastal dunes), dry forest (Caatinga) and tropical savanna (Cerrado). We asked the main following questions: 1) Is *K. tomentosa* dependent on pollinators to set fruits? 2) What species are its flower visitors and pollinators? We also discuss the association between the trichome elaiophore specialist *Centris hyptidis* Ducke and the *K. tomentosa* epithelial oil-glands.

## 2. Materials and methods

### 2.1 Study area and studied species

We conducted our study in 1999-2002 and 2013-2014 at the Guaribas Biological Reserve (06° 44' 25" S, 35° 08' 36" W) that comprises the municipalities of Mamanguape and Rio Tinto, Paraíba, Brazil. The climate is tropical with 1,512 mm annual rainfall. The rainy season usually lasts from March to August (Figure 1). The annual temperature ranges between 24–26°C (AESAs/PB, 2004). Sunrise in the area occurs at around 0500 h and 0530 h in the dry and rainy seasons, respectively.



**Figure 1.** Monthly rainfall in the Biological Reserve Guaribas, Mamanguape, PB, Brazil, throughout the periods 1999-2002 (mean) and 2013-2014. (Source: AESAs/PB).

The region lies on a tertiary sedimentary geological formation called *Formação Barreiras* which ranges between 50-100 km from the coast and extends from the southern Rio Grande do Norte to Rio de Janeiro (Barbosa et al., 2011). A type of savanna vegetation known as *tabuleiro* covers the region matched with lowland semi-deciduous forest. Considering the entire reserve, the elevation range is 60-204 m, mostly ranging from 100 m to 160 m (Barbosa et al., 2011). The elevation of our study locality ranged from 181 m to 193 m.

*Krameria tomentosa* is a shrub exclusively found in open vegetation throughout the region and the studied population comprises over 200 individuals with continuous flowering. Among the *Krameria* species, only *K. tomentosa* occurs in the area, but other oil-offering plants (malpighs) with seasonal flowering have also been recorded: *Byrsonima crassifolia* (L.) Kunth, *B. gardneriana* A. Juss., *B. sericea* DC., *Heteropterys nordestina* Amorim, *Stigmaphyllon paralias* A. Juss., *S. rotundifolium* A. Juss. and *Tetrapteryx phlomoides* (Spreng.) Nied. (Barbosa et al., 2011).

## 2.2 Floral morphology

Flower samples were collected and fixed in FAA (ethanol 95-100%, formaldehyde, acetic acid – 17:2:1) for morphological description, measurements and drawing. We measured the distance between the tips of the showy sepals (upper-lower tips and lateral tips) as flower dimension measurements, and the length of the petaloid petals, pistil and pairs of short and long stamens. The measurements were made on three flowers per individual (one per inflorescence; n=10) and taken in the field or in the laboratory with a digital caliper. To infer whether the right and left elaiophores are similar in size and thus likely similar in oil production, the glandular surface area of the elaiophores (n=12) was measured using Leica Application suite v4.2 software for the M205c Leica® stereomicroscope. A drawing was made using camera lucida with the flower immersed in diluted glycerin. Vouchers of the studied population were consigned to the SPF Herbarium (Universidade de São Paulo).

## 2.3 Floral biology

The time of flower opening was observed by marking a pre-anthesis bud of two inflorescences per individual (n=10). The flower longevity was studied in 71 first-day flowers distributed on 15 individuals (two to nine flowers per plant) on four different days. To avoid the fertilization effect on longevity, the buds were bagged in a tulle cloth to exclude pollinators. To quantify the pollen production the mean number of pollen grains per flower

was estimated by direct counting. The pollen grains of one anther of a short and long stamen per pre-anthesis bud (one per individual, n=12) were counted on a millimetric slide under a stereomicroscope. Three fresh flowers of eight individuals had their pollen grains stained with aceto carmine to check for pollen viability. The viability rate (*i.e.* pollen grains stained/total counted) was calculated by counting 200-300 pollen grains in the samples.

The stigmatic receptivity was tested every hour on one flower from three individuals in the first and second day of anthesis, between 0500 h and 1700 h, using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Dafni, 1992). The method was carried out on three days using first-day flowers and on one day using second-day flowers. To observe whether the pollen mass release occurs in the interval before stigmatic receptivity (see Results), the anthers were touched using forceps, simulating a floral visit.

## **2.4 Breeding system**

The *K. tomentosa* breeding system was evaluated in the dry (November 2013) and rainy (July 2014) seasons with the following controlled pollination treatments: cross-pollination, hand self-pollination, spontaneous self-pollination and open-pollination (control). Three flowers per individual (n=10) were used for each treatment in each season. In addition, to test whether pollen deposition on the stigma before its receptivity promotes fruit set, we performed cross-pollinations in the morning (0530-0630 h). When required, the flowers were bagged with tulle cloth (with openings of about one square millimeter in size) at the pre-anthesis bud stage and then rebagged after pollinating them to exclude pollinators. To avoid clones the maximum and minimum distances between the pollen donors and receivers were 136 m and 28 m, respectively in cross-pollinations. Wilted flowers that did not fall within six days after opening were assumed to produce fruits (see Results).

## **2.5 Flower visitors**

Two surveys were conducted to record the *K. tomentosa* flower visitors and their visits. Firstly, the bees were collected monthly on the flowers from April 1999 to March 2000 on three to four consecutive days and from May 2000 to May 2002 in a single day from 0500 h to 1700 h. The data are part of a survey for sampling the bee fauna in the area by Sakagami et al. (1967) method (Aguiar and Martins, 2003). In the second survey, the flower visitors as well as their frequency of visitation were sampled in 2013 and 2014. Regarding the continuous flowering in the population, the latter sampling was carried out in the dry

(October-December 2013 and January 2014) and rainy (August 2013, July-August 2014) seasons (Figure 1), with 10 and 9 days sampled, respectively. As an attractiveness measurement in those periods, we recorded the mean number of flowers on the first day of anthesis per plant (five different plants on four days;  $n=20$ ) twice in the dry season ( $23\pm 10.15$  flowers/plant in November 2013 and  $7\pm 7.3$  in January 2014) and once in the rainy season ( $11\pm 6.89$  flowers/plant in August 2014), to be able to relate the visits to the number of flowers available. The number of visits, the visitation time and resource collecting behavior were recorded between 0500 h and 1700 h, except for a single observation in August 2013 when only the occurrence (regarded as one visit) of flower visitors was recorded rather than their number of visits. We observed four different focal flowering plants every half hour (two plants observed at the same time during  $\sim 10$  min). At certain times, the remaining ten minutes were used to maximize sampling by walking along a  $\sim 1$  km trail among the *K. tomentosa* individuals. The occurrence index for each visitor species was taken as the ratio of the number of days it was recorded and the total number of sampled days. Voucher specimens were collected and deposited in the entomological collections DSE/UFPB and CEPANN/IB/USP.

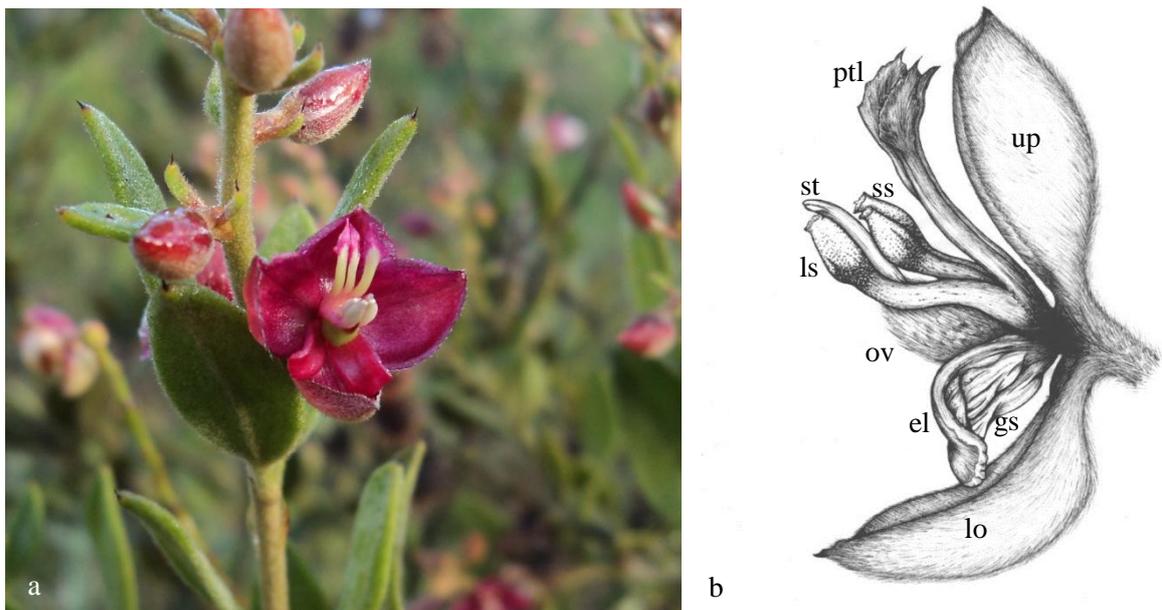
## 2.6 Statistical analysis

To check for differences between the area of the right and left elaiophores, a *t*-test for independent samples was used. A two-way analysis of variance (ANOVA) was applied to compare the stamen lengths regarding size (short and long) and position (right and left) as factors. The Wilcoxon test for non-parametric data and paired samples was used to compare the number of pollen grains produced per anther of short and long stamens. The Shapiro-Wilk test was used to check data normality. To compare the fruit set between the hand pollination treatment performed before stigmatic receptivity and cross-pollination, between open-pollination in the dry and rainy season, and among cross-pollination, hand self-pollination, spontaneous self-pollination and the control (in the dry season), the chi-squared test ( $\chi^2$ ) was used. The R 3.0.2 (The R Project for Statistical Computing) software environment was used to run the tests, except for the two-way ANOVA which was performed using Statistica 10.0 (Statsoft Inc.).

## 3. Results

### 3.1 Floral morphology and morphometry

*Krameria tomentosa* bears hermaphroditic, zygomorphic flowers (Figure 2a, b), supported by the peduncle and a pair of bracts, on terminal inflorescences as described by Simpson (1989). The conspicuous calyx is a set of four vinaceous sepals (upper, lower and two lateral sepals), pubescent on their dorsal portion. The measurements of the flower opening are given in Table 1. The corolla is formed by a pair of lateral rectangular petals, in whose outer surface are the elaiophores, and a group of two to three petaloid petals joined at their lower portion, each with a pink triangular apex. No difference was observed between the glandular surface area of right and left elaiophores ( $t=0.200$ ;  $df=22$ ;  $p=0.843$ ). The two-way ANOVA showed no interaction effect between size and position of stamens ( $F<0.001$ ;  $df=1$ ;  $p=0.981$ ), no significant position effect ( $p=0.642$ ), but a significant size effect ( $p<0.001$ ) (Table 1). The filaments and poricidal anthers are fused as a single unit (Figure 2a, b). The pistil is formed by a pubescent superior ovary with two ovules internally, but only one is functional; a vinaceous style with a tiny bilobed stigma is located among the anthers.



**Figure 2.** *Krameria tomentosa* flower (a, b). (b) Lateral view: (el) elaiophore, (gs) glandular surface, (ptl) petaloid petals, (ov) ovary, (st) stigma, (ls) long stamen, (ss) short stamens, (up) upper sepal, (lo) lower sepal. The lateral sepals were removed for the drawing. Drawing by L.T. Carneiro.

### 3.2 Floral biology

All sampled flowers were semi-open between 0430 h and 0530 h and quite open generally after 0600 h. The calyx was withered, closed and colorless at the end of the second

day of anthesis. Among the 71 flowers sampled, 31 (43.7%) flowers fell on the fourth day; 22 (31%) on the fifth day and 18 (25.3%) on the sixth day. The mean number of pollen grains produced per short and long anthers was 1,877 and 2,156, respectively, with significant differences between them ( $V=11$ ;  $p=0.027$ ) (Figure 3). Thus, considering this difference, a *K. tomentosa* flower produces approximately 8,000 pollen grains. Thereof, 83% ( $\pm 14.09$ ) were viable.

**Table 1.** Number of floral parts (N) and morphometry of the *K. tomentosa* flowers, including flower opening measurements, lengths (mm) of floral whorls and elaiophore area (mm<sup>2</sup>).

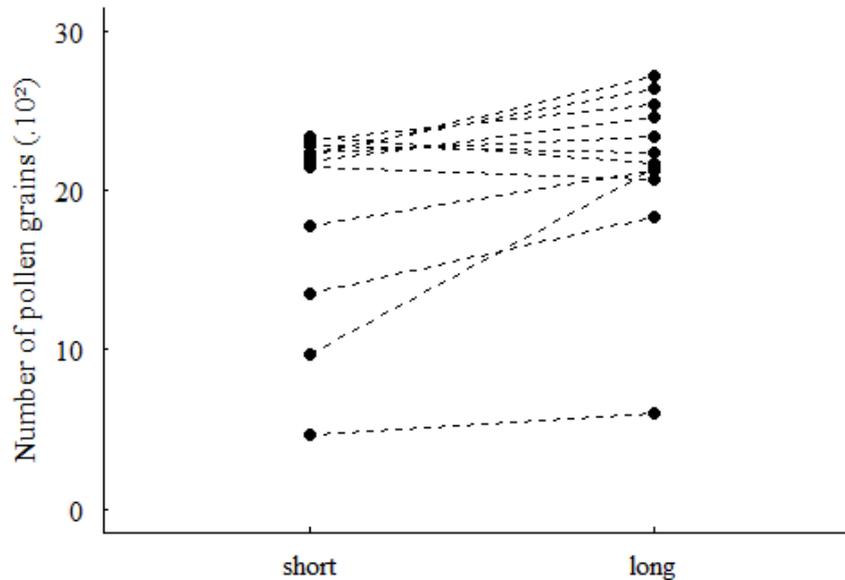
Floral parts and measurements	N	Dimension (mean $\pm$ SD)	Statistics
Calyx (sepals)	4		
upper-lower detachment		10.99 $\pm$ 1.43	
lateral detachment		12.05 $\pm$ 1.30	
Corolla (petals)	5		
petaloid petals length		6.05 $\pm$ 0.47	
Elaiophore area			
right		6.67 $\pm$ 0.44	$t=0.200$ ; $df=22$ ; $p=0.843$
left		6.70 $\pm$ 0.37	
Stamen length	4		
long		4.31 $\pm$ 0.31	$F=357.77$ ; $df=1$ ; $p<0.001$
short		3.20 $\pm$ 0.33	
Pistil length	1	4.82 $\pm$ 0.34	

The stigmas did not positively respond to the H<sub>2</sub>O<sub>2</sub> test in the first two hours of anthesis (0500 h and 0600 h). However, 77% of the tested stigmas ( $n=9$ ) were receptive at 0700 h. In the following hours, throughout the first day of anthesis, all sampled stigmas were receptive. The second-day flowers were no longer receptive. The pollen mass release was detected since anthesis onset. The anthers spontaneously released pollen throughout the first day of anthesis and pollen was still present on unvisited second-day flowers.

### 3.3 Breeding system

The natural fruit set in the *K. tomentosa* population was higher in the dry season than in the rainy season ( $\chi^2=4.35$ ;  $df=1$ ;  $p=0.037$ ). The fruit set in the whole experiment was influenced by the treatments ( $\chi^2=22.91$ ;  $df=3$ ;  $p<0.001$ ). No differences were detected among the cross-pollination, hand self-pollination and the control (dry season) ( $\chi^2=0.17$ ;  $df=2$ ;

p=0.916; Table 2). The fruit set of the spontaneous self-pollination treatment was smaller than the fruit set of the other treatments. Our data show that *K. tomentosa* is self-compatible, but requires pollen deposition by a biotic vector to set fruits. Fruits were produced when we manually pollinated the flowers before stigmatic receptivity, as well as in cross-pollination ( $\chi^2=1.73$ ; df=1; p=0.188; Table 2).



**Figure 3.** Number of pollen grains produced by short and long stamens (V=11; p=0.027; n=12). Paired data connected by a dashed line.

**Table 2.** Fruit set under different pollination treatments in *K. tomentosa*. Fl = number of flowers, Fr = number of initiated fruits, Fr/Fl = fruit set. Letters indicate statistical effects among the treatments.

Treatment	Fl	Fr	Fr/Fl
Cross-pollination	60	20	0.33 <sup>a</sup>
before stigmatic receptivity time	30	6	0.20 <sup>a</sup>
Hand self-pollination	60	22	0.36 <sup>a</sup>
Spontaneous self-pollination	60	2	0.03 <sup>c</sup>
Open pollination			
dry	30	11	0.37 <sup>a</sup>
rainy	30	4	0.13 <sup>b</sup>

### 3.4 Flower visitors

*Krameria tomentosa* flowers mainly attracted bees which foraged for oil or pollen. Twenty-one species of Apidae and one species of Syrphidae were recorded. Among the bees, twelve oil-collecting species visited the flowers (Tables 3 and 4). Five Meliponini and three Ceratinini species were recorded foraging for pollen. Regarding the visitation sampling (2013-2014), visits by *Centris* bees accounted for 52% of the total number of visits and those by *T. fulviventris* accounted for 40% (Table 4). On both visits by the syrphid fly we could not observe whether it foraged for pollen or only landed on the flower. We could also not see whether *A. huberi* collected oil or pollen.

Records of the flower visitors in *K. tomentosa* were sporadic. No visits were observed on three days (one in the dry season and two in the rainy season) of the 19 days of sampling. And no visits by *Centris* bees were recorded on seven days of the sampling, even in the period with several flowers available (November 2013). Regarding the days they were observed, the number of individuals/day ranged from 1 to 3 in the dry season and 1 to 10 in the rainy season.

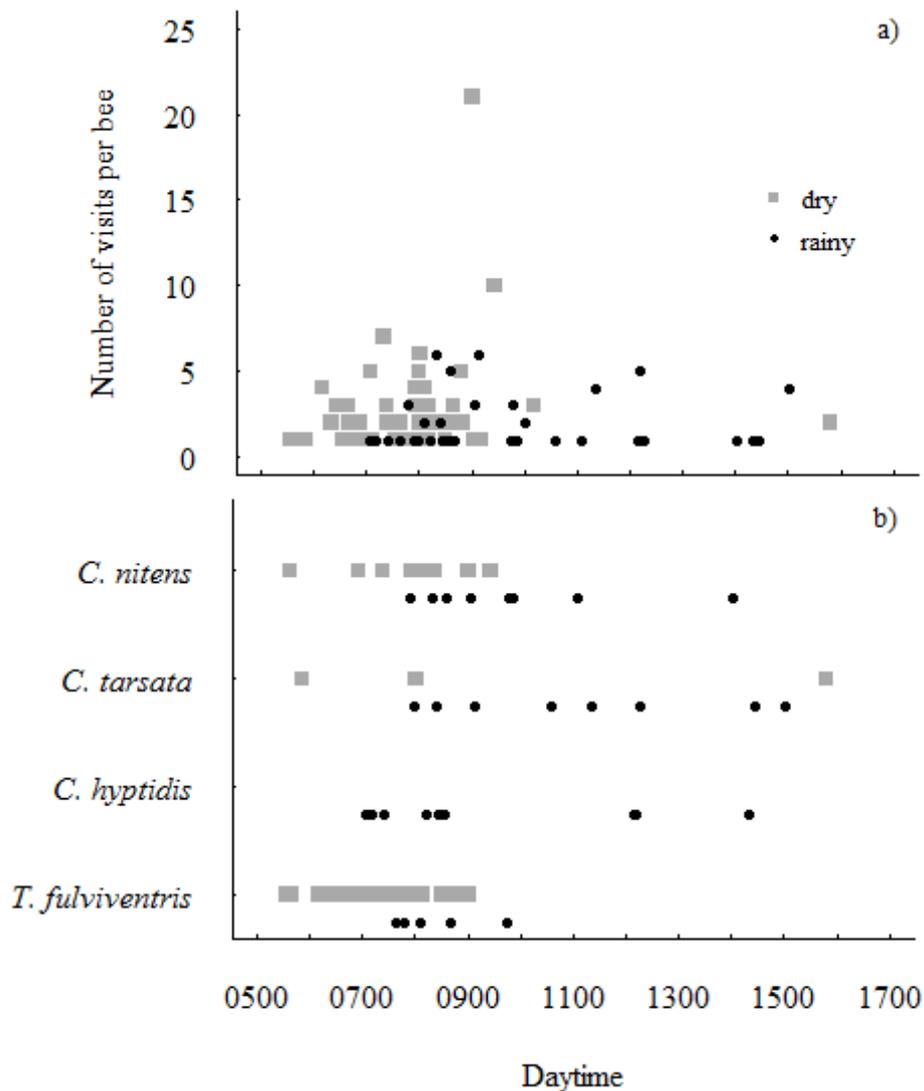
The floral visits were concentrated in the morning, initiated at approximately 0530 h and 0700 h in the dry and rainy seasons, respectively (Figure 4a); visits were performed mostly on first-day flowers. Among *Centris* bees, *C. nitens* was the most frequent in both seasons in the 2013-2014 sampling period (Table 4). *Centris* individuals rapidly collected floral oil (e.g. *C. nitens* spent ~6s, n=2) with their pairs of fore and mid legs for scraping the oil-secreting surface while they held the petaloid petals by their mandibles. In this sampling period, we recorded *C. hyptidis* only in August 2013-2014, which corresponds to the end of the rainy season in the region (Figure 1). However, *C. hyptidis* was the most recorded *Centris* bee on the *K. tomentosa* flowers in the 1999-2002 survey with records along the rainy period (Table 3). *Arhysoceble huberi* was the most common oil-collecting bee collected on *K. tomentosa* flowers in the 1999-2002 sampling, but only two visits by one individual were observed in the 2013-2014 period. The stingless bee *T. fulviventris* foraged on the flowers exclusively in the morning (Figure 4b) for pollen. This bee spent more time (~47s, n=8) at visiting the flowers than *Centris* and gathered the resource using its forelegs and mandibles. However, all the species touched the anthers and the stigma while visiting the *K. tomentosa* flowers and therefore they were regarded as potential pollinators.

**Table 3.** Number of individuals (N) per bee species collected on the *K. tomentosa* flowers during the 1999-2002 survey.

Bee species	N	Months recorded												
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
<b>APIDAE</b>														
<b>APINAE</b>														
Apini	<i>Apis mellifera</i> Linnaeus, 1758	9												
Centridini	<i>Centris (Centris) aenea</i> Lepeletier, 1841	4												
	<i>Centris (Centris) caxienseis</i> Ducke, 1907	6												
	<i>Centris (Centris) flavifrons</i> (Fabricius, 1775)	1												
	<i>Centris (Centris) nitens</i> Lepeletier, 1841	5												
	<i>Centris (Centris) spilopoda</i> (Moure, 1969)	1												
	<i>Centris (Hemisiella) tarsata</i> Smith, 1874	10												
	<i>Centris hyptidis</i> Ducke, 1908	24												
	<i>Centris (Paracentris) burgdorfi</i> Friese, 1900	1												
	<i>Epicharis (Epicharis) nigrita</i> (Friese, 1900)	1												
Meliponini	<i>Nannotrigona punctata</i> (Smith, 1854)	1												
	<i>Plebeia</i> sp.	1												
	<i>Trigona fulviventris</i> Guérin, 1837	26												
	<i>Trigona</i> aff. <i>fuscipennis</i> Friese, 1900	9												
	<i>Trigona spinipes</i> (Fabricius, 1793)	16												
Tapinotaspidini	<i>Arhysoceble huberi</i> (Ducke, 1908)	25												
	<i>Xanthopedia</i> aff. <i>larocai</i> Moure, 1993	1												
Tetrapediini	<i>Tetrapedia diversipes</i> Klug, 1810	2												
<b>XYLOCOPINAE</b>														
Ceratinini	<i>Ceratina (Crewella) maculifrons</i> Smith, 1854	6												
	<i>Ceratina (Crewella)</i> sp.1	3												
	<i>Ceratina (Crewella)</i> sp.2	2												

**Table 4.** Total number of visits per flower visitors of *K. tomentosa* in the dry and rainy seasons from the 2013-2014 sampling. Res = collected resource, OI = occurrence index.

Flower visitors		N Visits <sup>(OI)</sup>			Res
		Dry	Rainy	Total	
<b>APIDAE</b>					
APINAE					
Centridini	<i>Centris (Centris) nitens</i> Lepeletier, 1841	45 <sup>(0.60)</sup>	21 <sup>(0.56)</sup>	66 <sup>(0.58)</sup>	O
	<i>Centris (Hemisiella) tarsata</i> Smith, 1874	5 <sup>(0.20)</sup>	20 <sup>(0.56)</sup>	25 <sup>(0.37)</sup>	O
	<i>Centris hyptidis</i> Ducke, 1908	-	13 <sup>(0.22)</sup>	13 <sup>(0.10)</sup>	O
Tapinotaspidini	<i>Arhysoceble huberi</i> (Ducke, 1908)	-	2 <sup>(0.11)</sup>	2 <sup>(0.53)</sup>	?
Meliponini	<i>Trigona fulviventris</i> Guérin, 1837	72 <sup>(0.50)</sup>	8 <sup>(0.56)</sup>	80 <sup>(0.53)</sup>	P
	<i>Trigona spinipes</i> (Fabricius, 1793)	1 <sup>(0.10)</sup>	-	1 <sup>(0.05)</sup>	P
XYLOCOPINAE					
Ceratinini	<i>Ceratina (Crewella) maculifrons</i> Smith, 1854	9 <sup>(0.20)</sup>	-	9 <sup>(0.10)</sup>	P
<b>SYRPHIDAE</b>					
ERISTALINAE					
Volucellini	<i>Ornidia obesa</i> (Fabricius, 1775)	2 <sup>(0.10)</sup>	-	2 <sup>(0.05)</sup>	?



**Figure 4.** Number of visits per bee on two flowering *K. tomentosa* plants during ~10 min observation periods along 10 and 9 days in the dry and rainy season, respectively (a), and visitation time of oil-collecting *Centris* bees and pollen-collecting *T. fulviventris* (b) recorded in the 2013-2014 sampling.

#### 4. Discussion

*Krameria tomentosa* has a self-compatible breeding system dependent on oil- and pollen-collecting pollinators to set fruits. However, the majority of visits were by bees for oil gathering, among them *C. hyptidis* which has commonly been related to the trichome elaiophores of *Angelonia*. *Epicharis* is for the first time reported as visitor of *Krameria* flowers. Self-compatibility has been demonstrated to some extent in the genus. Simpson (1989) recorded less than 10% of seed set from hand self-pollination and cross-pollination in North American *K. lanceolata*, and highlighted a low seed-flower proportion in this monotypic group due to impressive fruit predation and spontaneous abortion of fruits, which

make evaluation of the breeding system difficult. Nevertheless, in this study, both hand self- and cross-pollination treatments produced more than 30% fruit set and no abortion after fruit formation was detected. In the population we studied, fruit predation is likely to affect half the fruits (L.T. Carneiro, unpublished data). No fruit without active transfer of pollen was produced, indicating the dependence on pollinators in *K. tomentosa*.



**Figure 5.** (a) *Centris hyptidis* before visiting a *K. tomentosa* flower and (b) *T. fulviventris* collecting pollen.

Stigmatic receptivity was initiated approximately two hours after flower opening, an interval in which pollen mass release by mechanical irritation could be observed. The *Krameria* poricidal anthers autonomously constrict, causing pollen mass extrusion (Simpson, 1989), throughout the first day of anthesis, which does not spontaneously contact the stigmatic surface. The strictly staminate phase is restricted to a short period at anthesis onset and, as the staminate and pistillate functions overlap, the pollen presentation appears to become higher. Even with their anthers and extruded pollen mass present near the stigma, autogamy does not occur in *Krameria tomentosa* flowers, as already mentioned by Simpson (1989) and Gimenes and Lobão (2006) for other *Krameria* species, including the Brazilian *K. bahiana*. Besides fruit set from both self- and cross-pollen deposition, our data show that the germination of the pollen deposited on the stigma before stigmatic activity is not inhibited and can lead to fruit set.

Both oil- and pollen-collecting bees were regarded as potential pollinators of *K. tomentosa* flowers in the site, as they are likely to deposit pollen on the stigma. This suggests that *K. tomentosa* is not exclusively dependent on *Centris* and other oil-collecting bees to set seeds and further experiments are necessary to measure the pollination efficiency by them and pollen-collecting bees such as *Trigona*.

In our study, the *Centris* bees sparsely visited *K. tomentosa* flowers for oil-gathering throughout the day, but mainly in the morning. A few visits were recorded during our sampling compared to the visitation pattern in *K. bahiana*, in which more than 500 visits by *C. leprieuri* and *C. tarsata* were observed in a sampling period of less than 12 days (Gimenes and Lobão, 2006). In other *K. tomentosa* populations (dune vegetation and another *tabuleiro* habitat, closer to the coast), few visits were observed as well (L.T. Carneiro, unpublished data). This pattern is not likely to be an outcome of a density effect as the *Centris* bees collected on *K. tomentosa* flowers were one of the predominant bee species surveyed in the area, with *C. nitens* as the most recorded among the female *Centris* bees (Aguiar and Martins, 2003). Few, sparse visits for oil-gathering appear to be common in this species. Nevertheless, this pattern did not reflect a low natural fruit set in *K. tomentosa* as demonstrated by the treatments (self- and cross-pollination and control/dry season). A low seed set in the control was obtained in the rainy period, probably due to the rain which prevented visitation. The rain and delayed sunrise were also likely to be factors which contributed to a delayed visitation pattern in the rainy season compared to the dry season (Figure 3).

Most of the Neotropical oil-flower taxa are visited by *Centris* bees for oil-gathering (Machado, 2004; Renner and Schaefer, 2010). *Centris aenea*, *C. caxiensis*, *C. flavifrons*, *C. nitens*, *C. spilopoda*, *C. tarsata* and *E. nigrita* have been recorded mainly on Malpighiaceae flowers – e.g. *Byrsonima* spp., *Banisteriopsis* spp., *Dicella bracteosa* (A. Juss.) Griseb., *Mascagnia cordifolia* (A. Juss.) Griseb. and *Stigmaphyllon lalandianum* A. Juss. (Gaglianone, 2003; Sigrist and Sazima, 2004; Ribeiro et al., 2008; Bezerra et al., 2009a; Dunley et al., 2009; Sazan et al., 2014) – including the West Indian cherry *Malpighia emarginata* D.C., an economically important crop in northeastern Brazil (Freitas et al., 1999; Vilhena et al., 2012). Visits on *K. tomentosa* flowers by *C. nitens* and *C. tarsata* were recorded in both the dry and rainy sampling periods (Table 4). Although *C. tarsata* was more often recorded on *K. tomentosa* flowers in the rainy period, increased nesting activity has been observed in the dry season in the area (Aguiar and Martins, 2002), indicating greater requirements for oil

gathering in that period. Displacement by competition between *Centris* bees in any season is not thought to occur due to the high number of *K. tomentosa* flowers available and not visited in the population. Perhaps *C. tarsata* explores Malpighiaceae species more often, which usually bear flowers mostly in the dry season (Bezerra et al., 2009a). Further studies concerned with the interaction between all the oil flowers – seven Malpighiaceae and *K. tomentosa* (Barbosa et al., 2011) – and *Centris* bees in the studied site could test this hypothesis.

Studies have regarded some Neotropical oil-collecting bees as exclusively associated with non-Malpighiaceae oil flowers (Vogel and Machado, 1991; Simpson et al., 1990; Sersic and Cocucci, 1999; Machado et al., 2002). *Centris hyptidis* is known as endemic to Brazilian tropical dry forest (Vivallo and Melo, 2009) and has been closely associated with the trichome elaiophores of *Angelonia* (*A. cornigera* and *A. pubescens*) floral sacs (Vogel and Machado, 1991; Machado et al., 2002). According to Vogel and Machado (1991), the combs present on its proportionally longer forelegs and midlegs are rudimentary and the anterior tarsal pads with unbranched spatulate setae are used to sopping up oil from the trichome elaiophores. In our study, this oil-collecting species was recorded on *K. tomentosa* flowers during the rainy sampling period, handling and gathering oil with both pairs of fore and midlegs, similar to the other two *Centris* bees recorded in 2013 and 2014. Presumably, *C. hyptidis* uses its reduced combs for scrapping *K. tomentosa* epithelial elaiophores. Barringer (1981) reported that at least three species of *Angelonia* occur close to the coast of northeastern Brazil, overlapping with the distribution of *K. tomentosa* and *C. hyptidis*. However, according to Barbosa et al. (2011), no *Angelonia* species occur in the area even near the eastern limit of the dry forest, a peripheral extension of the *C. hyptidis* distribution. Thus, *C. hyptidis* populations can persist in areas without *Angelonia*. On the other hand, Machado et al. (2002) highlighted that *C. hyptidis* never visited *Krameria* in a dry forest area where it co-occurs with *Angelonia*. However, the interaction between *Krameria* (*K. grandiflora*) and *C. hyptidis* has also been recorded in the Caatinga (Zanella, 2010), but we do not yet know about its oil-gathering efficiency as a way of comparing both oil-producing taxa. It is noteworthy that *C. hyptidis* has not been recorded on malpigh flowers at the study site (A. J. C. Aguiar, unpublished data).

The Malpighiaceae-*Centris* interaction is the oldest association among the South American oil-flower systems (Renner and Schaefer, 2010; Martins et al., 2014) and the pathways leading to oil foraging on young clades such as Krameriaceae, Plantaginaceae and

Calceolariaceae are not yet understood. Our report shows that the association between the morphology of the oil-collecting apparatus and the elaiophore type (epithelial or trichome) is not enough to determine ecological and evolutionary relationships in these systems. Moreover, there may be differences between malpigh and *Krameria* epithelial elaiophores that have not been tested yet but effect pollinators, such as cuticle components, resistance and hardness, and oil chemistry. Several *Centris* (*Paracentris* groups, *Penthemisia* and the *hyptidis* group) and Tapinotaspidini (*Arhysoceble* and *Caenonomada*) which are associated with oil gathering in Plantaginaceae (Simpson et al., 1990; Sersic and Cocucci, 1999; Martins et al., 2013) also exploit *Krameria* elaiophores (Simpson et al., 1977; Simpson, 1989; Zanella, 2010; Silva et al., 2012), but they do not apparently use malpigh floral oils. Further data on the interactions between oil-collecting bees and *Krameria* are needed. Studies on the geographic variations in oil-flower networks with ecological-evolutionary and chemical ecological approaches could offer new insights into those associations.

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**Oil-collecting bees associated to *Krameria grandiflora*: does functional significance of floral traits diverge between different pollinator-matching scenarios?**

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**Abstract:** An experimental field study was undertaken to assess the functional significance of the floral traits in variants of the mutualist interaction between oil-collecting bees and the floral oil-producing *Krameria grandiflora* (Krameriaceae). *Centris* bees interact with Krameriaceae flowers and entirely match their floral architecture, leading one to expect a narrow relationship between floral morphology and pollination success. The opposite is expected for a poor matched interaction performed by less effective pollinators and oil robbers. In this context, we compared the functional significance of the three *Krameria* specialized floral structures (sepals, petaloid petals and elaiophores) between scenarios of a perfect matching, created by *Centris* bees, and a poor matching performed by a Tapinotaspidini bee. The manipulative experiment consisted in excising the floral structures and combinations of them, recording the oil-gathering visitation and fruit set after exposure to natural pollination in the field. Our results did not support the hypothesis that the functional significance of floral traits differs under contrasting levels of pollinator matching. Petaloid petals were predicted to be the keystone trait for that divergence in pollinator matching, but like the sepals they had no effect on our response variables. However, the elaiophores were the main floral structure affecting oil gathering and fruit set, showing their significance for the interaction. In this study, we also provided a description of the interactions between oil-collecting bees and *K. grandiflora* across plant species range.

**Keywords:** dry diagonal / oil robbers / floral oil / *Centris* / *Caenonomada*

**Resumo:** Um estudo experimental de campo foi realizado para acessar a significância funcional dos caracteres florais em variantes da interação mutualística entre abelhas coletoras de óleo e a espécie produtora de óleo floral *Krameria grandiflora* (Krameriaceae). Espécies de abelha do gênero *Centris* interagem com as flores de Krameriaceae e se encaixam completamente a sua arquitetura floral, o que nos leva a esperar uma relação estreita entre morfologia floral e sucesso na polinização. O contrário é esperado para um fraco ajuste atribuído a polinizadores menos efetivos ou pilhadores de óleo. Nesse contexto, nós comparamos a significância funcional das estruturas florais especializadas presentes em *Krameria* (sépalas conspicuas, pétalas petaloides e elaióforos) entre cenários de encaixe perfeito promovido por *Centris* e um encaixe fraco dirigido por uma espécie de Tapinotaspidini. O experimento manipulativo consistiu na excisão das estruturas florais, incluindo suas combinações, e no registro da visitação para coleta de óleo e frutificação após exposição a polinização natural no campo. Nossos resultados não suportaram a hipótese de que a significância funcional dos caracteres florais difere sob níveis contrastantes de ajuste de polinizador. Esperava-se que as pétalas petaloides fossem a estrutura chave para a divergência em ajuste; porém, como as sépalas, as pétalas petaloides não afetaram nossas variáveis respostas. No entanto, os elaióforos foram a principal estrutura a influenciar a coleta de óleo e a frutificação, indicando sua significância para a interação. No presente estudo, nós também fornecemos uma descrição das interações entre abelhas coletoras de óleo e *K. grandiflora* ao longo de sua distribuição geográfica.

**Palavras-chave:** diagonal seca / pilhadores de óleo / óleo floral / *Centris* / *Caenonomada*

## 1. Introduction

Many plant traits evolve in response to selective forces exerted by interacting species, either to avoid them (*e.g.* herbivores) or keep them (*e.g.* pollinators) as interactors depending on their effect on plant fitness (Macior, 1971; Rausher, 1992; Fenster et al., 2004; Herrera and Pellmyr, 2009). In mutualisms as seed dispersal and pollination, plant traits associated to animal morphology, sensory physiology and foraging energetics are selected providing a better mutual fit (Chittka and Thomson, 2001; Harder and Johnson, 2009; Schiestl and Johnson, 2013; Wester and Lunau, 2016). By the way, the floral organizational diversity of animal-pollinated angiosperms might have arisen due to the adaptation to biotic pollen vectors, creating many complex floral structures (Faegri and van der Pijl, 1979; Endress, 2001; Fenster et al., 2004; Wilson et al., 2004; Armbruster et al., 2011; van der Niet and Johnson, 2012).

The maintenance of mutualists generally requires a joint operation of traits that exert complementary functions. In plant-pollinator interactions, the floral architecture components give the attraction (floral signaling) and the mechanical fit (traits involved in pollen transfer and deposition), combined with reward type and/or quality, that often constrain the morphological or behavioral range of the flower visitors. However, those interactions do not often result in an optimum matching (plant-pollinator fit) even in phenotypic specialized systems (*sensu* Ollerton et al., 2007), either due to floral trait variation (Nattero et al., 2010) or due to inappropriate pollinator behavior (*e.g.* cheaters and less effective pollinators; Pellmyr et al., 1996; Irwin and Brody, 1998). In this last situation, flower visitors usually exploit the floral rewards and provide none or less positive effect on plant fitness.

Matching in single plant-pollinator interactions can be geographically variable, depending on the pollinator local community and floral phenotype (Thompson, 2005; Nattero et al., 2010). In this geographic mosaic there might have pollinators which diverge in handling and/or response to stimuli from the floral phenotype, conferring different functions to floral traits among populations. These divergences can generate contrasting pollinator-mediated selection on floral traits and may be important for flower diversification (Castellanos et al., 2003; Medel et al., 2007). Manipulative experiments of floral architecture and phenotype have been useful tools to assess the adaptive value and multivariate selection on floral traits (Wilson, 1995; Herrera, 2001; Boberg and Ågren, 2009; Campbell, 2009;

Cuartas-Domínguez and Medel, 2010). Moreover, one can either infer on the functional significance of the floral traits (Helvesen and Helvesen, 1999; Dudash et al., 2011) or on plant-pollinator interaction stability, once the system experiences abrupt changes (Andersson, 1994). However, most of the experimental studies lack the evaluation of the manipulative effect in the phenotype on the pollinator behavior and visitation, besides the plant fitness (Kudoh and Whigham, 1998; Castellanos et al., 2004), giving only partial insights on mutualism responses.

In this study, we manipulated the floral architecture to assess the functional significance of the floral traits in variants of the mutualism between oil-collecting bees and the floral oil-producing *Krameria grandiflora* A. St.-Hil. (Krameriaceae). For this, we firstly surveyed the flower visitor species associated to *K. grandiflora* in wild populations across its geographical distribution and described their behavior on the flowers. Then, we selected contrasting and ideal scenarios in pollinator matching to perform the experimental study. The interaction between oil-collecting bees and *Krameria* species has been previously described as a very specialized mutualism because plants depend on the bees to fitness assurance and floral traits are highly associated to oil-gathering behavior (Simpson, 1989; Carneiro et al., 2015). The unique floral plan of Krameriaceae is the main attribute that confers the familial status to the group (only 18 *Krameria* species in the monotypic family; Simpson, 1989; Simpson et al., 2004), as it extremely diverges from the sister group Zygophyllaceae (Sheahan and Chase, 1996). The zygomorphic flowers bear a showy calyx and two specialized structures derived from their dimorphic corolla: a group of reduced petaloid petals and a pair of fleshy petals (elaiophores) (terminology provided by Vogel, 1974; Simpson, 1989). In turn, oil-collecting bees have highly specialized morphological structures (Neff and Simpson, 1981; Buchmann, 1987) and although there is no strong support for co-evolutionary relationships in this system, pollinator dependence only on *Krameria* oil flowers occurs in some populations, configuring locally narrow and stable interactions (Carneiro et al., 2015; Sabino et al., 2017).

The oil-collecting bees *Centris* (Centridini) have been usually known to be associated to the New World Malpighiaceae species (Anderson, 1982; Vogel, 1990; Mello et al., 2013); however, many species interact with Krameriaceae flowers (Simpson et al., 1977; Simpson, 1989; Gimenes and Lobão, 2006; Carneiro et al., 2015; Sabino et al., 2017). *Centris* bees entirely match *Krameria* floral architecture while visiting the flowers by holding the reduced petaloid petals with their mandibles and scraping the elaiophores with fore and midlegs,

always touching the reproductive organs (Simpson, 1989; Carneiro et al., 2015). Thus, one can assume that *Centris* oil-gathering behavior features a perfect matching in this system. On the other hand, a poor matching is realized by Tapinotaspidini bees like *Arhysoceble*, *Xanthopedia* and *Caenonomada* (see Results for behavior description). *Caenonomada* bees do not hold *Krameria* petaloid petals to collect oil and eventually touch the reproductive organs, which could provide low functional significance for this structure. Regarding these differences, we tested the system responses to a manipulative experiment in two *K. grandiflora* populations (perfect matched and poor matched) to evaluate the functional significance of the three specialized floral structures (sepals, petaloid petals and elaiophores). From this, we can infer on the potential divergences of selective forces in an intraspecific context (Herrera et al., 2006) within a specialized pollination system formed by oil-collecting bees as agents.

In this background, we hypothesized that the functional significance of the floral parts differs between the perfect matched and poor matched *K. grandiflora* populations. We expected that the three *Krameria* specialized floral traits contribute to pollination success and assure the oil-gathering behavior. We assumed that the calyx functions as the major attractive part while petaloid petals and elaiophores have the both mechanical-fit and attraction functions as they are extremely related to pollinator matching (pollen transfer and deposition) and besides being brightly colored structures. Moreover, the elaiophores produce the main floral reward in this system. Nevertheless, we also predicted that pollinator divergence could confer different effect to the floral units; mainly to petaloid petals which have no contribution as mechanical-fit trait to the interaction with *Caenonomada*. In this study, we also provided an evaluation of plant dependence on pollinators to set fruits and a list of novel records and descriptions of *Krameria*-bee interactions.

## **2. Materials and methods**

### **2.1 Plant species and reproductive biology**

*Krameria grandiflora* is a prostrate (to procumbent) perennial herb with flowering period ranging mostly from January to April. Flowers are larger (compared to other South American *Krameria* species); hermaphrodite and zygomorphic (Figure 1). There are some slight differences along its geographical range regarding reproductive and vegetative plant parts as mentioned by Simpson, 1989. In general, the calyx is formed by four spreading sepals

(rarely five). The dimorphic corolla divided into a group of three petaloid petals (rarely two or four), with pink/purple blades and greenish-yellow stems, and a pair of pink/purple elaiophores. *Krameria* elaiophores comprise a glandular epidermis in the dorsal surface of the fleshy petals covered by blisters which retains the oil (Milby, 1971; Simpson, 1982). The ovary produces two ovules but only one develops into seed (Simpson, 1989). Although several studies have shown that some *Krameria* species are dependent on pollinator to set fruits (Simpson, 1989; Gimenes and Lobão, 2006; Carneiro et al., 2015), there is no information on the *K. grandiflora* breeding system. A non-dependence on pollinators could negatively influence the effect of our functional significance experiment on pollination success. Thus, we performed a usual pollination experiment in one population and complementary pollinator exclusions in four populations. For the pollination experiment, we tagged 30 plants and bagged four pre-anthesis buds per plant to produce a paired experiment with three treatments and a control. The treatments consisted in hand-pollinating the flowers with exogenous pollen after emasculation (cross-pollination), with their own pollen (hand self-pollination) and keeping them bagged (autonomous self-pollination). As a control we left one flower exposed to natural pollination. Flowers used for the cross-pollination and hand-self-pollination treatments were re-bagged right after pollen deposition. As flowers remain open along one day (see Appendix S1), we re-bagged the control after flower closure in the late afternoon to maintain all treatments under the same conditions and avoid herbivores. After one week we recorded fruit formation. To complement the information we obtained from the pollination experiment, we prevented three flowers in 10 plants from visitation by bagging them in four populations and recorded if they yielded fruits one week later.

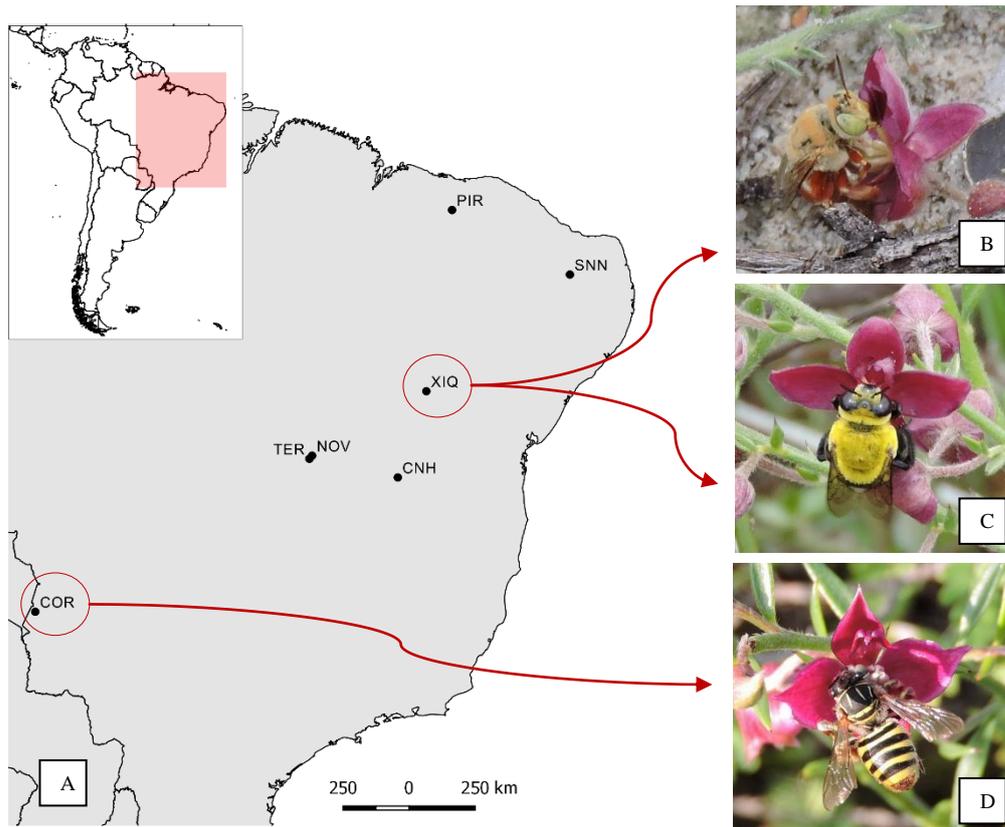
## **2.2 Plant-pollinator interaction sampling**

*Krameria grandiflora* occurs in isolated populations distributed in open habitats throughout the South American “dry diagonal” (see Werneck et al., 2011 for a broader overview), mostly in its wide-ranging portion, in the Caatinga dry forest. We conducted our surveys of flower visitor-*K. grandiflora* interactions along seven populations in the 2014-2016 flowerings (Figure 2A; see Appendix S2 for details). We recorded the flower visitors directly on *K. grandiflora* flowers between 0800 h and 1300 h (20 min per each half an hour) in two days/population/year. We performed visitation rate samplings only when flower visitors were abundant enough. In these cases, we observed 40 plants between 0800 h and 1300 h. Visitation rates were sampled in four plants during 20 min (5 min/plant) each half an

hour. We noted the reward gathered (oil and/or pollen) and described pollinator behavior. We classified pollinator behavior based on the combination of the contact with the reproductive organs (always or eventually), usage of the petaloid petals, reward gathered (oil or pollen) and bee size. We considered that flower visitors which always touched the anthers and the stigma and used the petaloid petals for oil gathering, performed a perfect pollinator matching. We obtained the intertegular distance from the specimens collected as size measurement (Cane, 1987). These specimens were identified and deposited in the entomological collection CEPANN/IB/USP, São Paulo, Brazil.



**Figure 1.** *Krameria grandiflora* flower and its floral parts. *Calyx*: Lt, lateral sepal; Lw, lower sepal; Up, upper sepal; *Corolla*: Pt, reduced petaloid petals; Ela, fleshy petal (elaiophore); gs, glandular surface; *Pistil*: ov, ovary; st, stigma; *Androecium*: Is, long stamen; ss, short stamen.



**Figure 2.** A: Localities of *Krameria grandiflora* populations surveyed. PIR, Parque Nacional das Sete Cidades, Piripiri-PI (4°05'S, 41°44'W); SNN, Estação Ecológica do Seridó, Serra Negra do Norte-RN (6°34'S, 37°15'W); XIQ, APA Lagoa de Itaparica, Xique-Xique-BA (11°01'S, 42°43'W); NOV, Nova Roma-GO (13°27'S, 47°05'W); TER, Teresina de Goiás-GO (13°34'S, 47°11'W); CNH, APA Rio Carinhanha, Carinhanha-BA (14°16'S, 43°49'W); COR, Parque Natural Municipal de Piraputangas, Corumbá-MS (19°14'S, 57°38'W). B-D: Oil-collecting bees associated to the populations (XIQ and COR) used in the manipulative experiment. B: *Centris* (*Centris*) *byrsonimae*; C: *Centris* (*Paracentris*) *xanthomelaena*; and D: *Caenonomada* aff. *unicalcarata*.

### 2.3 Manipulation of floral architecture

We performed our manipulative experiment in a perfect matched population (only visited by *Centris*) and in a poor matched population (mostly visited by *Caenonomada*) to evaluate the functional significance of sepals, petaloid petals and elaiophores considering oil-gathering visits and fruit set as response variables. We performed sets of five treatments in flowers paired in individual plants (sampling units) which consisted in the excision of sepals (S), petaloid petals (P), elaiophores (E), sepals + petaloid petals (S+P) and sepals + petaloid petals + elaiophores (S+P+E) (Figure 3). We left a sixth flower intact as a control. For fruit

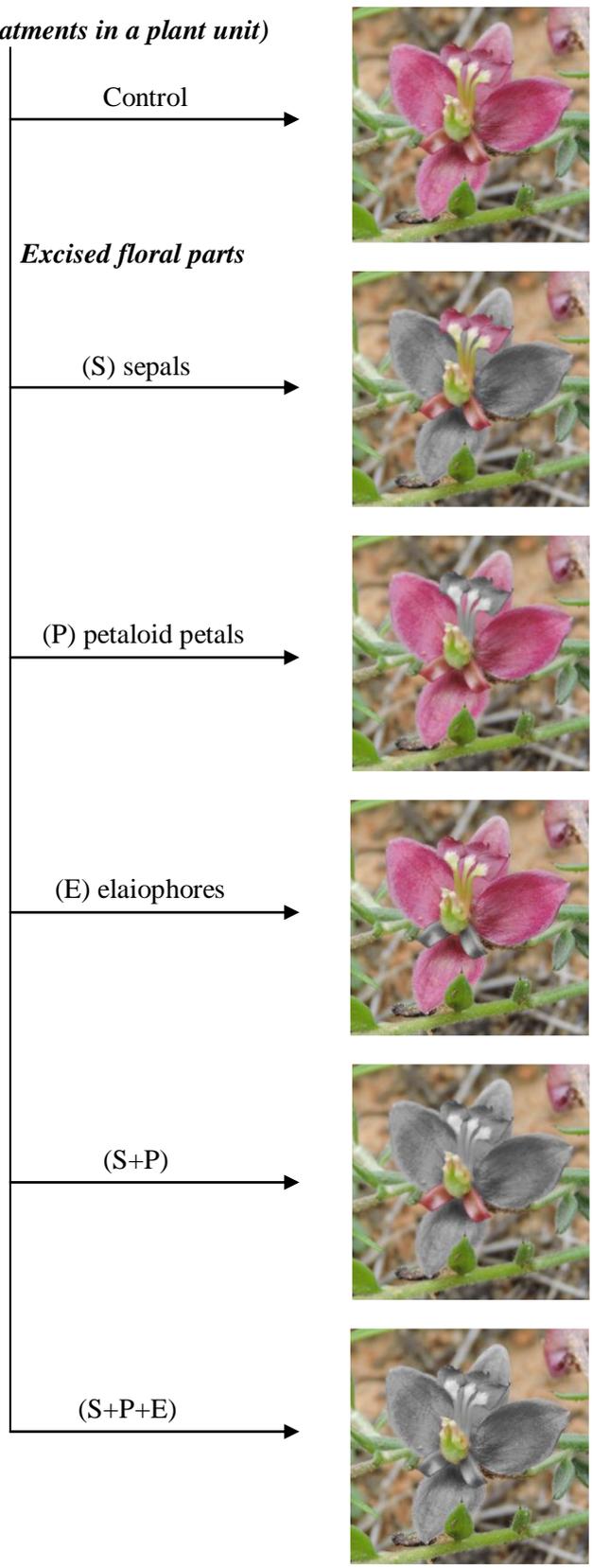
set, we produced 12 and 15 sets of treatments in the perfect matched and in the poor matched populations, respectively, and recorded fruit formation one week later. And for oil-gathering visits, the same treatments were performed in 15 and 10 plants in the perfect matched and in the poor matched populations, respectively. We recorded visits on five set of treatments per day during 25 min (5 min/set) each half an hour (1.5 h/day) between 09h30-11h00. This reduced period of sampling was chosen to diminish bee-learning effects (Lavery, 1994). We discriminated between visits with and without oil-gathering. All flowers were bagged in pre-anthesis until treatment manipulation and visitation sampling time to prevent them from previous visits. We tagged all manipulated flowers, including the control. We used a small adhesive paper attached in a string tied in their peduncle to recognize the treatments during visitation sampling and fruit formation records, as the peduncle does not drop with the flowers. We observed no bees attracted to tag components and assumed no interference by using them. Although the design was completely balanced at the start of the experiment, some flowers had to be eliminated because they were ripped off probably by passing animals. Individual plants were 3-5 meters away from each other. In this experiment there were also non-manipulated flowers in the individual plants.

## 2.4 Statistical analysis

To test the effect of treatments on the response variables in both the pollination experiment and the manipulative experiment we used generalized linear mixed models (GLMM) with binomial (for fruit set) and Poisson (for oil-gathering visits) distributions. In the pollination experiment we created a model that included the treatments as fixed effect and individual plant as random effect regarding the paired organization. We used the function *glmer* from the *lme4* package. In the manipulative experiment, we tested the effects of excisions and population (perfect and poor matching) on oil-gathering visits and fruit set. We produced four models and a null one, considering as random effect individual plants for fruit set and individual plants, day and time for oil-gathering visits. As fixed effect we included only treatment (1), only population (2), treatment plus population (3) and the interaction between them (4). For both set of models (fruit set and oil-visitation as response variables) we included the Bayesian inference in the GLMM using *blme* (*bglmer* function) package as it better fitted to our data.

**MANIPULATIVE EXPERIMENT**

*(paired treatments in a plant unit)*



**Figure 3.** Diagram showing the excision experiment set in two populations of *Krameria grandiflora* with different pollinator matching level. Excised floral parts are in grey.

To look for effects in our analysis we applied a model selection method using the corrected Akaike's Information Criterion (AIC<sub>c</sub>) (Burnham and Anderson, 2002). The model that minimizes AIC provides the most reliable fit to the data. We averaged the models that obtained  $\Delta\text{AIC} \leq 2$  (equally plausible) to rank their importance and obtain a single simplified model. Model selection were performed using the *MuMIn* package (Barton, 2016) and level confidence intervals were also estimated. All the procedures were performed in R software v3.3.1 (R Core Team, 2016).

### 3. Results

#### 3.1 Reproductive biology

In the pollination experiment, we found no effect of treatments on fruit set indicated by the averaged model parameters (Table S1). We obtained a low fruit set from the treatments (Table 1); however, the results suggested that *K. grandiflora* is self-compatible and dependent on pollinators to set fruits. The autonomous self-pollination treatment produced only one fruit (3.3%) and the highest fruit set rate were obtained through hand self-pollination (20%) and cross-pollination (16.7%). The control produced only 10% of fruits. No fruit formation was obtained from the complementary pollinator exclusions in the four populations, supporting the hypothesis of dependence on pollinators.

**Table 1.** Fruit set yielded from the pollination experiment performed on *Krameria grandiflora* flowers.

Treatment	Flowers	Fruits	% Fruit set
Cross-pollination	30	5	16.7
Hand self-pollination	30	6	20.0
Autonomous self-pollination	30	1	3.3
Control	30	3	10.0

#### 3.2 Pollinator-plant interactions and matching

We recorded 21 bee species on *K. grandiflora* flowers in the seven populations (see Table 2), mostly Centridini (*Centris*, 10 spp.) which foraged only for floral oil. These oil-collecting bees were the most observed on *K. grandiflora* flowers in five populations and the only oil bee taxa associated to the flowers in three of them (Table S2 for details). We also

recorded four species of Tapinotaspidini bees which foraged for both oil and pollen, depending on the species. Among them, *Caenonomada* aff. *unicalcarata* and *Arhysoceble huberi* were the most recorded in the study and the first was highly associated to the Corumbá population (COR, Figure 2D). Few sporadic exclusively pollen-collecting species were also recorded as shown in Table 2.

We identified four modes of pollinator behavior (one perfect matching and three poor matching) in *K. grandiflora* flowers: (1) *Centris* mode, (2) large sized Tapinotaspidini mode, (3) small sized Tapinotaspidini mode and (4) pollen-collecting bee mode. The *Centris* mode was the only one that perfectly matched the flower architecture by holding the petaloid petals while scraping both elaiophores with their fore and midlegs; always touching the reproductive floral organs (Figure 2B-D). In this mode, bees left a bite mark in the petaloid petals after visiting. The second mode comprises the behavior of large sized (but smaller than the smallest *Centris*) Tapinotaspidini bees (*Caenonomada* and *Tapinotaspoides*) which did not hold the petaloid petals and eventually touched anthers and stigma (poor matching; Figure 2D), but scrape the elaiophores with their fore and midlegs. When touching the reproductive organs, *Ca.* aff. *unicalcarata* leans on the flower by inserting anthers tips and stigma between its head and thorax for oil gathering. In the small sized Tapinotaspidini mode, bees were too small for holding petaloid petals and touching the reproductive organs during the oil-gathering, and remain only in one elaiophore. Thus, these bees only touched the anthers and stigma while collecting pollen. In this mode, the oil bees were considered oil robbers. Finally, the pollen-collecting bee mode comprises the behavior of bees that only gathered pollen and consequently contacted the reproductive organs, but did not match flower architecture.

### 3.3 Functional significance of floral traits

For oil-gathering visits as response variable, model (1) was the most plausible indicating that treatments had effect on it (Table S1). No divergence regarding populations was detected; therefore, the perfect matched population (only visited by *Centris*) and the poor matched one (highly associated to *Caenonomada*) responded similarly to the treatments. For oil-gathering visits, only the excisions of elaiophores (E) and of all floral parts (S+P+E) affected, providing a negative response to this variable (Table 3). The other treatments were similar to the control for this response variable (Table 4). In the treatment (E), most visits

**Table 2.** Flower visitors associated to *Krameria grandiflora* flowers in seven populations across its geographical range (see Figure 2A for local acronyms; in bold, populations used for the manipulative experiment). Filled boxes indicate presence and mean frequency of flower visitor: (■) = >75%; (▣) = 50-75%; (▤) = 25-49%; and (▥) = <25% (see Table S2 for details). Size<sub>ID</sub>: mean intertegular distance (mm); R: reward type (o = oil; p = pollen); PP: Petaloid petals held (y = yes; n = no); RO: Reproductive organs touched (a = always; e = eventually).

Flower visitors	Populations							Size <sub>ID</sub>	R	Matching	
	PIR	SNN	NOV	TER	<b>XIQ</b>	CNH	<b>COR</b>			PP	RO
<b>APIDAE</b>											
<b>CENTRIDINI</b>											
<i>Centris (Centris) aenea</i> Lepeletier, 1841	▣	▥					▥	4.75	o	y	a
<i>Centris (C.) caxiensis</i> Ducke, 1907		▥						4.30	o	y	a
<i>Centris (C.) byrsonimae</i> Mahlmann & Oliveira, 2012							▣	3.83	o	y	a
<i>Centris (Hemisiella) tarsata</i> Smith, 1874	▣	▣						3.55	o	y	a
<i>Centris hyptidis</i> Ducke, 1908		▥	▣	▥				3.68	o	y	a
<i>Centris hyptidoides</i> Roig-Alsina, 2000							▥	3.96	o	y	a
<i>Centris (Paracentris) burgdorfi</i> Friese, 1900*		▥						5.23	o	y	a
<i>Centris (P.) thelyopsis</i> Vivallo & Melo, 2009*			▥					3.46	o	y	a
<i>Centris (P.) xanthomelaena</i> Moure & Castro, 2001		▣					▣	4.35	o	y	a
<i>Centris (Trachina) perforator</i> Smith, 1874		▥						4.91	o	y	a
<b>TAPINOTASPIDINI</b>											
<i>Arhysoceble huberi</i> (Ducke, 1908)		▥						1.62	o/p	n	e
<i>Caenomada aff. unicalcarata</i> (Ducke, 1908)		▣			▣		▣	2.63	o	n	e
<i>Tapinotaspoides serraticornis</i> (Friese, 1899)			▥					2.23	o	n	e
<i>Xanthopedia globulosa</i> (Friese, 1899)					▥			1.80	o/p	n	e
<b>MELIPONINI</b>											
<i>Tetragonisca</i> sp.			▥					0.94	p	n	a
<i>Trigona</i> sp.*							▥	1.45	p	n	a
<i>Trigonisca</i> sp.*	▥							0.81	p	n	a
sp. 1							▣	1.44	p	n	a
sp. 2*							▥	1.13	p	n	a
<b>HALICTIDAE</b>											
sp.					▥			1.46	p	n	a
<b>MEGACHILIDAE</b>											
<i>Megachile</i> sp.*							▥	3.45	p	n	a

\* Single records in the whole study

**Table 3.** Model and averaged model explaining both oil-gathering visits and fruit set, respectively. Confidence intervals (CI) containing no zero are in bold, representing effect on the response variables. (S) – sepals removed; (P) – petaloid petals removed; (E) – elaiophores removed; (S + P) – sepals and petaloid petals removed; (S + P + E) – the three floral parts removed.

Effects	Response variable									
	Oil-visitation					Fruit set				
	Estimate	Std. Error	z-value	CI		Estimate	Std. Error	z-value	CI	
				2.5%	97.5%				2.5%	97.5%
Control (intercept)	-2.661	0.677	-3.930	-3.992	-1.330	-0.704	0.708	0.985	-2.103	0.696
(S)	0.197	0.345	0.571	-0.482	0.877	-0.132	0.689	0.191	-1.494	1.229
(P)	0.140	0.350	0.401	-0.547	0.828	0.037	0.668	0.055	-1.284	1.358
(E)	-1.270	0.544	-2.334	<b>-2.341</b>	<b>-0.201</b>	-3.209	1.072	2.967	<b>-5.328</b>	<b>-1.090</b>
(S+P)	-0.291	0.391	-0.743	-1.060	0.478	-4.285	1.422	2.986	<b>-7.098</b>	<b>-1.473</b>
(S+P+E)	-3.607	1.411	-2.556	<b>-6.381</b>	<b>-0.833</b>	-4.284	1.426	2.977	<b>-7.105</b>	<b>-1.463</b>
Population	-	-	-	-	-	0.347	0.682	0.506	-0.791	2.543

**Table 4.** Number of visits on treated flowers in the excision experiment and fruit set yielded. Data presented combining both populations as no divergence between them was detected. (S) – sepals removed; (P) – petaloid petals removed; (E) – elaiophores removed; (S + P) – sepals and petaloid petals removed; (S + P + E) – the three floral parts removed.

Treatment	Visitation		N flowers	Fruit set
	N visits	N oil-visits		
Control	16	15	25	12 (48)
(S)	19	18	22	9 (41)
(P)	19	17	26	11 (42)
(E)	18	4	25	1 (4)
(S+P)	11	11	23	0 (0)
(S+P+E)	0	0	20	0 (0)

**Table 5.** Number of visits per oil-collecting bee specie on the treated flowers in the excision experiment.

Oil-collecting bees	Total visits (Oil visits) per treatment					
	Control	(S)	(P)	(E)	(S+P)	(S+P+E)
Perfect matched						
<i>Centris byrsonimae</i>	5 (5)	5 (5)	6 (5)	9 (3)	4 (4)	0 (0)
<i>Centris xanthomelaena</i>	3 (3)	0 (0)	1 (1)	3 (1)	2 (2)	0 (0)
Poor matched						
<i>Caenonomada</i> aff. <i>unicarata</i>	8 (7)	14 (13)	12 (11)	6 (0)	5 (5)	0 (0)

were quicker, with no oil-gathering behavior (Table 5). For fruit set, both models (1) and (3) were equally plausible according to the model selection (Table 3). However, no different effect between populations was detected and the excision treatments were also the most plausible predictor affecting this variable. The treatment (E) yielded only one fruit within the whole experiment and for both (S+P) and (S+P+E) no fruit formation was observed. The treatments (S) and (P) had the same effect as control flowers (Table 4).

#### 4. Discussion

Geographical variation in pollinators across populations might generate differing selection and intraspecific divergence of floral phenotype (Thompson, 1997; 2005; Herrera et al., 2006; Gómez et al., 2009a; 2009b). This may be due to divergences in which pollinators respond to flower stimuli and match the floral architecture, differently affecting plant fitness. However, our results do not support the hypothesis that the functional significance of floral traits differs between levels of pollinator matching found in the pollinator-dependent *K. grandiflora*. Petaloid petals are predicted to be the keystone floral structure to the optimum matching in the *Krameria* system, because they provide the best fit to flowers. Accordingly, we expected that the excision of petaloid petals would weaken the interaction with *Centris* affecting oil gathering and fruit set; instead, these bees triggered the oil-gathering behavior like the poor matched *Caenonomada*. Therefore, both pollinators equally responded to our experiment, although *Caenonomada* proportionally visited more flowers with excised petaloid petals and sepals (Table 5).

Petaloid petals have different shape, size and tone relatively variable within the genus *Krameria* (Simpson, 1989). We provide the first study testing their function, but their utilization by *Centris* bees has been described for other *Krameria* species in which their oil-

gathering behavior is a standard (Simpson, 1989; Gimenes and Lobão, 2006; Carneiro et al., 2015). This group of reduced petaloid petals resembles in function the flag petal of the New World Malpighiaceae (Sazima and Sazima, 1989; Vogel, 1990). However, there is an evidence that the malpigh flag petal has functional significance on plant fitness as manipulated flowers (without this structure) yielded a low fruit set (Ferreira and Torezan-Silingardi, 2013). In *K. grandiflora*, plant fitness was not affected when the petaloids were excised. We could not assess whether there was a decrease in the oil-gathering efficiency by *Centris* when this structure was removed. If petaloid petals provide a better oil gathering for *Centris*, the evolution of these structures is likely to be related to this interaction. There is no evolutionary hypothesis on the evolution of Krameriaceae dimorphic corolla besides the intuitive selection mediated by *Centris*. In addition, we also could not assess whether there are differences in the responses regarding both *Centris* species separately within the perfect-matched population in the absence of petaloid petals. The small *Centris byrsonimae* visited more flowers with excised petaloid petals than the larger *C. xanthomelaena*; however, they provided not enough visits to our experiment to test for this difference (Table 5). If this were statistically asserted, the oil-gathering by larger *Centris* bees would be affected by petaloid petal absence, suggesting a tighter association in this interaction.

Like the petaloid petals, *Krameria* elaiophores are singular features of the genus with only slight differences among species (Simpson, 1982; 1989). Our results suggested that those glandular petals are keystones for the functioning of the system as they ensured plant fitness. The oil-collecting bees visited the manipulated flowers without elaiophores but most of the visits were quicker than a common oil-gathering visit, indicating that the bees perceived the elaiophore absence after landing. In a few visits, *Centris* species initiated the oil-gathering behavior in the absence of elaiophores. This might indicate that bees do not recognize this absence before landing on the flower. Something similar was found in the eglandular Malpighiaceae (*Banisteriopsis muricata* and *Heteropterys aceroides*) – *i.e.* naturally without floral oil glands –, that deceit oil bees which, soon after few scraping movements, ceased this behavior (Sazima and Sazima, 1989). Nevertheless, we have not accounted whether there was any manipulation effect of the elaiophore removal on fruit development. However, excisions of floral parts might not relate to failure in fruit set as tested by other manipulative studies (*e.g.* Kudoh and Whigham, 1998; Mojica, 2011)

Besides the mechanical-fit structure of *Krameria* flowers, there might be a strong visual signaling mainly provided by the showy calyx present in the genus. However, studies on the ultraviolet reflectance of *Krameria* flowers are lacking. In our experiment, when sepals were removed both *Centris* and *Caenonomada* bees landed and manipulated the flowers which yielded fruits. Simpson (1989) described a similar situation in which *Centris* visited *Krameria* flowers that had their sepals excised. Our results support the expectation that petaloid petals and elaiophores also function as visual cues in some extent as bees recognized the reward source when the showy calyx was excised. Nevertheless, showy floral parts are usually related to flower recognition by pollinators at long distances (Proctor et al., 1996). However, as the structure of our manipulative experiment did not account for the effect of the detectability from a distance, but at proximity because there were non-manipulated flowers in the sample units (display effect present), we could not detect whether the excision of the calyx would affect the initial recognition of reward source at long distance (Giurfa et al., 1996; Ibarra et al., 2002). Regarding the display effect and thus proximity, bees recognized the petaloid petals and elaiophores in the absence of sepals. Perhaps these structures are also related to scent emission which could reinforce flower recognition. Studies on *Krameria* scent are also lacking, but it is known that oil-collecting bees use diacetin (glycerol diacetate) as a reliable private-channel cue to detect oil-producing flowers (Schäffler et al., 2015). Finally, no fruit formation in manipulated flowers, both sepals and petaloid petals lacked (but elaiophores present), suggest a negative effect in pollinator fitting as bees reached and landed the flowers, combined with the low elaiophore recognition by them.

Here we observed divergences in pollinator assemblage and matching across *K. grandiflora* distribution even being a specialized pollination system as evidenced by other studies concerned on the multipopulation context of specialized pollination systems (e.g. Thompson, 1997; Herrera et al., 2006; Nattero et al., 2010; Ferreiro et al., 2015). The genus *Centris* was the most associated to *K. grandiflora* flowers as expected due to several records of *Centris-Krameria* across Neotropical environments (Simpson, 1989; Gimenes and Lobão, 2006; Carneiro et al., 2015). Among the associations with *Centris*, there is a special relationship between Krameriaceae and the subgenus *Paracentris* and *C. hyptidis* (Simpson, 1989; Carneiro et al., 2015; Sabino et al., 2017), generally related to oil-producing species with trichome elaiophores (Vogel and Machado, 1991; Machado et al., 2002; Martins et al., 2013; 2015). These bees have an inconspicuous oil-collecting apparatus thought to be not

appropriated to scrape epithelial elaiophores and restricted to forelegs (Vogel and Machado, 1991; Martins et al., 2013; 2015). However, these oil-collecting bees scrape *K. grandiflora* elaiophores to collect oil using their fore and midlegs. In this study, we recorded novel associations with two more *Paracentris* species (*C. thelyopsis* and *C. xanthomelaena*) and another from the “hyptidis” group (*C. hyptidoides*). These bees do not collect oil on Malpighiaceae epithelial elaiophores and ecological-evolutionary hypothesis regarding their sharing between Krameriaceae and Plantaginaceae are open to investigation.

The interactions between *K. grandiflora* flowers and Tapinotaspidini and *Centris* were simultaneously recorded in most of populations. Corumbá population was an exception as the association with *Caenonomada* was practically exclusive (Table S2). There are some studies that present the floral oil sources of Tapinotaspidini species (Vogel and Machado, 1991; Cocucci and Vogel, 2000; Rasmussen and Olesen, 2000; Machado et al., 2002; Cunha and Blochtein, 2003; Aguiar and Melo, 2009; Carneiro et al., 2015) and a few of them has considered these bees as illegitimate flower visitors or floral oil robbers, generally promoting mismatching and cheating due to their small size and behavior (Vogel and Machado, 1991; Martins et al., 2003). All Tapinotaspidini recorded in our study provided some level of cheating, mainly the small ones *Arhysoceble huberi* e *Xanthopedia globulosa* which collected oil without touching the reproductive organs. On the other hand, *Ca. aff. unicalcarata* might be a potential pollinator, although promoting a poor matching as it does not use the petaloid petals and eventually touched the anthers and stigma.

The present study illustrates that by testing the functional significance of floral traits, we can investigate their role in plant fitness and in pollinator behavior. Nevertheless, no divergence in the functional significance was found regarding two bee pollinators of *K. grandiflora* flowers experiencing different levels of matching which could mean that they act equally as selective forces. However, it would be particularly interesting to quantify selection mediated by these oil-collecting bees and assess whether it diverges in form and strength in varying pollinator scenarios. The interactions between *Krameria* and oil-collecting bees has been little explored since its description by Vogel (1974), and the following works (Simpson et al., 1977; Simpson, 1982; 1989), and therefore, further studies concerning this mutualism are promising for contributing with the understanding of oil system evolution in the Neotropical region.

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## Supplementary material

### Appendix S1

To carefully perform the pollination experiment we firstly estimated the period flowers remain available to pollinators and how much time they keep in the inflorescence when not previously visited, *i.e.* to know when considering fruit formation. For this, we bagged 29 floral buds (1-2 flowers in 25 plants) and followed them until dropping. All flowers started opened between 06h00 and 06h30 am and closed in the late afternoon in the first day of anthesis. Out of 29 bagged flowers, 41.4% dropped in 72h after opening; 51.7%, after 96h; and 6.9%, after 120h.

### Appendix S2

Flower visitors were recorded in seven wild populations across *K. grandiflora* geographical range. Our surveys were carried out in 2014 at COR and TER (see Figure 2A for population locations and names); in 2015 at COR, SNN and XIQ ; and in 2016 at CNH, COR, NOV, PIR, SNN and XIQ. Visitation rate samplings were performed in COR (2014 and 2015), TER, SNN (2015) and XIQ (2015). The other surveys consisted in capturing flower visitors directly on the flowers within each population because insects were not abundant enough to conduct a visitation sampling. As the locations belong to semi-arid environments, very low precipitations are common over the years, which can affect pollinator densities and foraging.

**Table S1.** Model selection for the pollination experiment and the effect of the excision experiment and populations (pollinator matching level) on oil-gathering visits and fruit set. Generalized linear mixed models (*glmer*) with binomial (fruit set) distribution were performed for the pollination experiment and generalized linear mixed models with Bayesian inference (*bglmer*) with Poisson (oil-gathering visits) and binomial (fruit set) distributions. AICc: corrected Akaike’s Information Criterion;  $\Delta$ AIC: Difference between one model and the best AIC; Weight: strength of evidence for each model.

Models	AICc	$\Delta$ AIC	Weight
Pollination experiment			
<b>Null</b>	<b>92.9</b>	<b>0.00</b>	<b>0.620</b>
<b>(1) Treatment</b>	<b>93.9</b>	<b>1.00</b>	<b>0.38</b>
Manipulative experiment			
Oil-visitation			
<b>(1) Treatment</b>	<b>372.0</b>	<b>0.00</b>	<b>0.660</b>
(3) Treatment + Population	374.1	2.12	0.229
(4) Treatment * Population	375.6	3.57	0.111
Null	396.7	24.70	0.000
(2) Population	398.7	26.64	0.000
Fruit set			
<b>(1) Treatment</b>	<b>118.5</b>	<b>0.00</b>	<b>0.574</b>
<b>(3) Treatment + Population</b>	<b>119.4</b>	<b>0.84</b>	<b>0.377</b>
(4) Treatment * Population	123.5	4.95	0.048
Null	156.3	37.77	0.000
(2) Population	157.4	38.92	0.000

**Table S2.** Bees recorded and their frequency (in parenthesis) per population/per year in seven *K. grandiflora* populations in 2014-2016 flowerings. MF: mean frequency.

Flower visitors	Populations													
	PIR		SNN		NOV		TER		XIQ		CNH		COR	
	2016	2015	2016	(MF)	2016	2015	2015	2016	(MF)	2016	2014	2015	2016	(MF)
<i>Centris aenea</i>	2 (0.33)	6 (0.15)		0.07			1 (0.12)	1 (0.08)	0.10	1 (0.07)				
<i>Centris caxienseis</i>		7 (0.17)		0.09										
<i>Centris byrsonimae</i>							2 (0.25)	8 (0.67)	0.46					
<i>Centris tarsata</i>	3 (0.5)	18 (0.45)	2 (0.14)	0.3	1 (0.06)	2 (0.07)					8 (0.11)			0.04
<i>Centris hyptidis</i>		5 (0.12)		0.06	5 (0.29)	9 (0.30)								
<i>Centris hyptidoidea</i>													2 (0.18)	0.06
<i>Centris burgdorfi</i>		1 (0.02)		0.01										
<i>Centris thelyopsis</i>					1 (0.06)									
<i>Centris xanthomelaena</i>		1 (0.02)		0.01			5 (0.62)	3 (0.25)	0.44	8 (0.57)				
<i>Centris perforator</i>		1 (0.02)		0.01										
<i>Arhysoceble huberi</i>			1 (0.07)	0.04	4 (0.23)	5 (0.17)					18 (0.24)			0.08
<i>Caenomada unicalcarata</i>		1 (0.02)	11 (0.79)	0.4	1 (0.06)	12 (0.40)					47 (0.65)	48 (1.00)	8 (0.73)	0.79
<i>Tapinotaspoides serraticornis</i>					3 (0.18)									
<i>Xanthopedia globulosa</i>														
Halictidae sp.						2 (0.07)								
<i>Tetragonisca</i> sp.					2 (0.12)									
<i>Trigona</i> sp.										1 (0.07)				
<i>Trigonisca</i> sp.	1 (0.17)													
sp.1										3 (0.21)				
sp.2										1 (0.07)				
<i>Megachile</i> sp.													1 (0.09)	0.03

**Geographic variation in oil-collecting bee assemblages  
influences trait correlation in oil-producing flowers**

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**Abstract:** Flowers are valuable systems for studying the phenotypic correlation structure of multiple traits as they represent a complex modular organ subjected to selective pressures provided by their pollinators. In this context, we aimed to evaluate whether oil-collecting bees influence the phenotypic integration and correlational structure of the *Krameria grandiflora* oil flowers. We obtained individual measurements of 13 floral traits comprising the specialized floral whorls: calyx and corolla. We compared the magnitude and pattern of phenotypic integration in a multipopulation approach. We sampled the oil-collecting bees on *K. grandiflora* flowers in six populations and created a similarity matrix of pollinator functional diversity using abundance and bee size. Relationships between the correlation patterns and pollinator functional diversity and composition, geographic distance and similarity in abiotic variables were determined. We found no significant association between correlation pattern and geographic distance and abiotic factors. However, we obtained a significant relationship between the patterns of phenotypic correlations and pollinator functional diversity and composition. These are the first evidences that differences in pollinator functional diversity within a specialized pollination system might affect the phenotypic correlation in flowers.

**Keywords:** floral integration / intraspecific variation / pollination / *Centris* / *Krameria*.

**Resumo:** As flores são sistemas valiosos para o estudo da estrutura de correlação fenotípica de caracteres múltiplos, pois representam um órgão complexo modular sujeito a pressões seletivas provenientes da interação com seus polinizadores. Nesse contexto, nós objetivamos avaliar se abelhas coletoras de óleo influenciam a integração fenotípica e a estrutura correlacional das flores de óleo de *Krameria grandiflora*. Nós obtivemos medidas individuais de 13 caracteres florais abrangendo dois verticilos florais especializados: o cálice a corola. Nós comparamos a magnitude e o padrão de integração fenotípica em uma abordagem multipopulacional. Nós amostramos as abelhas coletoras de óleo nas flores de *K. grandiflora* em seis populações e criamos uma matriz de similaridade de diversidade funcional de polinizador, usando abundância e o tamanho da abelha. Relações entre os padrões de correlação e diversidade funcional e composição de polinizadores, distância geográfica e similaridade em variáveis abióticas foram determinadas. Não encontramos associação entre o padrão de correlação e distância geográfica e fatores abióticos. No entanto, nós obtivemos uma relação entre os padrões de correlação fenotípica e a diversidade funcional e composição de polinizadores. Esta é a primeira evidência em que diferenças em diversidade funcional de polinizadores em um sistema de polinização especializado parece afetar a correlação fenotípica em flores.

**Palavras-chave:** integração floral / variação intraespecífica / polinização / *Centris* / *Krameria*.

## 1. Introduction

Species provide different adaptive responses to geographic variations in interacting partners and habitat (Gould and Johnston, 1972; Thompson, 1999; 2005; Eckert et al., 2008). This adaptive divergence may express changes in the phenotype or in the association of particular traits – *i.e.* phenotypic integration (Pigliucci, 2003) – which play a functional role in a given interaction (Armbruster and Schwaegerle, 1996; Conner, 1997; Armbruster et al., 2004; Pérez-Barrales et al., 2007; González et al., 2015). Studies on the integration of traits in a geographic mosaic context can provide insights on the processes controlling among-population divergences associated to past evolution (Wagner and Schwenk, 2000; Pigliucci, 2003). Trait covariation patterns relates to adaptation and constraints over evolutionary timescales . Therefore, trait covariation might reflect the phenotypic and genetic responses to natural selection, and the limits to such responses imposed by intrinsic constraints (Schlichting and Pigliucci, 1998; Pigliucci, 2003; Armbruster et al., 2004).

Flowers are highly organized modules (Armbruster et al., 2004) composed by interrelated whorls which interact with a variety of antagonist and mutualist animals across environments (Gómez et al., 2009). Many studies have suggested that pollinators exert correlational selection on floral arrangement (Conner and Via, 1993; Armbruster et al., 2004; Anderson and Busch, 2006; Bissell and Diggle, 2010) and this makes ideal study systems for the evolutionary inference on patterns of changing trait covariation within species range (Armbruster and Schwaegerle, 1996; Herrera, 2001; Murren, 2002). By the way, the earlier hypotheses on the patterns of phenotypic covariation reflecting responses to natural selection were set up within the pollination context (Berg, 1960). Berg's hypothesis considers the influence of the intimacy degree between plants and pollinators on trait covariation in flowers. Thus, selection on phenotypic integration within flowers would be stronger for specialist-pollinated than for generalist-pollinated species, due to the narrow selective pressures imposed by specialists. As plant species can experience different degrees of generalization and specialization across its geographical range (Dilley et al., 2000; Dupont and Skov, 2004; Ollerton et al., 2007), phenotypic integration should vary among populations (Herrera et al., 2002). Besides, trait relationships within floral whorls or intra-modules that play different functions in the flowers can also reflect responses to natural selection, although variation within these modules can be limited by developmental and genetic control (Armbruster et al., 1999; Conner, 2002; Ordano et al., 2008). Floral modules involved in pollen transfer and deposition (mechanical-fit traits) should be more integrated than floral traits related to the attraction of pollinators (Berg, 1960). For pollen transfer, traits are required to be more precise regarding the

position that pollen attaches on the body of the pollinator. Thus, selection should favor the correlation between floral traits involved in pollen transfer that display a narrower adaptive peak (Pérez-Barrales et al., 2014; Ordano et al., 2008). Moreover, this divergence in the magnitude of integration between intra-modules involved in pollinator attraction and pollen transfer should be higher in a specialized scenario.

Studies concerning on the variation in phenotypic integration of a single plant species throughout its range are scarce. Multipopulation-level approach on the pattern of phenotypic integration is also interesting to disentangle the relative contributions of genetic constraints and adaptation as the drivers of correlation and covariation among traits (Armbruster and Schwaegerle, 1996; Herrera et al., 2002; Armbruster et al., 2004). Although genetic drift and phenotypic plasticity can promote variation in floral correlations among populations (Schlichting, 1989; Waitt and Levin, 1993; Armbruster and Schwaegerle, 1996; Herrera et al., 2002; Maubecin et al., 2016), the evaluation of the association between the correlation patterns and the varying pollinator assemblages can provide a strong indication that natural selection might be a source of selective force affecting integration (Pérez-Barrales et al., 2014; González et al., 2015; Lázaro and Santamaría, 2016).

In this study, we evaluated the variation in floral phenotypic integration across the range of the oil-producing *Krameria grandiflora* A. St.-Hil. (Krameriaceae). This system can be a potential model to investigate the effects of variation in pollinator assemblage on floral phenotypic integration for two reasons. First, the species occurrence is scattered and spotty (Simpson, 1989), comprising to some extent the assumption that study populations should be independent. Second, the pollinator assemblages of *K. grandiflora* are geographically variable in morphology and composition even being taxonomically restricted (Chapter 2). The genus *Krameria* is specialized on floral oil offering for oil-collecting bees and comprises 18 species with featured modified floral whorls, extremely divergent from its sister group, Zygophyllaceae (Sheahan and Chase 1996; Simpson et al. 2004; see also Carlquist 2005). Flowers bear a showy calyx and a corolla with two distinct morphological units (Milby 1971; Simpson 1982; 1989; Carneiro et al. 2015; Figure 1A). The corolla is formed by a group of reduced petaloid petals with colored fan-shaped blades at their tip and two lateral fleshy petals (elaiophores hereafter) which bear a glandular outer surface that secrete fatty acids (Simpson et al., 1977; 1979). Studies on *Krameria* floral anatomy showed that petaloid petals are more structurally connected to the stamens than to elaiophores, indicating that both corolla parts might be morphologically decoupled (Milby, 1971; Dhillon, 1976). Pollination success depend on the oil-gathering behavior which consists in scraping

the elaiophore outer surface with fore and mid legs while bees hold the basis of the petaloid petals with their mandibles (Figure 1B-D; Simpson 1989; Carneiro et al. 2015).

Based on the phenotypic integration context and the system described above, we hypothesized that variation in the assemblage of oil-collecting bees influences the divergence in the pattern of phenotypic correlation of floral traits along *K. grandiflora* distribution range. We predicted that a low local variation in pollinator morphology negatively relates to the magnitude of floral integration, supporting Berg (1960) hypothesis. We then tested if the correlation patterns are influenced by abiotic factors and the geographic distances among populations. We also evaluated the presence of floral modules and tested whether functional integration fits the pattern of association among floral traits.

## **2. Materials and methods**

### **2.1 Study species**

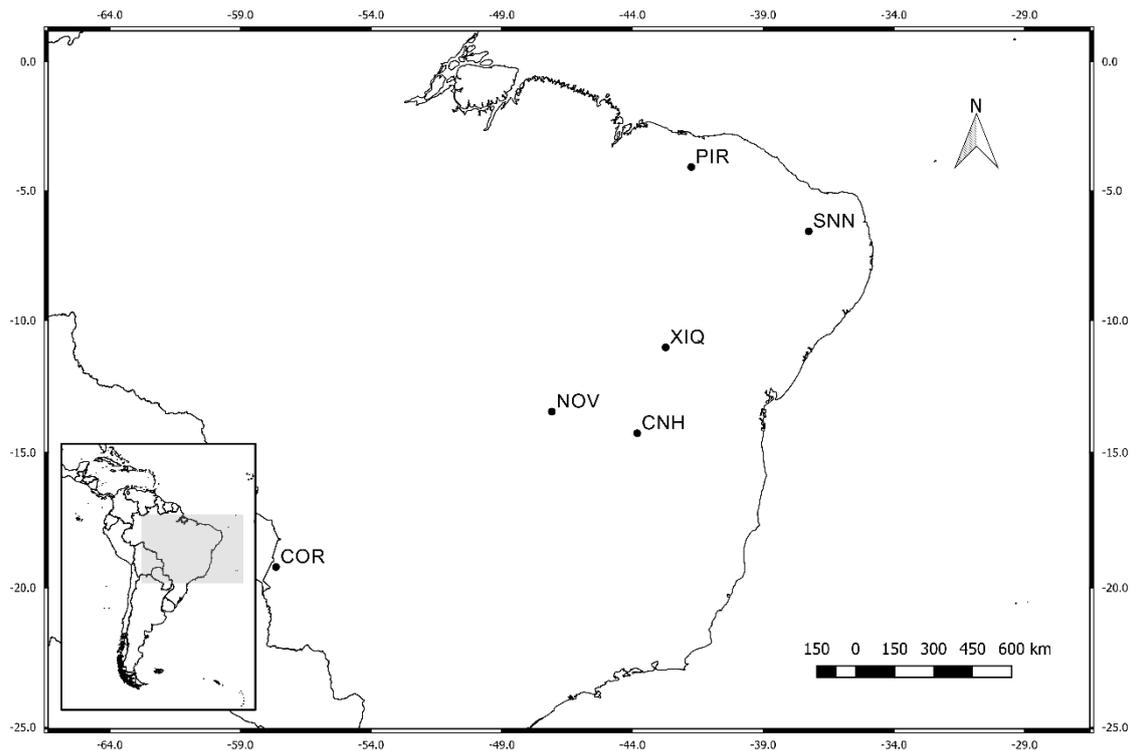
*Krameria grandiflora* is a prostrate (to procumbent) perennial herb that occurs in isolated populations distributed in open habitats throughout South American “dry diagonal” (Simpson 1989; see Chapter 2). Flowering period occurs mostly from January to April. Its hermaphrodite flowers last only one day opened and insect pollination is required for fruit set, even being self-compatible (see Chapter 2). As the entire eastern South American *Krameria* subclade (Simpson et al., 2004), *K. grandiflora* flowers have four showy sepals (upper, lower and two lateral ones; even though we observed on rare occasions individual flowers with a fifth sepal) and three free petaloid petals (Figure 1A). Throughout most part of its distribution, *K. grandiflora* is highly associated to oil-collecting bees from the genus *Centris* as other Krameriaceae species (Carneiro et al., 2015; Gimenes and Lobão, 2006; Simpson, 1989). However, *Caenonomada* (Tapinotaspidini), also an oil-collecting bee, is its main pollinator in the Southwestern portion of the distribution (Chapter 2). Variation in *K. grandiflora* pollinator assemblage also comprises differences in size among *Centris* species, which might reflect on divergent selection among populations.

### **2.2 Study sites and floral phenotypic variables**

Flowers were collected from 27 to 42 individual plants of six populations ( $n = 235$  plants; all populations combined) and preserved in FAA (formaldehyde-acetic acid-ethyl alcohol) solution. Selected plants were more than three meters apart from each other to avoid



**Figure 1.** *Krameria grandiflora* flower and its floral parts. **A.** Calyx: Lt, lateral sepal; Lw, lower sepal; Up, upper sepal; Corolla: bPt, petal laminae; sPt, petal stalk; Ela, fleshy petal (elaiophore); gs, glandular surface; Pistil: ov, ovary; st, stigma; Androecium: ls, long stamen; ss, short stamen; Operative distances: st-Pt, distance between the stigma and petaloid petal base; st-Ela, distance between the stigma and the distal border of the elaiophore. *Centris (Hemisiella) tarsata* (**B**), *C. (C.) caxiensis* (**C**) and *C. (Paracentris) xanthomelaena* (**D**) holding the three petaloid petals during oil gathering.



**Figure 2.** *Krameria grandiflora* populations included in this study. PIR, Parque Nacional das Sete Cidades, Piripiri-PI (n = 40); SNN, Estação Ecológica do Seridó, Serra Negra do Norte-RN (n = 27); XIQ, APA Lagoa de Itaparica, Xique-Xique-BA (n = 42); NOV, Nova Roma-GO (n = 42); CNH, APA Rio Carinhanha, Carinhanha-BA (n = 42); COR, Parque Municipal Piraputangas, Corumbá-MS (n = 42).

sampling clones or close relatives. We sampled the six populations in preserved habitats during a single flowering season (Feb-Apr/2016) to avoid the influence of climate change among years. As variation in floral traits among individuals and populations has been demonstrated to explain higher proportion of variance than within individuals in this species (Carneiro, L.T., unpublished data), we used the mean values of three flowers per each individual plant for 13 floral traits measured by LTC (Figure 1A). Floral traits represented characters related to the showy calyx and the dimorphic corolla. From calyx, we obtained the length and width of the upper sepal (UpL and UpW), lower sepal (LwL and LwW) and one lateral sepal (LtL and LtW). From corolla, we measured the height of the stalk of petaloid petals (sPtL), until the limit below the blade and regarding the middle one; the length and width of the blade of one lateral petaloid petal (bPtL and bPtW); and the length and width of

one elaiophore (ElaL and ElaW). We used measurements of a single elaiophore, lateral sepal and lateral petaloid petal instead of both units (right and left) as they are highly correlated (see Figure S1). In addition, we measured two operative distances: distances between the stigma and the base of petaloid blade (st-Pt) and between the stigma and the distal border of the elaiophore (st-Ela). The first relates to the point where oil-collecting bees hold the flowers and the other the maximum limit the bee legs must reach the elaiophore to oil gathering. Both distances might indicate the point the pollen is attached on pollinator thorax. We used scaled photographs and ImageJ 1.48v software (<https://imagej.nih.gov/ij/>) to obtain trait measurements (in millimeters).

### **2.3 Phenotypic integration and intraspecific comparisons**

To evaluate the association among floral traits we calculated the magnitude and pattern of phenotypic integration of *K. grandiflora* flowers for each population (Herrera et al., 2002; Pérez-Barrales et al., 2014). We determined Pearson's correlation coefficients between pairs of trait mean values and extracted the eigenvalues of the phenotypic correlation matrix created. The variance of the eigenvalues gives the integration index (INT), a measure of the magnitude of phenotypic integration (Wagner, 1984; Chevrud et al., 1989; Herrera et al., 2002; Baranzelli et al., 2014; Pérez-Barrales et al., 2014). The eigenvalue variance increases as the overall level of correlations increases (Cheverud et al., 1989). As we aimed to compare integration among populations and their sample size varied, we corrected the INT index so that observed differences in overall correlation were independent of sample size differences. For that, we subtracted the expected value of integration under the assumption of random covariation of traits ( $\text{Exp(INT)} = (\text{number of traits} - 1) / \text{number of samples}$  (*i.e.* number of individual plants) from the observed one and divided it by the potential maximum value of phenotypic integration in the given data set, which is equal to the number of traits. We multiplied the result by 100 to calculate the percentage of the maximum value possible. The corrected INT index ranges from 0% to 100%, representing a gradient of trait dependence, so that traits independently vary at zero. To compare the INT index among populations, we used a random resampling approach to obtain confidence intervals for it (Cheverud et al., 1989). We applied bootstrap procedure with 1000 permutations with replacement to detect significant differences. As pattern of integration, we calculated a matrix of similarity in correlation structure among populations using the correlation coefficients between population pairs. We calculated the significance of population divergence in correlation pattern

performing Mantel test with 1000 permutations. We performed the integration estimations, bootstrap and the correlation patterns comparisons using R software v3.3.1.

## 2.4 Intrafloral modularity

We used Dow and Cheverud (1985) approach for testing three *a priori* hypothesis of modularity (developmental, functional and morphological).and evaluating which best fits to our empirical phenotypic correlation matrix in each population. The developmental hypothesis (**T<sub>1</sub>**) regards that the floral traits derived from the same floral whorls (calyx or corolla) covary as integrated modules caused by developmental constraints. Thus, no effect of functional integration from pollinator-mediated correlational selection is included in this alternative, working as a null model. For the functional hypothesis (**T<sub>2</sub>**) we regarded the functional role of the *Krameria* specialized traits, related to oil-gathering behavior and pollination mechanism. Floral traits would be suites of an attraction module (sepals and petaloid petal blade) or mechanical-fit module (elaiophore, petaloid petal stalk and operative distances). We also included a third hypothetical alternative (morphological hypothesis, **T<sub>3</sub>**) that regards corolla dissociation into two morphological parts (petaloid petals and elaiophores), *i.e.* it follows a morphological criterion distinguishing three units: calyx, petaloid petals and elaiophores. We filled those hypothetical matrices with values of 1 for within module correlations and 0 for between modules (Cheverud et al., 1989). As operative distances have the influence of an unmeasured trait (pistil length), we used 0.5 for matrix elements when including them *within* module and *between* modules only when pistil length association was plausible. We compared the matrices with a matrix correlation and performed Mantel test for significance using *vegan* package in R software v3.3.1. To reduce the chances of obtaining type I errors, we applied the Bonferroni correction to the statistical significance dividing it by the number of hypothesis.

We used conditional independence graphs (Magwene, 2001) as a second modularity inference in addition to Cheverud et al. (1989) *a priori* approach to look for patterns of correlation strength for possible modules. Conditional independence graphs derive from partial inverse correlation matrices, which represent direct effects of correlated traits. We calculated the edge exclusion deviance using partial correlations coefficients between variables to test if close-to-zero partial correlations could be statistically removed from the independence graphs. Edges with deviances < 3.84 (corresponding with the 0.05 level in a  $\chi^2$

distribution with d.f.=1) were excluded. Then we estimated the edge strength and produced independence networks using the software Cytoscape v3.4.0.

## **2.5 Pollinators, geographic distances and abiotic variables**

We estimated whether populations that share similar pollinator functional diversity and composition are more similar on phenotypic correlation patterns. We recorded the pollinators associated to *K. grandiflora* flowers in the six populations along surveys carried out in 2014 (COR and NOV), 2015 (COR, SNN and XIQ) and 2016 (all populations). Pollinators were recorded between 8:00h and 13:00h (20 min per each half an hour) in two days per population each year. From the specimens collected, we obtained the mean intertegular distance per species, which represents a reliable measurement for dimension of bee body (Cane, 1987), and we also calculated the coefficient of variation ( $CV_{ID}$ ) of this variable for each population. While calculating the mean intertegular distance we regarded the population differences of pollinators. Thus, we created a proportional similarity matrix regarding the mean intertegular length per each species per populations weighted by their relative frequency (based on Herrera et al. 2002). We also created a similarity matrix for pollinator composition. All bees collected were identified and deposited in the entomological collection CEPANN/IB/USP, São Paulo, Brazil. We also tested whether geographic distances between populations and abiotic factors influence the phenotypic co-variation patterns in *K. grandiflora* flowers. We obtained the geographic distances between the six populations by plotting population coordinates in Google Earth software v7.1.7.2606 and drawing a straight line between them using the *ruler* tool. We used annual mean precipitation, annual mean temperature and local elevation as abiotic variables because we assumed that flowering events and floral traits are susceptible to them in semi-arid environments. We obtained the climatic variables from the INMET database (<http://www.inmet.gov.br/portal/>) compiled from stations close to the populations and regarding the 1995-2015 period. We correlated the abiotic similarity and the geographic distance matrices to the matrix of phenotypic correlations among populations and assessed their significance using Mantel test.

## **3. Results**

### **3.1 Intraspecific trait correlations, phenotypic integration and modularity**

The magnitude of phenotypic integration in the populations ranged from 7.94 (in NOV) to 20.66 (in CNH) and no differences between them were detected regarding the

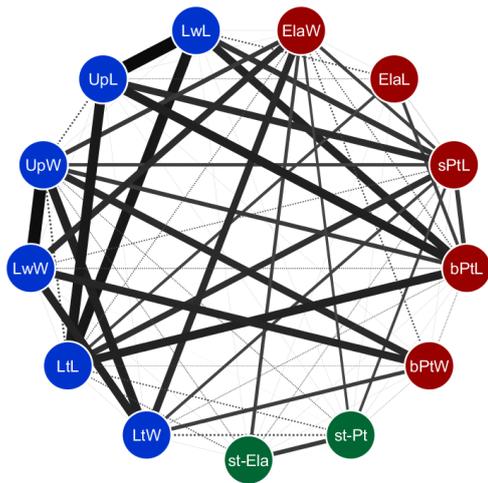
overlap in their confidence intervals (Table 1). The pattern of phenotypic integration represented by the phenotypic correlation structure differed between population pairs (all pairwise comparisons with statistical significance  $p < 0.01$ ) and matrix correlation coefficients ranged from 0.38 to 0.67 (Table 2). We show trait means and coefficients of variation (CV) in Table S1 and the correlation matrices of each population in Table S2.

In the modularity approach, most of the empirical phenotypic matrices significantly correlated to the morphological hypothesis ( $\mathbf{T}_3$ ; Table 3) suggesting that *K. grandiflora* flowers are formed to three modules morphologically defined by corolla, petaloid petals and elaiophores. One of them was also associated to  $\mathbf{T}_1$  and other also correlated to  $\mathbf{T}_2$  (Table 3). Conditional independence graphs for the six populations are shown in Figure 3 and present a gradient of significant edge strength of direct correlation between pair of traits. The percentage of significant edges ranged from 14.1% to 24.4%, out of 78 potential edges. These conditional independence graphs revealed a general pattern of stronger edges regarding pairs of calyx traits, also observed in the correlation networks (thicker and darker edges; Figure 3). Associations among corolla traits looked variable; however, the few significant edges between them did not represent an integrated unit compared to the calyx. This fits what the INT index for only corolla traits indicate (Table 1).

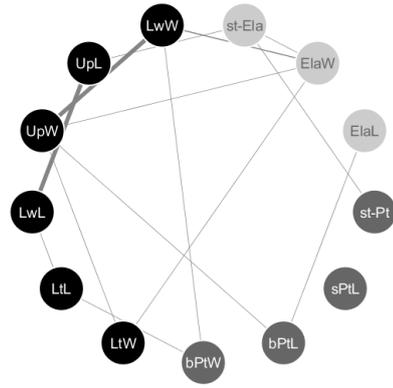
### 3.3 Pollinator assemblages and functional diversity

We recorded 13 oil-collecting species on *K. grandiflora* flowers in the whole study. Ten species belongs to the tribe Centridini that includes the largest one (*Centris burgdorfi*) and three Tapinotaspidini species, represented by small bees within the assemblages, like the smallest *Arhysoceble huberi* (Table 4). As all species contacted anthers and stigma at some extent (Chapter 2), we assumed that all species listed are potential pollinators.

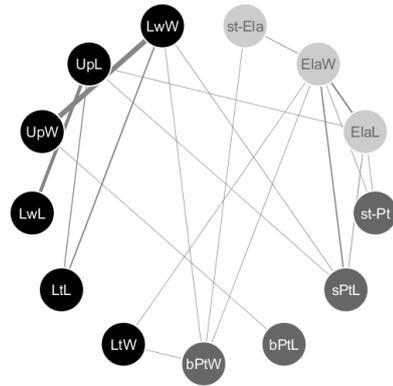
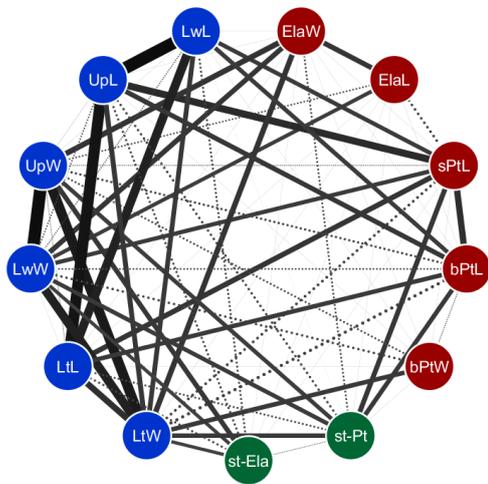
Pollinator assemblages diverged between populations, with species richness ranging from two in PIR and CNH to nine in SNN. The lowest variation in bee size was obtained for CNH ( $CV_{ID} = 7.8$ ) and the highest for COR ( $CV_{ID} = 35.8$ ) (Table 1). The most widespread oil collecting bees recorded were *C. tarsata* and *C. aenea*, shared by four populations. *Centris burgdorfi*, *C. hyptidoides* and *C. thelyopsis* were the rarest ones in the whole study (Table 4).



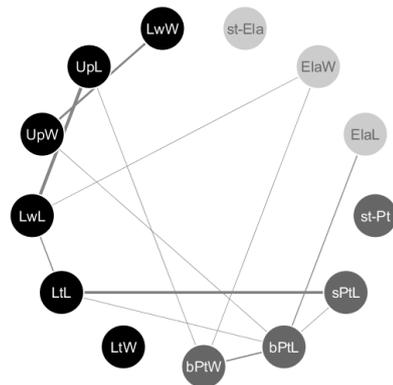
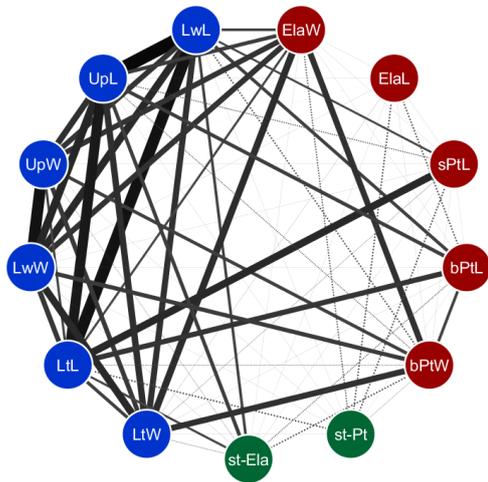
PIR

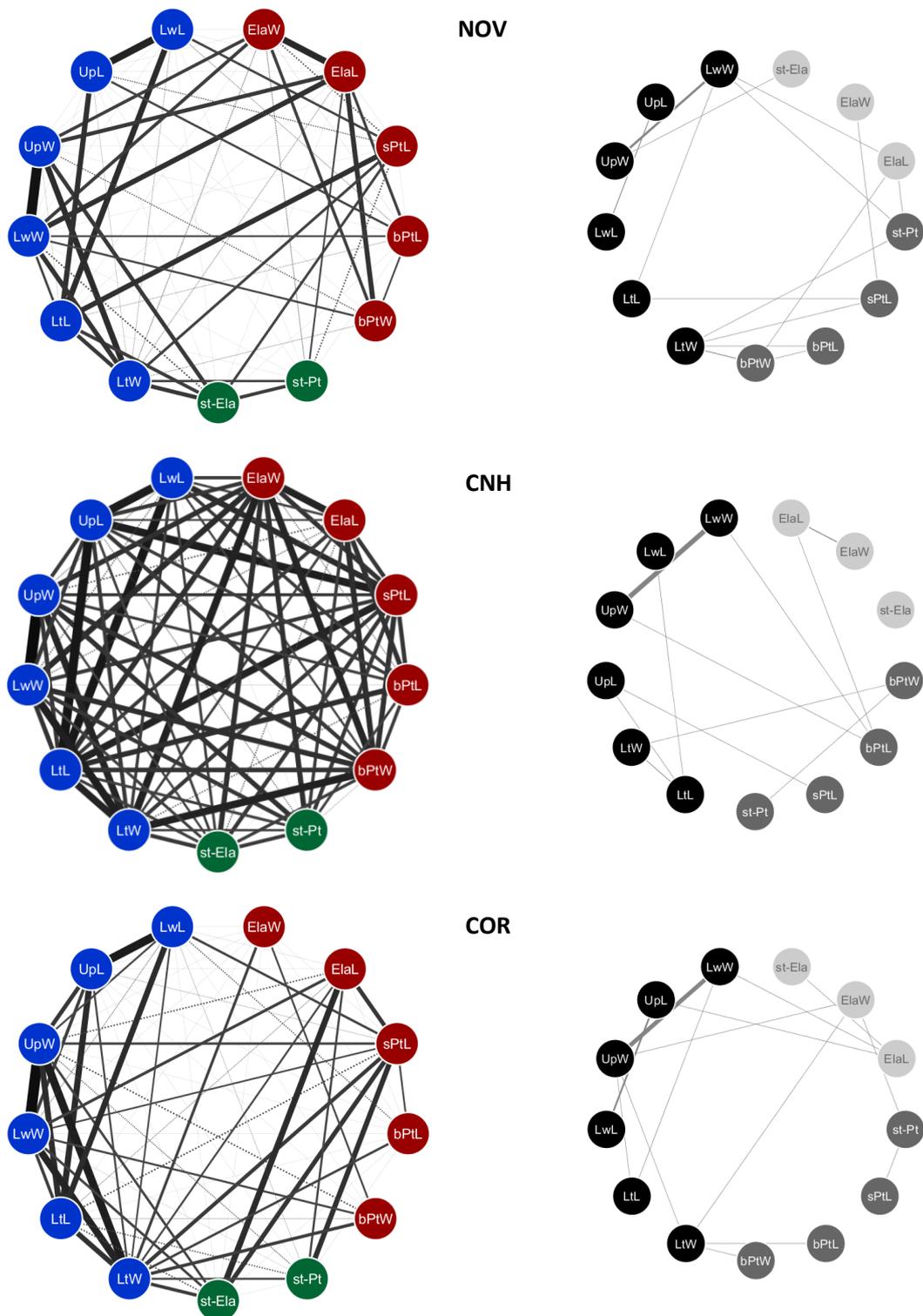


SNN



XIQ





**Figure 3.** Phenotypic correlation (*colored/left*) and conditional independence networks (*in gray scale/right*) networks for the six *Krameria grandiflora* populations. *Left:* calyx traits in blue; corolla traits in red; and operative distances in green. Thicker and darker edges represent higher  $r$ -values. Solid edges indicate significant correlation coefficients ( $p < 0.05$ ) and dotted edges indicate non-significant correlations. *Right:* floral traits grouped into calyx, corolla and petaloid petals. Only significant edges are plotted and edge width represent strength values. For trait names, see Figure 1.

**Table 1.** Intraspecific variation in the magnitude of phenotypic integration (corrected INT index), abiotic variables, coefficient of variation of pollinator intertegular distance ( $CV_{ID}$ ) and pollinator richness (PRich) of *K. grandiflora* populations. CI: confidence intervals; Elev: elevation; Precip: annual mean precipitation; Temp: annual mean temperature.

Population	Magnitude - INT (%)				Abiotic variables			Pollinator	
	INT	CI (95%)	INT (calyx)	INT (corolla <sup>*</sup> )	Elev (m)	Precip (mm)	Temp (C°)	$CV_{ID}$	PRich
PIR	12.02	8.91-22.34	23.70	5.86	156	1385	27.5	14.59	2
SNN	11.24	8.18-21.57	28.36	4.98	216	654.7	28.4	31.16	9
XIQ	11.85	8.38-20.88	36.02	4.56	406	608.4	26.7	12.61	3
NOV	7.94	5.75-16.51	16.87	10.15	391	1371	24.4	29.08	6
CNH	20.67	13.09-30.97	32.20	23.77	452	795.2	25.6	7.81	2
COR	9.30	6.77-18.72	27.10	2.95	167	996.7	25.9	35.85	4

<sup>\*</sup>Operative distances not included.

**Table 2.** Intraspecific correlation matrix, pollinator functional diversity matrix, geographical distances and similarity matrix of abiotic variables regarding six *K. grandiflora* populations.

<i>Lower: similarity matrix of correlation among populations (<math>p &lt; 0.01</math>), Upper: pollinator functional diversity (proportional similarity matrix based on Bray Curtis).</i>						
	PIR	SNN	XIQ	NOV	CNH	COR
PIR		0.42	0.13	0.08	0.13	0.04
SNN	0.62		0.11	0.40	0.11	0.41
XIQ	0.46	0.56		0.00	0.58	0.00
NOV	0.45	0.59	0.41		0.00	0.39
CNH	0.67	0.57	0.58	0.55		0.00
COR	0.39	0.58	0.45	0.47	0.38	

<i>Lower: geographical distances, Upper: Euclidian similarity matrix of abiotic variables.</i>						
	PIR	SNN	XIQ	NOV	CNH	COR
PIR		732.70	815.81	235.44	659.87	388.45
SNN	568.67		195.57	737.32	274.66	345.45
XIQ	775.11	778.06		762.71	192.39	455.93
NOV	1190.75	1318.91	544.65		578.98	436.20
CNH	1149.46	1114.20	378.23	364.43		349.01
COR	2406.39	2613.70	1840.84	1295.89	1571.75	

**Table 3.** Correlations between empirical phenotypic matrices and three hypothetical ones of intrafloral modularity for each population ( $p < 0.016$  in bold – significance regarding Bonferroni correction). T<sub>1</sub>: developmental; T<sub>2</sub>: functional; and T<sub>3</sub>: morphological.

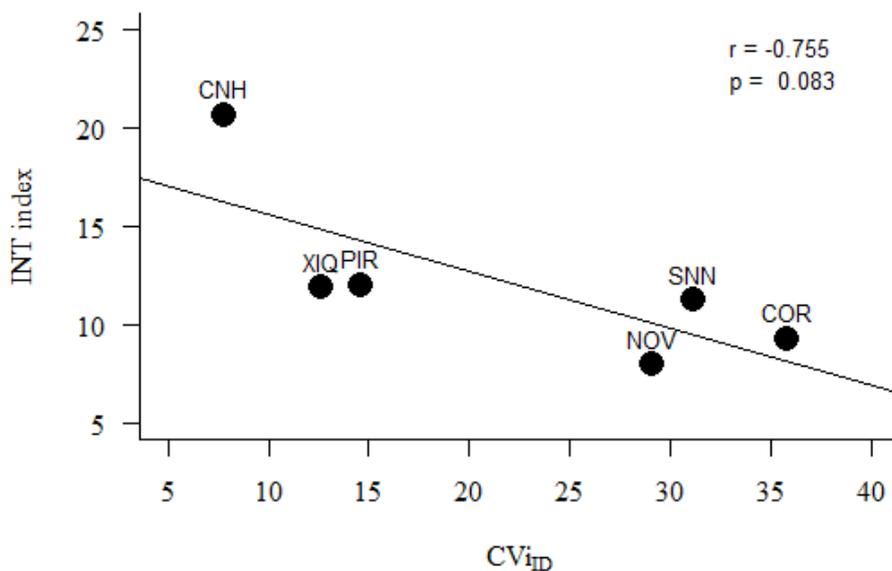
Population	Hypothesis - $r$ ( $p$ -value)		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
PIR	0.051 (0.244)	0.274 (0.030)	0.122 (0.145)
SNN	0.111 (0.116)	0.261 (0.023)	<b>0.372 (0.002)</b>
XIQ	<b>0.260 (0.005)</b>	<b>0.368 (0.011)</b>	<b>0.478 (0.001)</b>
NOV	0.216 (0.047)	0.172 (0.076)	0.188 (0.062)
CNH	<b>0.293 (0.009)</b>	0.258 (0.033)	<b>0.313 (0.009)</b>
COR	0.211 (0.018)	0.284 (0.026)	<b>0.367 (0.002)</b>

**Table 4.** Mean body size and relative frequency of oil-collecting bees recorded on *K. grandiflora* flowers in six populations. Mean<sub>ID</sub>: mean of intertegular distance.

Oil-collecting bees	Populations – Mean <sub>ID</sub> (Relative frequency)					
	PIR	SNN	XIQ	NOV	CNH	COR
<b>APIDAE</b>						
<b>CENTRIDINI</b>						
<i>Centris (Centris) aenea</i>	4.01 (0.40)	4.91 (0.07)	4.94 (0.10)		4.62 (0.11)	
<i>Centris (C.) caxiensis</i>		4.30 (0.09)				
<i>Centris (C.) byrsonimae</i>			3.83 (0.46)			
<i>Centris (Hemisiella) tarsata</i>	3.26 (0.60)	3.59 (0.30)		3.70 (0.07)		3.51 (0.04)
<i>Centris hyptidis</i>		3.78 (0.06)		3.62 (0.33)		
<i>Centris hyptidoides</i>						3.96 (0.05)
<i>Centris (Paracentris) burgdorfi</i>		5.23 (0.01)				
<i>Centris (P.) thelyopsis</i>				3.46 (0.03)		
<i>Centris (P.) xanthomelaena</i>		4.74 (0.01)	4.37 (0.44)		4.14 (0.89)	
<i>Centris (Trachina) perforator</i>		4.91 (0.01)				
<b>TAPINOTASPIDINI</b>						
<i>Arhysoceble huberi</i>		1.54 (0.04)		1.63 (0.22)		1.58 (0.15)
<i>Caenonomada aff. unicalcarata</i>		2.56 (0.40)		2.73 (0.25)		2.65 (0.76)
<i>Tapinotaspoides serraticornis</i>				2.23 (0.10)		

### 3.4 Influences on phenotypic integration

*Krameria grandiflora* populations with similar patterns of phenotypic co-variation significantly showed greater similitude in pollinator functional diversity (Mantel test:  $r = 0.55$ ,  $p = 0.014$ ) and composition ( $r = 0.52$ ,  $p = 0.021$ ). We also found a non-significant tendency to a negative relationship between the INT index and  $CV_{ID}$  ( $r = -0.75$ ,  $p = 0.083$ ; Figure 4). There was no significant relationship between the similitude in patterns of phenotypic co-variation and geographic distance among populations ( $r = -0.27$ ,  $p = 0.792$ ) and abiotic factors ( $r = -0.125$ ,  $p = 0.693$ ).



**Figure 4.** Correlation between the coefficients of variation in bee size per population ( $CV_{ID}$ ) and the magnitude of phenotypic integration (INT index).

### 4. Discussion

Trait correlations is expected to vary geographically within species range, sometimes responding to changes in selective pressures exerted by interacting partners across communities (Armbruster and Schwaegerle, 1996; Herrera et al., 2002; Pérez-Barrales et al., 2007; González et al., 2015). Our results support this idea as we found evidences that the intraspecific variation in the pattern of phenotypic correlation in the *K. grandiflora* oil flowers

relates to the variation of pollinator assemblage. Populations of *K. grandiflora* with similar variation in morphology and composition of oil-collecting bees displayed a similar trait correlation, regardless similarity in abiotic factors and geographic distance among populations. Therefore, our results suggest that the geographic divergence in functional diversity and composition within a single pollinator functional group (oil-collecting bees) may influence the variation of trait correlation patterns in a plant species.

Variation of phenotypic correlations among conspecific populations might be an effect of natural selection, drift or plasticity rather than genetic constraints, although genetic constraints can be ultimately adaptive (Armbruster et al., 2004). On the contrary, gene flow should prevent intraspecific divergence (Slatkin, 1987). We found differences in the phenotypic correlation matrices among *K. grandiflora* populations; however, they are neither related to geographic distance among populations nor to abiotic factors. Influence of geographic distances was not expected as *K. grandiflora* occurs scattered and isolated across its range (Simpson, 1989). This indicates that the populations are independent, although there is no information on the phylogeographic patterns of species distribution. Moreover, there might be no apparent effect of gene flow influencing the patterns of trait correlation found in this study. Plasticity have been attributed to affect phenotypic correlations (Schlichting, 1989; Waitt and Levin, 1993); however, our results did not support the hypothesis that environment changes account for variation of phenotypic correlations in *K. grandiflora* flowers, as no significant correlation was found between the correlation pattern and abiotic factors. Random drift is also another evolutionary process attributed to changes in multivariate patterns across populations (Herrera et al., 2002; Maubecin et al., 2016). However, our results indicate that selection might overcome drift as the variation in pollinator is associated to floral phenotypic correlations across *K. grandiflora* geographical range.

The association between the among-population variation of trait correlation and functional diversity and composition of interacting species suggests that the adaptive component is important for trait covariance (Armbruster et al., 2004; Pérez-Barrales et al., 2007; Ordano et al., 2008). Several studies have demonstrated that trait variation can be attributed to selection pressures mediated by interacting species in a geographic mosaic of selective regimes (Herrera et al., 2006; Anderson and Johnson, 2009; Gómez et al., 2009; Cosacov et al., 2014; Nogueira et al., 2015; Ferreiro et al., 2017). However, the influence of biotic agents on the spatial variation of phenotypic integration and correlation has been little

explored (Herrera et al., 2002; Pérez-Barrales et al., 2007; 2014; Rosas-Guerrero et al., 2011; Gómez et al., 2014; Pérez-Barrales et al., 2014; González et al., 2015).

Regarding plant-pollinator interactions, the few studies available support the adaptive influence of variation in pollinator assemblage on phenotypic integration or correlation patterns (Rosas-Guerrero et al., 2011; Pérez-Barrales et al., 2014; González et al., 2015; Lázaro and Santamaría, 2016). Moreover, this pattern has been evidenced within pollination systems that comprise more than one pollinator functional group. For example, Perez-Barrales et al (2014) demonstrated this pattern between *Narcissus* species (Amaryllidaceae) and their varying pollinator assemblage which included species of Diptera, Hymenoptera and Lepidoptera (both butterflies and moths). In the same way, González et al (2015) found that populations with similar trait correlation pattern shared similar pollinator functional diversity that also comprised Diptera, Hymenoptera and Lepidoptera. Thus, a high pollinator morphological and behavioral variation has been considered in these studies, which reflect a large extent of selective pressures on floral phenotype across a range. In this study, we found trait correlation patterns influenced by a geographical range comprising local specialization and generalization within a single functional group, indicating that slight differences of pollinator morphology might produce effect on the floral trait correlation.

To fit Berg's hypothesis that specialist scenarios provide a stronger selective pressure on floral integration in contrast to generalist, the magnitude of phenotypic integration (INT index) and the variation of pollinator size ( $CV_{ID}$ ) should display a significant negative association. However, our results showed only a marginal tendency to a negative correlation between INT index and  $CV_{ID}$ ; maybe limited by the number of populations included in the study. Nevertheless, populations with a low  $CV_{ID}$  may be rare throughout *K. grandiflora* distribution which also could limit this inference. The population with the highest INT index (CNH) was related mainly to one pollinator (*C. xanthomelaena*; 89% of relative abundance) and the populations with the lowest INT indices (COR and NOV) were associated to Tapinotaspidini bees, which perform a poor matching in *K. grandiflora* flowers (Chapter 2), and small sized *Centris*. Intermediated INT indices were determined for populations (PIR, SNN and XIQ) with large and small sized *Centris* as pollinators. This pattern might fit the hypothesis that large *Centris* play a role as selective force to the floral phenotype in *K. grandiflora* due to their optimum matching to the flowers (Chapter 2). The disparity found in CNN population in magnitude of phenotypic integration in the whole flower and in corolla

traits separately (related to the mechanical fit) compared to other populations could be due to the absence of small *Centris* bees relaxing the selective force on phenotypic integration. The presence in similar proportion of *C. xanthomelaena* and *C. byrsonimae* (a smaller *Centris*), in XIQ might give support to this. Nevertheless, further investigations are required to evaluate all these speculations on the relationship between the correlation pattern of *K. grandiflora* floral traits and oil-collecting bees.

Morphological modifications suffered by *Krameria* floral whorls throughout the evolutionary history of its ancestral lineages are intriguing and have made the evaluation of the relationship with sister groups difficult to determine (Simpson, 1989; but see Sheahan and Chase, 1996). These modifications are related to the showy calyx and the decoupled corolla that functions as attraction and mechanical-fit traits, respectively (see Chapter 2 for a broad discussion). Thus, we predicted that corolla is more integrated than calyx as floral traits that interact to favor accurate placement of pollen grains on and deposition by pollinators (*i.e.* mechanical-fit traits) affect more plant fitness than traits related to advertisement functions (Conner and Via, 1993; Armbruster et al., 1999; Ordano et al., 2008). However, the Pearson's correlation coefficients, the magnitude of integration per floral whorl and the stronger correlations obtained from the conditional independence procedure showed an opposing pattern for all populations. Thus, the *K. grandiflora* calyx is more integrated than its corolla. This result indicates that the association of corolla traits is labile compared to calyx traits and two explanations can be set for it. First, the *K. grandiflora* corolla traits might experience more intensively the effect of multiple selective directions exerted by local variation in pollinator size. This may be supported by the high magnitude of corolla integration in CNH, with low  $CV_{ID}$  and the low magnitude in other populations with more variation in pollinator size. And second, the lower integration observed for the corolla may be an effect of its decoupling into two morphological units, supported by the association between the phenotypic correlations and the morphological hypothesis ( $T_3$ ). Both explanations can be connected; however, the first explanation seems more plausible due to the variation in corolla integration found across populations.

Recent studies have demonstrated that geographical variants of oil-collecting bees can either mediate divergent selection on floral rewards either associate to phenotypic variation in oil flowers (Cosacov et al., 2014; Ferreira et al., 2017). In another approach, we provided new evidence that variation within oil-collecting bee assemblage throughout the range of the oil-

producing *K. grandiflora* influences the phenotypic correlation pattern of its floral traits. Following the assumption that phenotypic and genetic correlation matrices are proportional in plants (Waitt and Levin, 1998), the geographical pattern of floral integration displayed by *K. grandiflora* may present a short cut of the realized evolutionary outcomes mediated by pollinators as selective drivers. Further investigations on the historical process involved in *K. grandiflora* segregation could provide insights on the timescale in which selective pressures could act for divergence and better disentangle the contributions of natural selection and genetic drift to variations in floral integration.

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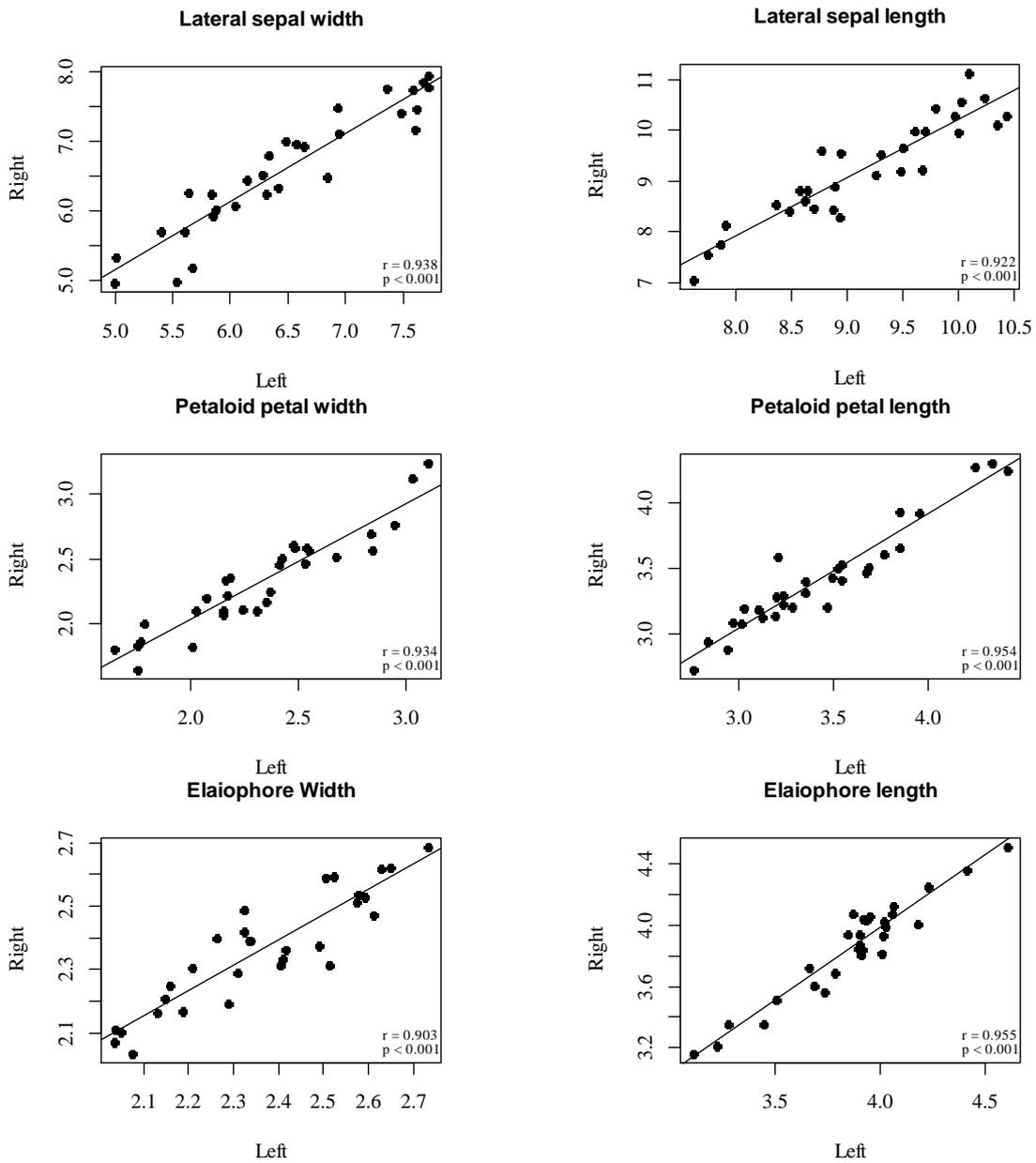
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## Supplementary material

**Figure S1.** Pearson's correlation coefficients between widths and lengths of right and left elaiophores, lateral sepals and petaloid petals. The measurements were obtained from one flower of 10 plants in three populations ( $n = 30$ ) using high resolution scaled photographs and ImageJ 1.48v software.



**Table S1.** Trait means and coefficient of variation (CV) from six populations (sample sizes in parenthesis) of *Krameria grandiflora*. For trait names, see Figure 1.

Populations (sz)	Floral traits (mm)												
	Calyx				Corolla						Operative distances		
	UpL	UpW	LwL	LwW	LtL	LtW	ElaL	ElaW	sPtL	bPtL	bPtW	st-Pt	st-Ela
PIR (40)	8.854 (9.225)	5.290 (11.680)	9.436 (9.507)	5.342 (10.866)	9.775 (7.650)	6.594 (8.734)	4.025 (8.186)	2.421 (6.863)	3.900 (11.204)	3.290 (11.295)	2.277 (10.501)	2.729 (15.141)	4.025 <sup>bc</sup> (10.421)
SNN (27)	8.530 (6.925)	4.902 (12.930)	9.018 (6.197)	5.396 (10.581)	8.713 (6.952)	5.821 (8.985)	3.423 (8.704)	2.139 (10.454)	3.608 (7.398)	2.969 (9.415)	2.094 (11.805)	3.113 (12.946)	5.279 (7.607)
XIQ (42)	8.216 (8.221)	5.271 (10.989)	8.805 (8.284)	5.924 (9.511)	9.152 (6.680)	6.560 (8.093)	3.689 (5.669)	2.084 (8.946)	3.559 (8.280)	3.985 (8.008)	2.718 (9.816)	2.701 (14.154)	5.678 (7.421)
NOV (42)	9.792 (9.114)	4.956 (10.617)	10.519 (8.189)	5.095 (9.064)	10.286 (7.129)	6.132 (8.187)	3.772 (7.598)	2.203 (7.225)	4.228 (7.507)	2.978 (8.913)	1.949 (13.812)	2.960 (11.645)	6.358 (8.099)
CNH (42)	7.990 (6.520)	4.739 (12.062)	8.435 (6.528)	5.193 (10.116)	8.963 (7.203)	6.238 (10.002)	3.324 (7.823)	2.075 (10.525)	3.67 (10.505)	3.493 (6.370)	2.374 (12.449)	2.619 (18.536)	5.629 (8.005)
COR (42)	8.851 (6.315)	4.710 (12.428)	9.575 (6.780)	4.974 (11.200)	9.256 (5.658)	5.531 (10.988)	3.487 (8.930)	2.056 (7.936)	3.597 (10.064)	3.240 (13.673)	1.991 (11.068)	2.670 (16.656)	5.677 (7.935)

**Table S2.** Pearson's correlation matrices for each *K. grandiflora* population.

	st.Pt	st.Ela	ElaW	ElaL	sPtL	PIR		LtW	LtL	LwW	LwL	UpW
						bPtL	bPtW					
st.Pt												
st.Ela	0.44											
ElaW	0.34	0.39										
ElaL	0.16	0.05	0.16									
sPtL	0.35	0.25	0.38	0.23								
bPtL	0.18	0.07	0.27	0.38	0.44							
bPtW	0.22	0.16	0.29	-0.05	0.05	0.24						
LtW	0.31	0.20	0.59	-0.01	0.36	0.25	0.41					
LtL	0.29	0.27	0.26	0.36	0.54	0.66	0.23	0.17				
LwW	0.20	0.19	0.57	-0.14	0.27	0.26	0.62	0.65	0.14			
LwL	0.12	0.08	0.08	0.29	0.51	0.64	-0.07	-0.01	0.76	-0.03		
UpW	0.26	0.25	0.45	-0.10	0.39	0.44	0.59	0.68	0.31	0.87	0.10	
UpL	0.11	0.20	0.19	0.24	0.58	0.69	0.09	0.14	0.75	0.17	0.90	0.30

cont.

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	st.Pt	st.Ela	ElaW	ElaL	sPtL	SNN		LtW	LtL	LwW	LwL	UpW
						bPtL	bPtW					
st.Pt												
st.Ela	0.29											
ElaW	0.30	0.18										
ElaL	0.04	0.06	0.48									
sPtL	0.47	0.11	0.07	0.35								
bPtL	0.45	-0.02	0.13	0.20	0.55							
bPtW	0.20	0.01	0.04	-0.02	0.12	0.26						
LtW	0.45	0.43	0.50	0.16	0.36	0.38	0.45					
LtL	0.31	0.29	0.19	0.03	0.49	0.44	0.16	0.54				
LwW	0.43	0.46	0.48	0.41	0.43	0.29	0.34	0.68	0.16			
LwL	0.20	0.22	0.07	-0.05	0.43	0.44	0.07	0.46	0.68	0.26		
UpW	0.43	0.52	0.50	0.29	0.25	0.32	0.31	0.75	0.24	0.88	0.19	
UpL	0.34	0.28	0.11	-0.06	0.59	0.47	0.11	0.47	0.81	0.28	0.88	0.22

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cont.

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	st.Pt	st.Ela	ElaW	ElaL	sPtL	XIQ bPtL	bPtW	LtW	LtL	LwW	LwL	UpW
st.Pt												
st.Ela	-0.06											
ElaW	0.27	0.12										
ElaL	0.29	0.02	0.14									
sPtL	0.28	0.13	0.14	0.16								
bPtL	0.21	0.25	0.11	0.29	0.21							
bPtW	0.22	0.29	0.53	0.13	0.16	0.36						
LtW	0.19	0.24	0.57	0.22	0.23	0.19	0.53					
LtL	0.29	0.32	0.32	-0.01	0.60	0.49	0.24	0.38				
LwW	0.12	0.21	0.51	0.17	0.21	0.05	0.43	0.60	0.47			
LwL	0.14	0.35	0.34	-0.06	0.32	0.38	0.28	0.53	0.83	0.61		
UpW	0.11	0.23	0.50	0.20	0.08	-0.06	0.38	0.45	0.33	0.83	0.50	
UpL	0.11	0.40	0.41	0.05	0.26	0.40	0.25	0.55	0.75	0.63	0.92	0.54

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cont.

	NOV											
	st.Pt	st.Ela	ElaW	ElaL	sPtL	bPtL	bPtW	LtW	LtL	LwW	LwL	UpW
st.Pt												
st.Ela	0.40											
ElaW	0.26	0.12										
ElaL	0.31	0.20	0.60									
sPtL	0.30	0.49	0.30	0.13								
bPtL	0.14	0.27	0.21	0.38	0.08							
bPtW	-0.03	-0.12	0.39	0.51	-0.19	0.31						
LtW	0.35	0.38	0.26	0.24	0.37	-0.04	0.24					
LtL	0.18	0.52	0.11	0.12	0.53	0.24	-0.09	0.45				
LwW	0.06	0.29	0.41	0.53	0.05	0.31	0.33	0.50	0.35			
LwL	0.02	0.32	0.15	0.15	0.35	0.25	-0.05	0.24	0.57	0.18		
UpW	0.20	0.33	0.40	0.46	0.07	0.18	0.28	0.57	0.22	0.83	0.04	
UpL	0.02	0.35	0.08	0.08	0.31	0.26	0.03	0.27	0.60	0.19	0.86	0.05

cont.

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	st.Pt	st.Ela	ElaW	ElaL	sPtL	CNH bPtL	bPtW	LtW	LtL	LwW	LwL	UpW
st.Pt												
st.Ela	0.43											
ElaW	0.53	0.53										
ElaL	0.40	0.28	0.65									
sPtL	0.44	0.46	0.50	0.46								
bPtL	0.36	0.16	0.27	0.51	0.47							
bPtW	0.23	0.40	0.62	0.56	0.52	0.41						
LtW	0.35	0.45	0.67	0.36	0.45	0.29	0.67					
LtL	0.38	0.38	0.53	0.43	0.66	0.54	0.49	0.64				
LwW	0.46	0.38	0.57	0.27	0.44	0.22	0.54	0.68	0.49			
LwL	0.21	0.37	0.41	0.35	0.57	0.41	0.34	0.39	0.75	0.28		
UpW	0.51	0.34	0.53	0.30	0.40	0.38	0.51	0.63	0.47	0.90	0.26	
UpL	0.27	0.36	0.45	0.32	0.69	0.40	0.45	0.45	0.78	0.46	0.72	0.44

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cont.

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	COR											
	st.Pt	st.Ela	ElaW	ElaL	sPtL	bPtL	bPtW	LtW	LtL	LwW	LwL	UpW
st.Pt												
st.Ela	0.19											
ElaW	-0.18	0.06										
ElaL	0.34	0.58	-0.04									
sPtL	0.52	0.49	0.08	0.46								
bPtL	0.28	0.33	0.02	0.43	0.44							
bPtW	-0.07	-0.13	0.32	-0.07	-0.11	0.19						
LtW	0.33	0.41	0.37	0.32	0.44	0.43	0.40					
LtL	0.28	0.29	0.14	0.25	0.30	0.55	0.23	0.52				
LwW	0.22	0.36	0.15	0.38	0.31	0.41	0.33	0.65	0.39			
LwL	0.23	0.20	0.09	0.21	0.35	0.55	0.01	0.31	0.57	0.25		
UpW	0.27	0.35	0.07	0.30	0.35	0.45	0.30	0.70	0.57	0.87	0.32	
UpL	0.18	0.19	0.08	0.02	0.23	0.50	0.08	0.32	0.59	0.40	0.73	0.46

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## Conclusões gerais

Entender como as espécies exercem pressões seletivas uma sobre as outras e o quanto implicam em mudanças morfológicas e diversificação têm sido os principais desafios da biologia evolutiva. A partir desse estudo, mostrei que o padrão de correlações entre caracteres florais (integração floral) difere em nível intraespecífico, e essa variação se relaciona com a divergência geográfica na assembleia de polinizadores que interage localmente com as populações de *K. grandiflora*. Esse resultado suporta a hipótese de que muito da variação de atributos morfológicos pode ser explicada pela divergência em pressões seletivas exercidas por espécies interagentes.

Mais especificamente mostrei que a influência da divergência na assembleia de polinizadores sobre o padrão de integração do fenótipo pode ser proporcionada por variações geográficas minuciosas na morfologia e diversidade de espécies pertencentes a um único grupo funcional de polinizadores, o que parece refletir diferentes cenários com variação no grau de generalização/especialização mesmo em um sistema de polinização considerado especializado.

Além disso, a variação geográfica na assembleia de polinizadores também consistiu em cenários divergentes em comportamento e ajuste à morfologia floral, o que também poderia refletir em pressões seletivas distintas. Embora um efeito da diferença nesses cenários sobre a funcionalidade do fenótipo floral não tenha sido detectado experimentalmente, essas diferenças podem contribuir como pressão seletiva sobre os caracteres, pois o desajuste morfológico contribuindo com o fitness, pode gerar efeito sobre a correlação entre caracteres. Nesse sentido, estudos de seleção fenotípica sobre os caracteres florais em cenários de ajuste e desajuste poderiam elucidar seus efeitos.

O presente estudo evidencia, portanto, que a abordagem multipopulacional em escala geográfica das interações entre espécies proporciona uma compreensão mais ampla sobre as dinâmicas seletivas, uma vez que os processos evolutivos geradores de mudança agem no nível abaixo de espécies.