Trade off between quantity and size of pollen grains in the heterantherous flowers of *Senna pendula* (Fabaceae)

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**ABSTRACT**
Heteranthery, the presence of different types of stamens in a flower, may reduce the conflict between pollinators and plants by ensuring the resource for the pollinator without drastically affecting the availability of viable male gametes for fertilization, according to the division-of-labor hypothesis. We investigated whether the poricidal anthers of *Senna pendula*, a buzz-pollinated heterantherous species, present morphological and physiological differences among pollen grains from the three sets of stamens. We compared quantity, ornamentation, size and fecundity of pollen from long, medium and short stamens. The short feeding stamens produced larger but fewer pollen grains than the long pollinating stamens, which produced smaller pollen grains but in higher quantity. The total pollen volume of pollinating and feeding stamens per flower, however, was the same. The medium stamen produced less-fertile small pollen grains and seems to play no specific role in bee feeding and pollination. Our results indicate differential allocation of pollen for pollinating and feeding stamens mediated by heteranthery. The differences in volume versus quantity of pollen grains fit the division-of-labor hypothesis well for heterantherous pollen-only flowers with poricidal anthers.

**Keywords:** bees, buzz-pollination, division-of-labor, heteranthery, pollen economy, *Senna pendula*, stamen dimorphism

**Introduction**
Flowers that offer only pollen to pollinators face the dilemma to attract and reward their pollinators and at the same time to protect and guarantee pollen for sexual reproduction (Klinkhamer & Jong 1993; Westerkamp 1996). To deal with this dilemma such pollen flowers have evolved several strategies to minimize pollen loss, among them heteranthery (Vogel 1978; Buchmann 1983).

Heterantherous flowers have stamens that differ in size, shape and/or position in a flower (Vallejo-Marín et al. 2009). Several heterantherous species have a set of stamens with attractive coloration easily accessible to pollinators, while the other set usually has stamens of larger size, cryptic coloration and is located in a position that corresponds to the position of the stigma (Barrett 2010; Vallejo-Marín et al. 2014). The stamen dimorphism of heteranthery is involved in a “division-of-labor” between stamens (Darwin 1862; Müller 1883), one group contemplate the demand for food of pollinators (feeding stamens), and the other pollen export for pollination (pollinating stamens). Division-of-function has been empirically demonstrated for several heterantherous plant species, such as, *Solanum rostratum* Solanaceae (Vallejo-Marín et al. 2009); *Monochoria korakowii* Pontederiaceae (Tang & Huang 2007); *Senna*...
The presence of heteranthery in flowers is strongly correlated with poricidal anther dehiscence and abundance of floral nectaries (Vallejo-Marín et al. 2010). Flowers with both, heteranthery and poricidal anther dehiscence occur in at least 11 plant families, among them Fabaceae, Melastomataceae and Solanaceae (Vallejo-Marín et al. 2010). The flowers with these characteristics are pollinated mainly by bees that extract pollen by vibrating anthers, a phenomenon known as buzz-pollination (Michener 1962; Vogel 1978; Buchmann 1983). The diversification of several clades of plants with poricidal anther dehiscence coincides with the appearance of buzz pollinating bees (Cardinal et al. 2018), which may be the result of a convergent evolution of both heteranthery and poricidal anther dehiscence (Buchmann 1983; Luca & Vallejo-Marín 2013; Russell et al. 2017).

Flowers of the genus Senna have the typical floral traits associated with heteranthery: the flowers usually present poricidal anther dehiscence, enantioistyly and absence of floral nectaries (Buchmann 1974; Irwin & Barneby 1982). Flowers of Senna show a considerable diversity of arrangements in floral symmetry and heterantherous types. Marazzi et al. (2007) differentiate four types of stamens: (1) three uppermost stamens modified in staminodes (except Senna section Psilorhegma), (2) a set of four short-sized “feeding” middle stamens, (3) a set of three long-sized “pollinating” lowermost stamens, in which (4) the medium stamen may be reduced or even absent. The lowermost stamens show the greatest diversity in position, size and anther morphology in the genus Senna.

The division of anther function has already been reported for heterantherous representatives within this genus (Dulberger 1981; Marazzi et al. 2007; Luo et al. 2009; Amorim et al. 2017; Mesquita-Neto et al. 2017). Heteranthery could accompany structural and qualitative differences in pollen (Lloyd 2000), but only a few studies indicated such differences in pollen from pollinating and feeding stamens (Carvalho & Oliveira 2003; Luo et al. 2009).

We studied Senna pendula a shrub widely distributed in South America and frequently cultivated as ornamental (Irwin & Barneby 1982). The flowers of this species have three sets of stamens: two long, one medium-sized and four short. Based on the hypothesis that pollen grains might differ in morphology and function related to the division-of-labor of stamens, we asked: (1) Are there differences in pollen grain morphology (ornamentation, shape, volume) and pollen quantity (number of grains) from the three sets of stamens? (2) Are there differences in the fecundity of pollen from the three sets of stamens?

Materials and methods

Study area

The field study was conducted from March to July 2014, in a native population of Senna pendula (Humb. & Bonpl.ex Willd.) H.S.Irwin & Barneby in the nature reserve Serra do Curral (19°57′S 43°54′W, altitude 1200-1380 m), located in the metropolitan area of Belo Horizonte, Minas Gerais, Brazil. The climate is tropical, with mild winters and hot and rainy summers. Precipitation is concentrated in the rainy season from October to March with an annual average of 1,490 mm (Assis 2012). The vegetation is characteristic of the transition between Atlantic Forest and Cerrado.

Study species

Senna pendula blooms from March to May, and fruits ripen from April to July. The shrub reaches a height of 2 m and has inflorescences with three to 12 flowers in racemes. The corolla has five golden-yellow non-concave petals and pistils oriented to the right or to the left, a condition known as enantioistyly. The androecium has two long lateral symmetric stamens (“pollinating stamens”), one centralized median-stamen, four short-central stamens (“feeding stamens”) and three staminodes (Fig. 1).

Pollen morphology, size and quantity

We counted the pollen grains for each set of stamens of S. pendula using twenty flower buds in pre-anthesis from ten individual plants (two buds from each plant), stored in 70 % ethanol. The anthers were macerated in Eppendorf® flasks and prepared according to Lloyd (1972). The pollen grains were counted in a Neubauer chamber (Maeda 1985). The amount of pollen of the different stamen types was compared using analysis of variance (ANOVA) with general linear model procedure (GLM, Poisson distribution) (R Core Team 2017).

The pollen grains of the different types of anthers were stored in glacial acetic acid (N = 10 individuals, 10 flowers) to compare ornamentation and size of pollen grains from the three stamen types. We confected microscope slides with glycerinated gelatin, sealed with paraffin and analyzed the pollen grains in light microscope with 1000x magnification (N = 30 grains per type of stamen).

The pollen grain volumes were determined (μm³) from radial measurements taken of the polar and equatorial diameters of 50 non-acetylated pollen grains using a light microscope (1000x magnification). We adjusted the values obtained from the measures to a known geometric shape (oblate-spheroidal), whose volume was calculated by the following equation: \( V = \frac{4}{3} \pi a^2 b \), where \( V \) is the volume (μm³), \( a \) is the radius of the polar diameter and \( b \) the radius of the equatorial diameter.
We multiplied the average grain volume by the pollen number of each type of stamen and calculated also the total volume of pollen grains for the three sets of stamens. We used ANOVA, with GLM procedure (Inverse Gaussian distribution), using the stats package in R version 3.3.3 (R Core Team 2017), to determine differences between volume and quantity of the pollen grains of the three groups of stamens. We calculated the pollen/ovule ratio for *S. pendula*, dividing the average number of ovules in a flower (N = 10 individuals, 20 flowers) by the average number of pollen grains per flower (N = 10 individuals, 10 flowers; Cruden 1977). We considered the number of samples (N) as the number of individuals of plants. When more than one flower per plant was used, we considered the average value of within-plant flowers as one sample.

**Breeding system and fruit set**

To determine the breeding system of *S. pendula*, controlled hand pollination was performed. Flower buds were bagged and flowers hand-self-pollinated (N = four plants, 16 flowers) or maintained bagged (spontaneous self-pollination) (N = two plants, five flowers). We performed hand-cross pollination with pollen from long (N = four plants, 52 flowers), medium (N = four plants, 61 flowers) and short stamens (N = four plants, 51 flowers) to verify fertility of pollen from the three types of stamens. Pollen donors were at least 100 meters distant from pollen receiving plants. Hand cross- and hand self-pollination treatments were conducted in new open flowers in the morning. Moreover, non-bagged marked flowers were maintained accessible to flower visitors. We compared fruit set (number of fruits/number of flowers) and seed set (number of seeds/fruit) of the different treatments. The statistical significance the fruit and seed set were determined by the chi-square test and ANOVA (GLM procedure, Poisson distribution), respectively. The GLM-ANOVA was performed in stats package in R version 3.3.3 (R Core Team 2017). Sample number (N) was the number of plant individuals.

**Floral visitors**

To evaluate the behavior of floral visitors, observations were performed on different individuals between 8h00min and 17h00min. After floral visits, the insects were collected, mounted, labeled, identified and included in the entomological collection of Universidade Federal de Minas Gerais. We noted which species of bees vibrate flowers to collect pollen and touch the stigmas.
Results

Pollen morphology, ornamentation and quantity in the different anther types

The tricolporate, oblate-spheroidal pollen grains of *Senna pendula* exhibited no differences in the microreticulate ornamentation at 1000x magnification under the light microscopy. However, short-stamens produced pollen grains with larger volume (mean ± SD: 11,810.1 ± 4,549.2 μm³), about 37% larger than pollen grains from long (7,644.2 ± 1,014.0 μm³) and medium-stamens (7,926.6 ± 1,120.9 μm³) (Fig. 2; GLM, F = 34.7, p ≤ 0.001, Inverse Gaussian distribution).

Single long stamens have more pollen grains (mean ± SD: 15,062.5 ± 56,657.4) than the medium (3,812.5 ± 2,515.5) and short stamens (4,812.5 ± 3,225.9) (GLM, F = 49.9, p ≤ 0.001, Poisson distribution; Fig. 3). Considering the total set of stamens in a flower, on average, 56% of the total number of pollen grains of a flower are allocated to long, 7.5% to medium and 36.5% to short stamens (GLM, F = 75.4, p ≤ 0.001, Poisson distribution).

However, relating the number of grains per stamens to the respective volume of the pollen grains (Fig. 2, GLM, F = 34.7, p ≤ 0.001, Inverse Gaussian distribution), the set of long and short Stamens have no difference in pollen volume per flower (Fig. 3, GLM, p = 0.93).

Breeding system and fruit set and fecundity of pollen from different stamens

Hand self-pollination experiments set no fruits. Fruit set using pollen from long and short stamens after hand cross-pollination was similar, both were 25% (X² = 0.02, p = 0.62) and higher than the 8% seed set sired by pollen from medium size stamens (long versus medium: X² = 4.68, p ≤ 0.05; short versus medium: X² = 4.89, p ≤ 0.05; Tab. 1). However, seed set in the mature fruits after hand cross-pollination with pollen from the three stamen types did not differ (Tab. 1). A flower produced, on average, 53,187.5 ± 19,258.9 pollen grains (Nplants = 10) and 103.8 ± 7.97 ovules (Nplants = 10), a pollen/ovule ratio of 512.4:1.

Table 1. Breeding system of *Senna pendula*. Fruit and seed set in flowers previously bagged in the bud stage: spontaneous self-pollination (flowers maintained bagged), hand self-pollination (pollination with pollen of the same flower), hand cross-pollination (pollination with pollen of other plant individuals), and open (non-bagged control) treatments. Hand cross-pollination treatments were conducted with pollen from long, medium or short stamens. Number (percent) of fruits set and mean (± SD); (Nplants) represents the number of plant individuals tested per treatment. Values with the different superscript letters indicate significant differences between fruit (p ≤ 0.05; Mantel-Haenszel Chi-square) and seed sets (GLM, F = 0.69, p = 0.51, Poisson distribution).

<table>
<thead>
<tr>
<th>Pollination treatment</th>
<th>Nplants (No. Flowers)</th>
<th>Fruit set (%)</th>
<th>Seed set (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous self-pollination</td>
<td>2 (5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hand self-pollination</td>
<td>4 (16)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Open pollination</td>
<td>5 (155)</td>
<td>13±(8)</td>
<td></td>
</tr>
<tr>
<td>Hand cross-pollination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long stamen</td>
<td>4 (52)</td>
<td>13±(25)</td>
<td>34±12.6±</td>
</tr>
<tr>
<td>Medium stamen</td>
<td>4 (61)</td>
<td>5±(8)</td>
<td>33.8±20.3±</td>
</tr>
<tr>
<td>Short stamen</td>
<td>4 (51)</td>
<td>13±(25)</td>
<td>35.8±25.8±</td>
</tr>
</tbody>
</table>

Floral visitors

The floral visitors to *Senna pendula* were mainly bees of the tribes Bombini, Centridini, Xylocopini, Exomalopini and Meliponini. The large bees of *Xylocopa (Neoxylocopa) frontalis*, *Xylocopa (Neoxylocopa) griseascens*, *Bombus (Fervidobombus) pauloensis*, *Centris (Trachina) fusca*, and *Centris (Melacentris) collaris* collected pollen by anther vibration. The females of these species hovered at close distance in front of the flowers before they decided to visit a flower or not. Their flower visits were uniformly in a specific position with both long lateral stamens positioned right and left-handed to the bee’s dorsal surface. While the ventral surface faced the short stamens, the dorsal surface contacted the stigma. Vibrations were applied to the flower in this position removing pollen simultaneously from all stamens. During the flower visits, the dorsum (mesoscutum, scutellum and metanotum) of the bees touched the stigma.

Females of *Melipona bicolor, Exomalopsis collaris*, and *Pseudaugochlora graminea* landed in the center of the flower, positioned the abdomen close to the anther opening and individually vibrated short, medium and long stamens. These bees contacted the stigma only occasionally.
Flower visits of workers of *Paratrigona subnuda* did not vibrate the flowers or the anthers, and collected pollen inserting their mouthparts into the anther pores of the different stamen types. Workers of this bee species spent long periods in a single flower removing pollen grains from their mouthparts with the fore legs and transferring them to the corbicula.

**Discussion**

Flowers of *S. pendula* show differential allocation of pollen grains to the long and short stamens. Short feeding stamens contain fewer but larger pollen grains, while the long pollination stamens have more but much smaller grains. These differences in volume versus quantity of pollen grains fit the division-of-labor hypothesis among stamens, as suggested by Müller (1881) and Darwin (1862).

The division-of-labor hypothesis predicts that the long stamens contain pollen destined for pollination and the short ones pollen for bee feeding (Darwin 1862; Müller 1881). In our studies, large carpenter bees (*Xylocopa*), bumblebees (*Bombus*) and oil-collecting bees (*Centris*) were the effective pollinators, which vibrated all anthers simultaneously in a single position defined by the complex morphology of the zygomorphic to slightly asymmetric flowers of *S. pendula* (Westerkamp 2004). The dorsum of the large bees receives the ejected pollen from the long stamens during the applied vibration; this part is a "safe site" for pollen deposition, because this region gets into contact with the stigma and the bees rarely remove these pollen loads (Westerkamp 2004; Westerkamp & Classen-Bockhoff 2007; Amorim et al.)

**Figure 3.** Median and percentiles (25–75 %) of number of pollen grains (upper) and volume of pollen (bottom) in long, medium and short stamen (left side "stamen") and by stamens groups within flowers (right side "flower") in *Senna pendula*. Number of pollen grains (top) and volume of pollen (bottom). Different letters represent significant differences between the stamen groups indicated by GLM: number of pollen per anther ($F = 38.1$, $p \leq 0.001$) and per flower ($F = 31.4$, $p \leq 0.001$), Poisson distribution; volume of pollen per flower ($F = 23.2$, $p \leq 0.001$) and per anther ($F = 3.1$, $p \leq 0.05$), Inverse Gaussian distribution; Nplants = 10 (20 flowers).
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Differently, pollen from the four short central feeding stamens is ejected onto the large bee's mesepisternum, a region from which it is frequently removed and transferred to the scopa on the hind legs. Moreover, the large bees only associate the feeding stamens with presence of food and fail to visit flowers whose short (but not medium and long stamens) are removed as demonstrated in *Senna reniformis* (Mesquita-Neto et al. 2017), which has the same flower morphology as *S. pendula*. The deposition of pollen in different body parts of the large and buzzing-bees, mediated by heteranthery, is characteristic for division-of-labor of stamens in heterantherous flowers (Vallejo-Marín et al. 2009; Tong & Huang 2018).

*Senna pendula* belongs to clade VII (sensu Marazzi et al. 2007), in which most of the representatives have monosymmetric flowers that possess upper and lower petals with similar shapes or have lower petals slightly longer and thinner than the upper ones (Marazzi & Endress 2008). In the latter, the pollen jet of the long stamens is directed to an elastic concave petal then it is again deflected from a second upper petal until the pollen grains are finally deposited on the bee's dorsum. In this pollination mechanism, denominated ricochet (Westerkamp 2004; Amorim et al. 2017), two petals are involved in the deflection of the pollen jet and the two long stamens are directed ventrally to one of the concave petals. The ricochet mechanism associated with the strongly concave petals and the deflected pollen grains from the pollination stamens occurs in numerous species of *Senna* and in different clades (Marazzi et al. 2007; Amorim et al. 2017). In *S. pendula*, the five petals are elliptical to oval, possess only weak concavity in the upper petals and do not interfere the direction of the pollen jet from the long stamens, which are here positioned dorsally and almost symmetrically left and right-hand of a large buzzing bee. Pollen in *S. pendula*, thus is ejected directly onto the bee's dorsum from the long stamens during the flower visit, similar to *S. reniformis* (Mesquita-Neto et al. 2017), without deflection by any petal (sensu Westerkamp 2004; Amorim et al. 2017). Because the long pollination stamens have the same relative position to the bee's body in floral morphs with left and right-sided pistil in *S. pendula*, enantioistyly in this species has not the role to promote cross-pollination (see Gottsberger & Silberbauer-Gottsberger 1988; Mesquita-Neto et al. 2017), and the stigma of right and left-sided pistils might indiscriminately contact pollen grains from both pollination stamens on the dorsal surface of large bees.

Poricidal dehiscence of anthers and rigorous self-incompatibility makes *S. pendula* dependent of large buzz-pollinating bees that move between conspecific plants to produce fruits. Fruit and seed production after hand cross-pollination with pollen from pollination and feeding stamens reached similar levels in *S. pendula*. Several studies have demonstrated differential fertility among heteromorphic stamens (Fægri & Pijl 1979; Sarala et al. 1999; Luo et al. 2009; Mesquita-Neto et al. 2017), while others found similar rates of fructification between pollen from pollination stamens and feeding anthers (Tang & Huang 2007; Gross & Kukuk 2001). Thus, the differential fecundity of pollen from different types of stamens is not necessarily associated with heteranthery.

**Different pollen allocation to feeding and pollinating stamens economizes gametes**

The pollen/ovule ratio was lower than that predicted by Cruden (1977) for obligatory xenogamic plants. It is expected that flowers that offer only pollen as a reward for pollinators produce more pollen than flowers that offer nectar or multiple rewards, because pollen in the former is also used to feed the bees (Vogel 1978; Cruden 2000). In this context, lower pollen production than expected might be an evolutionary response due to a high efficiency in pollination (Cruden & Jensen 1979) associated with pollen economy mediated by heteranthery (also see Ferreira & Araújo 2016).

Heterantherous flowers that provide a high quantity of its pollen to pollinators may reduce available male gametes for fertilization (Vogel 1978; Buchmann 1983; Vallejo-Marín et al. 2010). *Senna pendula* seems to have another way to mediate the dilemma: about 40% of the total pollen number in the flower is available to pollinators in the feeding stamens. High proportions of pollen for feeding are also found in other species of *Senna* (*S. reniformis*, Mesquita-Neto et al. 2017; *S. bicapsularis*, *S. alata*, Luo et al. 2009). Papaj et al. (2017) showed that storage of a minimum quantity of pollen in the feeding stamens is necessary to prevent bees to also collect pollen from the pollination stamens. Therefore, it seems to be necessary that pollination by large bees requires that the flowers allocate significant amounts of pollen to the feeding stamens. The total pollen grain volume of the four short feeding stamens is equivalent to the total pollen volume of the two long pollination anthers. However, the pollination anthers bear 50% more pollen grains than feeding anthers. For bees, the volume of pollen grains represents better its nutritional value than the number of grains (Silveira 1991). While a greater food volume may satisfy pollen-collecting bees, a high number of viable pollen grains is fundamental for pollen allocated to the reproduction of the plant. Probably there is an advantage for the plant to produce fewer but larger pollen grains for bee consumption. Several studies attribute the pollen number/size relationship as trade-off by the way that the reduction in the number of pollen grains should be associated with an increase in the size of pollen grains (eg. Charnov 1982; Vonhof & Harder 1995).

A negative correlation between pollen grain size and pollen protein content was found in buzz-pollinated plants by the way that smaller grains tend to have higher protein richness (Roulston et al. 2000). If we assume that the volume of a pollen grain is a reliable negative indicator of the nutrient content, and the bees have to collect a minimum
quantity of pollen to feed their larvae, the found differences here in pollen volume and pollen grain numbers seem to be well explained: *S. pendula* offers larger grains but with less essential nutrients in the short feeding stamens and save protein-rich pollen in long pollinating anther. To our knowledge, there is still no information on differences of the nutritional content of pollen grains from feeding and pollination stamens in heterantherous buzz-pollinated species. We hypothesize that pollen grains from pollinating anthers contain more high-value nutrients (P, N, proteins) than the possibly more energy-rich pollen grains from the feeding stamens in species with porical anthers. Measuring pollen quality in these species may help to understand the role of specialized feeding and resource allocation.

**The role of medium-sized stamens and staminodes**

In *S. pendula*, the small pollen grains of medium-sized stamens had much lower viability than the larger ones of the feeding stamens. The medium stamen seems to play no role in division-of-labor, and the decrease of pollen fertility indicates loss of the reproductive function, alike the morphologically similar *S. reniformis* (Mesquita-Neto et al. 2017). In some species and among several clades within the genus *Senna*, the medium stamen can be strongly reduced, sterile or absent (Irwin & Barneby 1982; Marazzi et al. 2006; 2007). We interpret the lower fertility of pollen from the medium stamen in *S. pendula* as related to an evolutionary process of stamen reduction as demonstrated in other species of *Senna* clade VII.

The staminodes, like the central short stamens, might possess a signaling function. In *S. pendula* they show bright white reflectance to the human eye, similar to the region surrounding the apical open pores of the anthers (Fig. 1A). This visual contrast, possibly enhanced by uv-absorbance, might amplify the signaling function of short stamens, indicating the presence of food for effective pollinators. This could be verified experimentally by removing staminodes and with the use of spectrophotometry.

We conclude that pollen from short feeding stamens and long pollinating stamens in *S. pendula* exhibit different quantity and size but similar fecundity. Whereas pollen from short feeding stamens have considerably larger size than pollen from long pollination stamens, those of the latter are smaller and more numerous, which is advantageous for pollination. The indicative of differential allocation of pollen grains for pollination and feeding of pollinators can be considered as manifestation of the division-of-labor function in pollen rewarding flowers with heteromorphic stamens.

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