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Testing functional hypotheses on poricidal anther dehiscence and heteranthery in buzz-pollinated flowers

Agnes S. Dellinger, Léa Pöllabauer, Milan Loreti, Janis Czurda & Jürg Schönenberger

Poricidal anther dehiscence is characteristic of pollen-rewarding flowers specialized on buzz-pollinating bees. Pollen dosing as a strategy of avoiding excessive pollen loss as a reward to the bees is believed to have played a major role in the evolution of buzz-pollination. Such dosing strategies should, however, be flexible and allow dispensing schedules to be adjusted to pollinator visitation frequency and flower age in order to avoid risks of dispersing too small amounts of pollen. In addition, many pollen-rewarding flowers have evolved different stamen types within the same flower (heteranthery), usually involving at least one showy and one cryptic type. Heteranthery is commonly explained by 'division-of-labour' between stamen types with one type depositing pollination pollen on the bee's back (which cannot be reached by grooming) and the other depositing feeding pollen on the bee's belly. Recently, however, differential pollen dosing between stamen types as a strategy to maximize male fitness has been proposed as an alternative driver of the evolution of heteranthery.

We used three pollen rewarding species (*Senna reticulata* (Fabaceae), *Adelobotrys adscendens*, *Conostegia subcrustulata* (both Melastomataceae)) with poricidal anthers to test 1) whether pollen dosing strategies change with flower age and 2) whether the different stamen types of heterantherous species differ in dosing strategies. In *A. adscendens*, 3) we also tested whether pollen from two different stamen types is deposited in different areas of the pollinator's body (mimicked by a microscope slide) as proposed by the 'division-of-labour' hypothesis.

Pollen was dosed in all three species, but pollen release increased with flower age only in *S. reticulata*. Differential dosing by the different stamen types was strong in both heterantherous species. In *A. adscendens*, we could not detect pollen deposition in different areas of the pollinator's body, but significantly more pollen was released by the cryptic (feeding) stamen type. Our results suggest that heteranthery may indeed be understood as a complex combination of spatial and temporal 'division-of-labour'. As proposed by other authors, heteranthery may function both in differential pollen placement (spatial aspect) but also in differential dosing between stamen types via 'shift-working' (temporal aspect).

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Eine porizide Antherenöffnung charakterisiert Blüten, die auf Vibrationsbestäubung spezialisiert sind und Pollen als einzige Bestäuberbelohnung anbieten. Um nicht zu viel Pollen als Belohnung zu verlieren, haben sich in vibrationsbestäubten Blüten spezialisierte Mechanismen zur Pollendosierung entwickelt. Diese Dosierungsmechanismen sollten jedoch so flexibel sein, dass auch bei niedriger Frequenz von Blütenbesuchen und in älteren Blüten nur kleine Mengen an Pollen in den Blüten zurückbleiben. Zusätzlich weisen viele vibrationsbestäubte Blüten unterschiedliche Staubblatttypen auf (Heterantherie), zumeist einen auffälligen Typ und einen unauffälligen Typ. Heterantherie wird oft mit einer „Arbeitsteilung" erklärt, bei der ein Staubblatttyp Pollen für die Bestäubung unerreichbar auf dem Rücken der Biene ablädt (Befruchtungsstaubblätter), und der andere Futterpollen auf dem Bauch der Biene deponiert (Beköstigungsstaubblätter). Unterschiedliche Dosierungsstrategien zwischen den zwei Staubblatttypen wurden als alternative Gründe für die Evolution von Heterantherie vorgeschlagen. An drei vibrationsbestäubten Pflanzenarten (*Senna reticulata* (Fabaceae), *Adelobotrys adscendens*, *Conostegia subcrustulata* (beide Melastomataceae)) untersuchten wir 1) ob sich die Pollendosierungsstrategien mit dem Älterwerden der Blüte verändern und 2) ob sich die zwei Staubblatttypen heterandrischer Blüten in ihren Pollendosierungsstrategien

unterscheiden. An *A. adscendens* testeten wir außerdem, 3) ob Pollen der unterschiedlichen Staubblatttypen gemäß der Hypothese der Arbeitsteilung an unterschiedlichen Körperstellen des Bestäubers (repräsentiert durch einen Objektträger) deponiert wird. Alle drei Arten dosierten ihren Pollen, aber nur Staubblätter von *S. reticulata* setzten beim Älterwerden mehr Pollen frei. In beiden heterandrischen Arten bestanden große Unterschiede in der Pollendosierung zwischen den zwei Staubblatttypen. Bei *A. adscendens* konnten wir keine Hinweise auf Pollendeposition an unterschiedlichen Stellen feststellen, jedoch setzten die unscheinbaren Beköstigungsstaubblätter deutlich mehr Pollen frei. Unsere Ergebnisse legen damit nahe, dass Heterantherie eine komplexe Kombination aus räumlicher (Pollendeposition an unterschiedlichen Körperteilen des Bestäubers) und zeitlicher (schnellere Pollenfreisetzung bei einem der beiden Staubblatttypen) Arbeitsteilung zwischen den unterschiedlichen Staubblatttypen darstellen könnte.

Keywords: buzz-pollination, poricidal anthers, artificial buzzing, pollen dosing, division-of-labour, Melastomataceae, Fabaceae.

Introduction

Pollination, i.e. the successful transfer of pollen (containing the male gametes) to stigmas (receptive female organs), is a quintessential step in plant reproduction. Various vectors for pollen transfer exist, including animals, wind and water, or pollination may also occur autonomously by self-pollen-transfer within flowers (Proctor et al. 1996). Pollination by animals is believed to be a major driver of diversification in angiosperms (flowering plants, van der Niet et al. 2014). Approximately 80 % of angiosperms rely on animal pollinators, including insects, mammals, birds and reptiles (Ollerton et al. 2011). Animals visit flowers for various reasons, most importantly to obtain food or shelter or to find mates (Proctor et al. 1996). The most common food rewards offered by flowers are pollen and nectar (Simpson & Neff 1981). Offering pollen as a reward is risky, however, as excessive pollen consumption by flower visitors reduces pollen available for pollination and ovule fertilization (De Luca & Vallejo-Marín 2013). Hence, selection for optimizing efficient pollen transfer and minimizing pollen loss has led to the evolution of a tremendous diversity of pollination strategies (Westerkamp 1997, Castellanos et al. 2006, Hargreaves et al. 2009, Minnaar et al. 2018).

Buzz-pollination by bees represents one such strategy (Buchmann 1983, Bernhardt 1996). Buzz-pollinated flowers usually only offer pollen as a reward. The pollen is concealed in elongated tubular anthers, which open at a small pore at the tip of the anther (Buchmann 1983). Normally, the only way to excise pollen from such tubular anthers is by applying high-frequency vibrations to the stamens. Only certain bee and bumblebee species can produce vibrations at the required frequencies (240–405 Hz, DeLuca et al. 2013, Cardinal et al. 2018). Despite this pollination strategy being functionally very specialized, buzz-pollination has evolved many times independently across angiosperms and is currently found in approximately 6 % of species (Endress 1994, Vallejo-Marín 2019). In some groups, like the genus *Solanum* (approx. 1500 sp.) or the family Melastomataceae (ca. 5000 sp.), buzz-pollination is even the dominant pollination strategy, likely representing extraordinary evolutionary success (Macior 1971, Renner 1989, Berger et al. 2016, Dellinger et al. 2019).

The tubular, poricidal anther structure of buzz-pollinated flowers is interpreted as the result of selection to minimize pollen loss to pollinators (Endress 1996, Amorim et al. 2017, Vallejo-Marín 2019). Pollen can be strictly dosed through the anther pore and

may be dispensed to many different pollinators throughout the flower's life-span (anthesis, Harder & Barclay 1994). In theory, dispensing small doses of pollen to several pollinators may increase chances of successful outcross-pollen transfer (Harder & Wilson 1994, LeBuhn & Holsinger 1998, Castellanos et al. 2006, Muchhala et al. 2010). The balance between optimal and overly strict dosing can be delicate, however. If pollinator visitation frequencies are very low, strict dosing is disadvantageous (Castellanos et al. 2006). Also, at the end of anthesis, no pollen should remain in the anthers. The few studies that have addressed these questions indicate that flowers can adjust their dosing strategies to pollinator visitation frequencies and change dispensing schemes over the course of anthesis (Larson & Barrett 1999, Sargent 2003, Castellanos et al. 2006). To maximize reproductive success, flowers would hence dose pollen more strictly at the beginning of anthesis, and dispense it more freely at the end (Larson & Barrett 1999). In buzz-pollinated flowers, the only outlet of pollen is the stamen pore. Thus, we hypothesize that adjustments of dosing may happen primarily by gradual enlargement of the stamen pore during anthesis.

Flowers may not only dose pollen within single stamens, but also between stamens within a flower, i.e. when presenting structurally different types of stamens. This condition is referred to as 'heteranthery' (Endress 1994, Vallejo-Marín 2010). Buzz-pollination, pollen rewards and heteranthery are significantly correlated across angiosperms (Vallejo-Marín 2010). The evolution of heteranthery has been explained by a 'division-of-labour' hypothesis, stating that different stamen types within a flower can carry different functions (Müller 1881, Luo et al. 2008, Vallejo-Marín et al. 2009, Mesquita-Neto et al. 2017). Specifically, it has been suggested that one stamen type may be involved in producing fodder pollen ('feeding' stamens) as pollinator reward while the other type produces pollination pollen ('pollination' stamens, Müller 1881). The different stamen types are often aggregated in different areas of the flower and differ in size so that they can deposit pollen on different parts of the pollinator's body (Luo et al. 2008, Amorim et al. 2017). Ideally, pollen from 'feeding' stamens should be deposited on body parts the bee can easily groom while pollen from 'pollination' stamens should be deposited on 'safe sites' like the bee's back (Luo et al. 2008). Recently, Kay & Jogesh (2017) proposed an alternative functional explanation for the evolution of heteranthery in that it may also provide a means of pollen dosing. Following this reasoning, one would presume that pollinators first exploit the showy stamens (e.g. more vividly coloured) and only later forage on the less attractive ones. In contrast to the 'division-of-labour' hypothesis, however, pollen from both stamen types would function in pollination and in feeding pollinators (Kay & Jogesh 2017).

We selected three buzz-pollinated plant species (*Senna reticulata* (Willd.) H.S.Irwin & Barneby (Fabaceae), *Adelobotrys adscendens* (Sw.) Triana, and *Conostegia subcrustulata* (Beurl.) Triana (both Melastomataceae)) to assess the adaptive significance of poricidal anthers in functioning as dynamic pollen dosing mechanisms. First, we artificially buzzed flowers of all three species to test whether pollen dosing changes over the course of anthesis, i.e. if older flowers release more pollen than younger flowers. Second, in the two heterantherous species (*S. reticulata*, *A. adscendens*), we tested whether the different stamen types differ in their pollen dosing strategies. Finally, in the heterantherous species *A. adscendens*, we assessed whether the different stamen types indeed deposit pollen in different areas of the pollinator's body as assumed by the 'division-of-labour' hypothesis.

Material and Methods

Study site and study species

Fieldwork for all three study species was conducted in a lowland tropical rainforest site in Costa Rica, i.e. in the area of the Tropical Research Station La Gamba, between February 13th and 22nd 2018. *S. reticulata* are shrubs or treelets with many multi-flowered inflorescences (Parolin 2001). Flowers are relatively large (diameter ca. 2 cm), pentamerous with free petals which form cup-shaped corollas (Amorim et al. 2017). *S. reticulata* is heterantherous and has four different stamen types (Fig. 1A): two long, lateral fertile stamens ('pollination stamens'), one central median stamen (often infertile), four short, central fertile stamens ('feeding stamens') and three sterile reduced staminodes (Marazzi et al. 2007). Recent pollination experiments have given support to the 'division-of-labour' hypothesis in *Senna* and complex co-functioning between petals and stamens in a ricochet pollination mechanism (Amorim et al. 2017). *A. adscendens* grows as a liana up to ca. 20 m into the canopy and also presents several densely flowered inflorescences. Flowers are ca. 1 cm in diameter, pentamerous and also have cup-shaped corollas (Fig. 1B, Dellinger et al. 2019). *A. adscendens* flowers are heterantherous and have two stamen types, five long, narrow stamens with corrugated thecal walls (Fig. 1D) and five short, wider stamens with smooth thecae (Fig. 1E, Dellinger et al. 2019). The function of these stamens has never been studied, but following 'division-of-labour' theory, we hypothesize that the long stamens may be 'pollination' stamens and the short ones 'feeding' stamens. *C. subcrustulata* grows as shrubs or trees with multi-flowered inflorescences but has smaller, hexamerous flowers (ca. 0.7 cm in diameter) with twelve isomorphic stamens (Fig. 1C, Kriebel 2016).

From our own observations prior to the start of experiments, we know that flowers of all species are anthetic for one day only. Flowers open in the early hours of morning (ca. 5 am) and wither in the late afternoon (*S. subcrustulata*, ca. 5 pm) or close in the early evening (*A. adscendens*, *C. subcrustulata*, ca. 7 pm).

Experiment 1 – Testing the effect of flower age and heteranthery on pollen dosing

In order to test if pollen dosing strategy changes over the course of anthesis, we performed artificial buzzing experiments on the three study species. For this purpose, several inflorescences were selected in four individuals of *S. reticulata* and *C. subcrustulata* each, and in two individuals of *A. adscendens*. In the selected inflorescences, all open flowers were removed and discarded and the entire inflorescences were bagged with mesh-nets (mesh size < 1mm) to prevent pollinator visitation. The following day, newly opened flowers were picked at three different times to capture different anthetic stages: shortly after sunrise (6:00–7:30, young), at midday (12:00, medium) and in the late afternoon before daily rainfall (16:30–17:00, old). In each species, a total of 25 flowers were collected per anthetic stage. 20 of these flowers were used for artificial buzzing experiments, and five flowers were used for pore size measurements (see section 'Pore size measurements'). Flowers were transferred to the air-conditioned laboratory of the Tropical Research Station La Gamba immediately after collection to perform artificial buzzing experiments. Following the method of Amorim et al. (2017) developed for studying pollination in *Senna*, we modified an electric toothbrush (Philips Sonicare Flexcare Platinum HX9111/20) by exchanging the brush with a sewing needle to apply artificial vibrations to flowers.

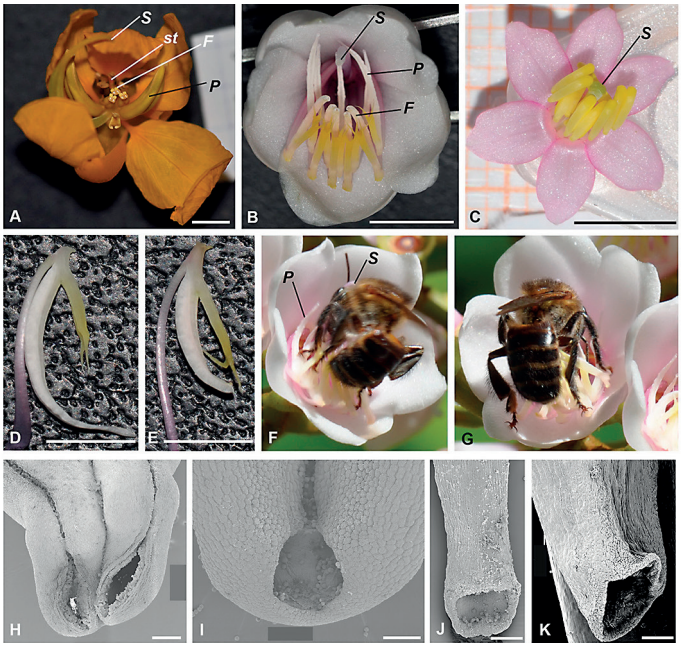

Fig. 1: Flowers of the three study species, details of stamen dimorphism and pollination of *Adelobotrys adscendens* by *Melipona costaricensis*, and SEM images of stamen pores. A) Enantiostylous (S) and partially asymmetric flower of *Senna reticulata* with two large pollination stamens (P, lateral) and four centrally arranged feeding stamens (F) and staminodes (st), the curved petals possibly function in the ricochet-mechanism of pollen transfer (as documented for other *Senna* species, see Amorim et al. 2017). B) Flower of *A. adscendens* with dimorphic and monosymmetrically arranged stamens. C) Flower of *Conostegia subcrustulata* with isomorphic stamens arranged in a weakly monosymmetric pattern; note green, capitate stigma. D) Long stamen (hypothesized pollination stamen) of *A. adscendens*. E) Short stamen (hypothesized feeding stamen) of *A. adscendens*. F), G) *A. adscendens* buzzed by its main pollinator, *M. costaricensis*; note how the bee crouches above the entire androecium, the short stamens pointing at its belly and the long stamens at its head. H) Stamen pore of pollination stamen of *S. reticulata*. I) Stamen pore of *C. subcrustulata*. J) Stamen pore of long (pollination) stamen of *A. adscendens*. K) Stamen pore of short (feeding) stamen of *A. adscendens*. P – pollination stamen, F – feeding stamen, S – stigma, st – staminodes. Scale bars: A–E: 0.5 mm, H: 200 µm, I–K: 100 µm. – Abb. 1: Blüten der drei Untersuchungsarten, Details der unterschiedlichen Staubblatttypen und Bestäubung bei *Adelobotrys adscendens* durch *Melipona costaricensis* sowie SEM Bilder von Staubblattporen. A) Enantiostylie (S) und teilweise monosymmetrische Blüte von *Senna reticulata* mit zwei großen Bestäubungsstaubblättern (P, seitlich), und vier zentralen Futterstaubblättern (F) und Staminodien (st), die gekrümmten Petalen fungieren möglicherweise als Reflektoren beim Pollentransfer (wie bei anderen Arten der Gattung *Senna* dokumentiert, Amorim et al. 2017). B) Blüten von *A. adscendens* mit zwei Staubblatttypen in monosymmetrischer Anordnung. C) Blüte von *Conostegia subcrustulata* mit nur einem Staubblatttyp, der schwach monosymmetrisch angeordnet ist, Stigma grün. D) Langes Staubblatt (wahrscheinlich mit Bestäubungsfunktion) von *A. adscendens*. E) Kurzes Staubblatt (wahrscheinlich mit Fütterfunktion) von *A. adscendens*. F), G) *M. costaricensis*, Hauptbestäuber von *A. adscendens*; die Biene krümmt sich über das gesamte Andrözium, die kurzen Staubblätter sind auf den Bauch, die langen Richtung Kopf gerichtet. H) Staubblattpore eines Bestäubungsstaubblattes von *S. reticulata*. I) Staubblattpore von *C. subcrustulata*. J) Staubblattpore des langen Staubblatts von *A. adscendens*. K) Staubblattpore des kurzen Staubblatts von *A. adscendens*. P – Befruchtungsstaubblätter. F – Beköstigungsstaubblatt. S – Stigma. st – Staminodien. Maßstabsbalken: A–E: 0,5 mm, H: 200 µm, I–K: 100 µm.

The basic and peak frequency of vibrations produced by this toothbrush are in the range of frequencies produced by buzzing carpenter bees (basic frequency 246.2 Hz, peak frequency 246 Hz, Amorim et al. 2017). Before artificial buzzing, we removed all petals from the flower. To vibrate all stamens equally, the needle was then inserted in the flower's floral base (just below the superior ovary), in a 90° angle to the flower's median plane (Amorim et al. 2017). Each flower was vibrated for 0.5 to 0.75 seconds at highest intensity (level three of the toothbrush). All ten stamens were then collected into a 1.5 ml Eppendorf tube filled with 700 µl of 70 % ethanol for later pollen counting. In addition, 10 freshly opened, unvisited (young) flowers were collected for each species and put into 70 % ethanol without further manipulation to serve as references for pollen counts (see below, referred to as "virgin" from here onwards).

Experiment 2 – Heteranthery as 'division-of-labour' (differential pollen placement)

Differential pollen placement has been shown for some *Senna* species by artificial buzzing and using surrogate bees (Amorim et al. 2017). We adopted a similar approach to testing the 'division of labour' versus the 'pollen dosing' hypothesis in heterantherous *Adelobotrys adscendens*. As for the pollen dosing experiment, we bagged inflorescences of two individuals with mesh-nets and collected freshly anthetic flowers the next morning (between 7:30 and 9:30, n = 57). Flowers were transferred to the laboratory immediately after collection. We then split flowers into three treatment classes: i) intact flowers (n = 20), ii) flowers with long stamens only (n = 19; we removed short stamens with forceps), iii) flowers with short stamens only (n = 18; we removed long stamens with forceps). We then prepared microscope slides by marking a 2 cm cross in the centre of the slide (Fig. 2A). A single flower was positioned above the slide, with the flower centre (pedicel) above the intersection of the cross and the style in the lower half of the cross (Fig. 2B, C). We then inserted the needle of the electric toothbrush in the hypanthium base as described above and artificially buzzed the flower for approximately 1 second at highest intensity (Fig. 2C). We photographed microscope slides under a stereomicroscope, always using the same settings. We prepared photos for further analyses using Photoshop (CC-Version 19.x). To improve photo quality, images were masked with an 80 % sharpening filter, converted into a negative image and any colouration of the background was removed. Further analyses were performed in the R-package *EBImage* (Pau et al. 2010): pollen images were converted into black-and-white-images and the coordinates of the black pixels, representing pollen grains, were extracted. For each treatment class, all images were combined to later obtain the average pollen grain distribution. We used the R-package *spatstat* (Baddeley et al. 2013) to convert the pixel distributions into point-pattern-datasets and calculated convex-hulls around the pixel (pollen) areas. We used Kernel-density functions with Diggle's edge correction to estimate pixel (pollen) densities in the polygons (Diggle 1985). To evaluate average pixel distributions, we drew 100 random subsets of the mean number of pixels of each treatment group (intact 14,184 pixels, short only 12,520 pixels, long only 2,419 pixels) from the combined pixel distributions and calculated the average density across these subsets. In addition, to only assess differences in pollen placement (not masked by differences in average pollen deposition), we drew another 100 random subsets of 1,000 pixels each from the three treatment groups. We then plotted and visually compared pollen grain distributions from the three treatments and the two subsets. We further tested for spatial clustering of pollen grains using the Clark-Evan's test, which

compares the observed distribution of points to the expected distribution under a completely random Poisson process (Clark & Evans 1954).

We also counted pollen grains remaining in the stamens after artificial buzzing following the methods described below.

Pollen counting

We quantified the amount of pollen grains remaining in the anthers after artificial buzzing using a particle counter (Topas Particle Counter FAS362B, also see Vallejo-Marín et al. 2009). In the two heterantherous species, we randomly selected stamens of each type (one 'feeding' and two 'pollination' stamens in *Senna reticulata* and two stamens of each type in *Adelobotrys adscendens*) per flower for pollen counting. In *Conostegia subcrustulata*, we selected two stamens at random. We put single stamens into fresh 1.5 ml microcentrifuge tubes filled with 1,000 µl of purified water and squeezed them with an Eppendorf micropestle (SigmaAldrich) to rip open stamens to excise pollen. We then placed the tubes

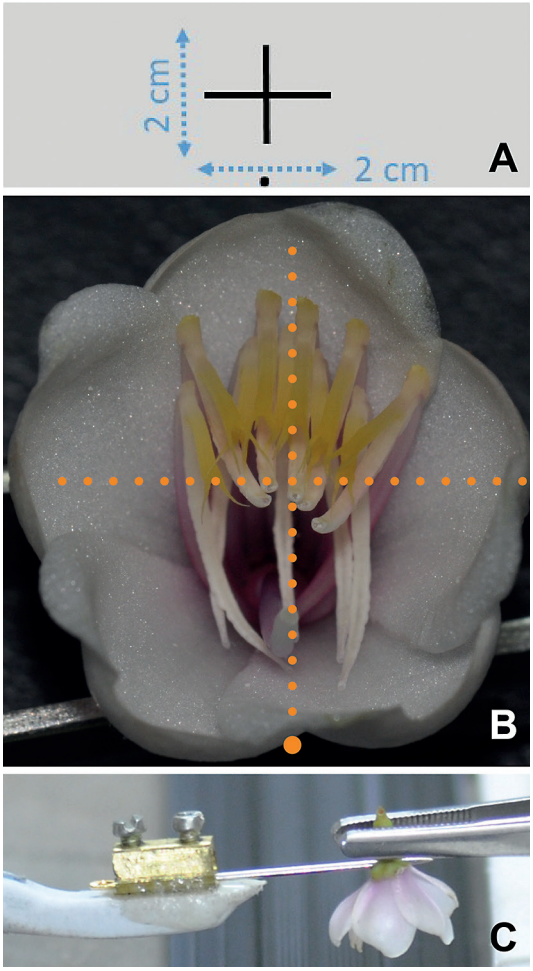

Fig. 2: Setup of experiment 2. A) Microscope slide with 2x2 cm cross marked, the flower center was arranged above the cross, the dot was used to always arrange flowers with appendages pointing to the upper side of the slide and the style towards the dot at the lower side. B) Intact flower, the orange cross indicates the flower arrangement above the cross marked on the microscope slide, the dot is shown on the side of the style. C) Setup of artificial buzzing, the flower is held upside down over the microscope slide, the needle is inserted into the hypanthium and tweezers are used to prevent the flower from turning sideways. – Abb. 2: Aufbau von Experiment 2. A) Objektträger mit 2x2 cm Kreuz, über welchem das Zentrum der Blüte positioniert wurde; der Punkt diente dazu, die Blüte stets so auszurichten, dass die Staubblattanhängsel in Richtung des oberen Randes des Objektträgers deuteten und der Griffel Richtung Punkt am unteren Rand wies. B) Intakte Blüte, das orange Kreuz zeigt, wie die Blüte über dem Kreuz am Objektträger ausgerichtet wurde, ebenso ist der Punkt markiert. C) Künstliche Vibration: Die Blüte wird mit der Kronenöffnung nach unten zeigend über den Objektträger gehalten, die Nadel der adaptierten elektrischen Zahnbürste steckt im Hypanthium, die Pinzetten dienen zum Stabilisieren der Blüte bei der künstlichen Vibration.

into an ultrasonication bath for 5 minutes to break up pollen clumps and assure complete pollen release from the stamens. We then injected 100 µl of each sample into the particle counter (duration of count per sample 10 seconds). The FAS362B counter can distinguish 62 size classes between 2 and 200 µm. In order to only analyse size classes in the size range of pollen grains (and not possible residual plant material from sample preparation), we measured pollen grain sizes under a light microscope prior to counting. We also used histograms to plot the frequency distribution of all counted particles to cross-check that the size classes representing pollen grains would be selected. Based on measurements and visual inspection of the count data, we chose the following size classes: *S. reticulata* 17.96–28.55 µm, *A. adscendens* 7.75–11.58 µm and *C. subcrustulata* 10.7–15.4 µm (starting at 9.4 µm for freshly opened, virgin stamens, see Results). We summed up particle counts for the chosen size classes to obtain total pollen number per count. The total number of pollen grains was multiplied by 10 to obtain the total amount of pollen grains per stamen.

Pore size measurements

For each species, pore sizes were measured for two stamens from five flowers for the three anthetic stages (n = 30 stamens per species). In heterantherous *S. reticulata* and *A. adscendens*, we selected one stamen per type from each flower. We prepared single stamens for Scanning Electron Microscopy (SEM) by dehydration over an ethanol series, critical point drying (CP Autosamdri-815), coating with gold (Sputter Coater (SCD 050)) and mounting them onto aluminium stubs. Samples were scanned and photographed in a JEOL JSM-6390 at 10 kV. Pore surface area was measured with the software Fiji/ImageJ (version 1.51w, Schindelin et al. 2012).

Statistical analyses

All statistical analyses were performed in R (R Developmental Core Team 2018). We first calculated the mean number of pollen grains remaining in single stamens after buzzing at different anthetic stages (Tab. 1). For *S. reticulata* and *A. adscendens*, we tested whether the two stamen types differed significantly in pollen amount of virgin flowers using GLMMs (Generalized Linear Mixed Effects Models, *lmerTest*, Kuznetsova et al. 2017), treating stamen type as fixed factor and flower ID as a random effect; data on plant individuals was not recorded.

To assess pollen dosing, we subtracted the number of pollen grains remaining after artificial buzzing in young, median or old stamens from the number initially present in virgin stamens. We used the maximum number of pollen grains detected in virgin stamens as reference for subtraction. In the heterantherous species, we used respective maximum values for the different stamen types. We chose the maximum reference value over the mean since some pollen removal estimates were negative (i.e. fewer pollen grains in virgin than in artificially buzzed flowers; for similar problems, see Muchhala & Thomson 2010). Using the maximum reference value, two buzzed samples in *A. adscendens* and *C. subcrustulata*, respectively, and eight in *S. reticulata* still had higher pollen counts than the reference maximum; these samples were excluded from analyses. For possible methodological shortcomings, see Discussion. To test for changes in pollen dosing over anthesis, we ran GLMMs on the amount of pollen removed from stamens by artificial buzzing, setting the three different anthetic stages and stamen type as fixed factors and flower ID as random effect, using a Poisson family for the model and the initial amount of pollen present in

each stamen type as offset. For *C. subcrustulata*, we ran a linear model on the amount of pollen removed with the different anthetic stages as fixed factor. We calculated the proportion of removed pollen to compare pollen dosing rates between species and stamen types.

Differences in pore area between the three anthetic stages were evaluated using ANOVAS (homogeneity of variance tested by Levene's test and normality by Shapiro-Wilk-test). We did not test for changes in pore area over anthesis for the two stamen types separately due to small sample sizes.

To assess differences in pollen release between the two stamen types in *A. adscendens* (experiment 2), we ran a GLM with the initial number of pollen grains for each stamen type as offset and a quasipoisson family.

Results

Pollen grains are small in all species, largest in *S. reticulata* (20.3–28 µm) and smaller in *C. subcrustulata* (10–14.7 µm) and *A. adscendens* (8.0–11.8 µm).

We found a maximum of 100,940 pollen grains in individual virgin stamens of *S. reticulata*, 58,730 in *A. adscendens* and 32,975 in *C. subcrustulata* (Tab. 1 for averages). Virgin pollinating (large) stamens contained eight times more pollen than virgin feeding (small) stamens in *S. reticulata* (GLMM: *t-value 8.70, df = 15, p < 0.01*) and 1.4 times more pollen in *A. adscendens* (GLMM: *t-value 5.32, df = 17, p < 0.01*, Tab. 1). The staminodes did not differ significantly in pollen amount from small feeding stamens in *S. reticulata* (GLMM: *t-value –1.08, df = 19, p = 0.291*) and were not considered in subsequent analyses.

Tab. 1: Mean number (and standard deviation) of pollen grains in virgin stamens and pollen grains remaining after artificial buzzing (experiment 1) per stamen type in the different anthetic stages. – Tab. 1: Durchschnittliche Anzahl (und Standardabweichung) der Pollenkörner in unmanipulierten Staubblättern und nach künstlicher Vibration (Experiment 1) je Staubblatttyp und unterschiedlichem Anthesezeitpunkt.

species	stamen type	mean (sd) no. of pollen grains				
		virgin	young	medium	old	pore area (µm²)
S. reticulate	pollinating	63,109 (24,060)	34,819 (28,562)	16,906 (19,922)	23,693 (25,099)	1,558.4 (434.6)
	feeding	7,919 (2,478)	7,848 (2,863)	7,292 (5,502)	5,632 (4,069)	1,490.9 (895.7)
	staminodes	1,253 (732)	1,920 (774)	1,210 (729)	840 (–)	–
A. adscendens	pollinating	45,302 (7,505)	37,089 (15,326)	36,058 (11,103)	33,921 (9,471)	14,364.3 (3,479.9)
	feeding	32,069 (7,498)	31,980 (10,771)	31,611 (8,276)	27,474 (8,392)	27,178.9 (5,082.9)
C. subcrustulata	–	20,980 (7,843)	15,693 (8,746)	16,907 (6,384)	14,312 (8,278)	34,741.5 (9,690.5)

Experiment 1 – Testing the effect of flower age and heteranthery on pollen dosing

In *S. reticulata*, we found pollen dosing both through flower age and heteranthery (Fig. 3A). More than 50 % of pollen grains were released on average by artificial buzzing at any anthetic stage (young 51 %, medium 68 %, old 70 %). Pollination stamens released on average 30 % more pollen than feeding stamens. Both stamen types released significantly less pollen at the young stage than at older stages (pollination stamens: z-value –2.38, p = 0.02; feeding stamens: z-value –4.03, p < 0.001). Medium and old-stage stamens of both

types did not differ in the amount of pollen released (pollination stamens: z-value –0.92, p = 0.36; feeding stamens: z-value 0.318, p = 0.75).

In *A. adscendens*, we only found pollen dosing through heteranthery, but not flower age (Fig. 3B). Long stamens released significantly more pollen (41 %) than short stamens (36 %; z-value 76.39, p < 0.01, Fig. 3B). Pollen release did not change significantly over anthesis in either stamen type, however (long stamens: young vs. medium: z-value –0.32, p = 0.75; medium vs. old: z-value 0.15, p = 0.88; short stamens: young vs. medium: z-value –1.05, p = 0.29; medium vs. old: z-value 1.19, p = 0.23).

In isomorphic *C. subcrustulata*, we did not find pollen dosing through flower age (Fig. 3C, F = 1.46, df = 2, p = 0.24). Artificial buzzing released ca. 56 % of pollen in young, 49 % in medium and 60 % of pollen in old flowers.

Pore area did not increase over anthesis in *S. reticulata* (*F 0.007, df = 2, p = 0.993*) and *A. adscendens* (F 1.08, df = 2, p = 0.35). Pore area increased 1.5 times from young to old flowers in *C. subcrustulata* (F 12.7, df = 2, p < 0.01; young 25.051 µm², old 39.337 µm²). In *S. reticulata*, the two stamen types had similar pore areas while in *A. adscendens*, pore areas of short (feeding) stamens were almost twice the size of pore areas of long (pollinating) stamens (Tab. 2, Fig. 1 H-K).

Tab. 2: Average pore area (µm²) per stamen type for all anthetic stages. – Tab. 2: Durchnittliche Porenfläche (µm²) pro Staubblatt für alle Zeitpunkte der Anthese.

species	type	young	middle	old
S. reticulata	pollinating	1,484 (549)	1,670 (112)	–
	feeding	1,547 (1,048)	1,295 (842)	1,484 (731)
A. adscendens	pollinating	16,453 (5,433)	14,058 (2,654)	13,089 (1,479)
	feeding	26,542 (2,955)	29,659 (6,853)	24,508 (1,621)
C. subcrustullata	-	25,051 (3,370)	38,087 (9,295)	39,337 (8,027)

Experiment 2 – Heteranthery as 'division-of-labour' (differential pollen placement) or dosing strategy

Image analyses showed that pollen from *A. adscendens* flowers was deposited in similar areas on microscope slides across all treatments (intact flower, short (feeding) stamens only, long (pollination) stamens only, Fig. 3). In all treatments, we found significant clustering of pollen grains (highest pollen density) in the centre of the slide and gradual decrease in pollen density around this centre (Clark-Evan's Test: intact: *R = 0.005, p < 0.01*; long stamens only: *R = 0.002, p < 0.01*, short stamens only: *R = 0.003, p < 0.01*). Interestingly, we found that short (feeding) stamens contributed ca. 75 % of total pollen deposited while long (pollinating) stamens only contributed around 25 % of total pollen deposited, despite the latter having more initial pollen present (Fig. 4A). This observation was confirmed when testing for significant differences in the relative amounts of released pollen between the two stamen types: on average, short stamens released more than twice as much pollen as long stamens (32 % of total pollen released compared to 14 %; *t-value –3.77, df = 40, p < 0.01;* Fig. 3). When sub-setting the three treatments to 1,000 pollen grains each to only assess differences in pollen deposition density, we found that pollination stamens deposited pollen in higher densities in the centre of the slide while short stamens distributed pollen more evenly across the slide (Fig. 4B).

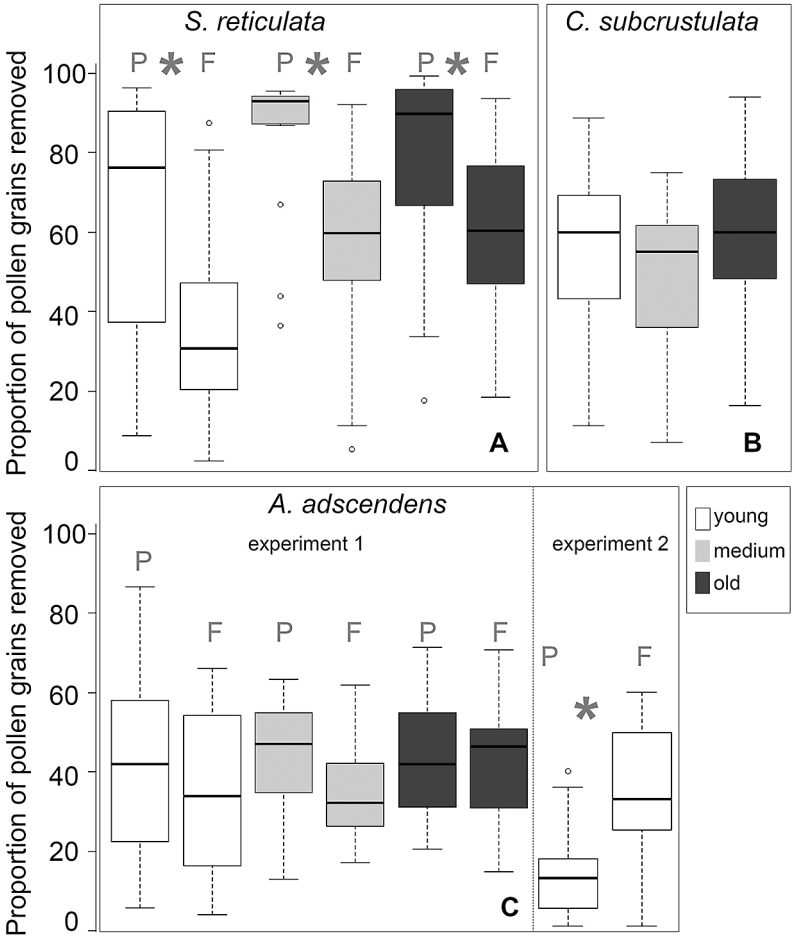

Fig. 3: Box-whisker-plots representing proportion of pollen grains removed at different anthetic stages and from different stamen types (P – pollination stamens, F – feeding stamens). A) Pollination stamens released significantly more pollen than feeding stamens in *S. reticulata*, but only feeding stamens released significantly more pollen grains at later than younger anthetic stages (see text). B) There was no significant difference in pollen release in *C. subcrustulata*. C) There was no significant difference in pollen release between anthetic stages or stamen types in experiment 1 in *A. adscendens,* but in experiment 2, short (feeding) stamens released significantly more pollen grains. P - pollination stamens, F - feeding stamens, * marks significant differences between stamen types. – Abb. 3: Die Box-Whisker-Plots zeigen die relativen Pollenmengen, die zu unterschiedlichen Zeitpunkten der Anthese aus den Blüten bzw. den unterschiedlichen Staubblatttypen entfernt wurden (P – Befruchtungsstaubblätter, F – Beköstigungsstaubblätter). A) Bei *S. reticulata* setzten die Befruchtungsstaubblätter signifikant mehr Pollen frei als die Beköstigungsstaubblätter, doch nur die Beköstigungsstaubblätter setzten zu späteren Anthesezeitpunkten signifikant mehr Pollen frei als zu früheren Anthesezeitpunkten. B) Bei *C. subcrustulata* gab es keine signifikanten Unterschiede in der Pollenfreisetzung zwischen den unterschiedlichen Anthesezeitpunkten. C) Bei *A. adscendens* wurden in Experiment 1 weder signifikante Unterschiede in der Menge des freigesetzten Pollens noch Unterschiede zwischen den Anthesezeitpunkten festgestellt. In Experiment 2 jedoch setzten die kurzen Beköstigungsstaubblätter signifikant mehr Pollen frei. P – Befruchtungsstaubblätter, F – Beköstigungsstaubblätter, * zeigt signifikante Unterschiede zwischen den Staubblatttypen an.

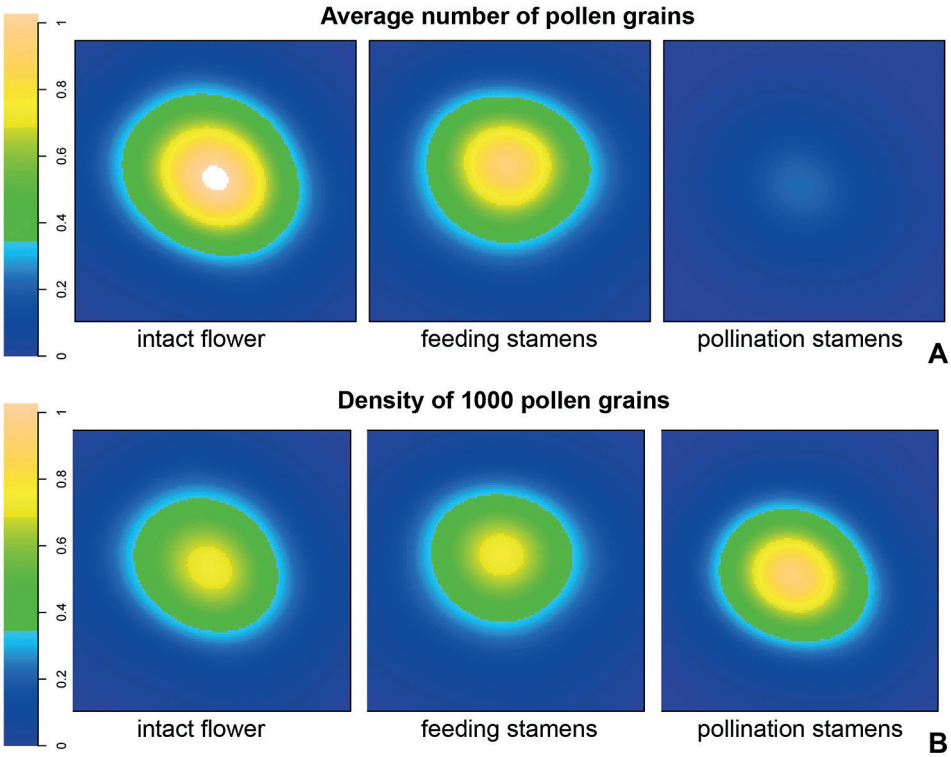

Fig. 4: Pollen densities deposited by artificial buzzing of intact *A. adscendens* flowers and flowers with feeding or pollination stamens only; the scale indicates pollen density (0 – lowest density, 1 – highest density). A) The average number of pollen grains deposited per treatment is shown, with high densities in the centre of the microscope slide by intact flowers and flowers with only feeding stamens and low densities in the same area by flowers with only pollination stamens. B) The average density of deposition of 1,000 pollen grains is shown for the three treatments, with highest densities deposited in the centre of the slide by pollination stamens. – Abb. 4: Pollendichten, die durch die künstliche Vibration von intakten und manipulierten Blüten von *A. adscendens*, auf dem Objektträger abgelagert wurden; der Maßstab zeigt die Pollendichte an (0 – niedrigste Dichte, 1 – höchste Dichte). A) The mittlere Anzahl an Pollenkörnern die je Experimentansatz (intakte Blüte, nur Befruchtungsstaubblätter, nur Beköstigungsstaubblätter) abgeladen wurde. Bei intakten Blüten und Blüten mit Beköstigungsstaubblättern befinden sich die höchsten Dichten an Pollenkörnern im Zentrum der Objektträger, während diese Zone bei Blüten nur mit Befruchtungsstaubblättern eine sehr niedrige Dichte an Pollenkörnern aufweist. B) Die mittlere Ablagerung von 1000 Pollenkörner je Experimentansatz, wiederum befindet sich die höchste Dichte im Zentrum des Objektträgers, wobei die bedeckte Fläche durch die Befruchtungsstaubblätter am größten ist.

Discussion

In this study, we investigated the functional role of poricidal anthers as pollen dosing mechanisms in relation to flower age and heteranthery in three buzz-pollinated plant species. Our results demonstrate that pollen removal from poricidal anthers is restricted as only 36 % to 70 % of pollen grains were removed when flowers were buzzed artificially. In the three species studied, we detected three different patterns: differences in dosing through heteranthery and flower age (*S. reticulata*), differences in dosing through heteranthery only (*A. adscendens*) and no differences in dosing (*C. subcrustulata*).

Following Pollen Presentation Theory (PPT), a strict dispensing mechanism such as poricidal anthers should adjust pollen release to pollinator quantity (abundance) and quality (efficiency in transferring conspecific pollen; Harder & Barclay 1994, Harder & Wilson 1994, Castellanos et al. 2006). While freshly opened virgin flowers are expected to release only small doses of pollen (provided that visitation rates are high), old virgin flowers, which are at risk of not being visited a second time, should release larger amounts of pollen. Few experimental studies have tested these ideas, but support came from buzz-pollinated *Dodecatheon* where older flowers released about 20 % more pollen than young flowers (Harder & Barclay 1994). This pattern was found in *S. reticulata* only (Fig. 3A). In this species, average pollen release was high (often more than 60 % during a single buzz, Fig. 3A) while it was generally below 50 % in *A. adscendens* and intermediate in *C. subcrustulata*. These findings suggest that *S. reticulata* doses pollen less strictly at any anthetic stage. In addition, pollen release significantly increased in later stages. This increased pollen release could be a response to relatively low pollinator visitation rates or high pollinator efficiency (Castellanos et al. 2006). We have observed irregular visits of *Xylocopa* sp. and halictid bees to *S. reticulata*, but detailed data on visitation rates and pollinator efficiency in La Gamba are lacking. Snow & Roubik (1987) reported that large bees could remove about 15–25 % of pollen grains in *S. reticulata* while halictids removed negligible amounts only. To fully understand pollen dispensing schedules in this species, field observations of natural pollinators should be combined with artificial buzzing experiments and assessments of pollen deposition on stigmas. Likewise, the lower amounts of pollen released by *A. adscendens* and *C. subcrustulata* are difficult to interpret at the current stage of knowledge. We have observed *Melipona costaricensis* as the main pollinator of *A. adscendens* in La Gamba, and preliminary results suggest that visitation frequencies are around 1.8 visits per flower per hour between 6 am and 12 pm (Dellinger, unpublished results). Thus, visits are possibly frequent enough to justify the release of relatively small doses of pollen only. In *C. subcrustulata*, we found a significant increase in anther pore size in older flowers, possibly indicating an adjustment to less strict dosing in older flowers. However, this increase in pore size was apparently not enough to significantly increase pollen release as assessed by our buzzing experiments (Fig. 3).

Significant differences in initial pollen amount (virgin stamens) and pollen release patterns between the two stamen types in *S. reticulata* and *A. adscendens*, respectively, support the hypothesis that heteranthery serves as a within-flower dosing strategy (Kay & Jogesh 2017). In *S. reticulata*, the large pollination stamens contained significantly more pollen than the small feeding stamens, and they also released significantly more pollen than the small stamens during a single buzz (Fig. 3). These results suggest that pollination pollen is released quickly and upon the first visits a flower may receive, assuring pollen export and increasing male fitness. Both male and female fitness may benefit from the

more gradual pollen release from feeding stamens: the continued (and increasing) release of small doses of the pollen reward may assure further bee visits and each visit increases the chance of exporting and receiving pollen to/from different flowers or individuals (Muchhala et al. 2010). In *A. adscendens*, the long stamens contained significantly more pollen than the short stamens (Tab. 1). While we found significantly higher pollen release by the large stamens in experiment 1, results of experiment 2 are opposed: the short stamens released twice as much pollen (Fig. 3B, Fig. 4). Given that pore area of short stamens is almost twice as large as the pore area of long stamens and pollen can thus be released more easily (Fig. 1J, K, Tab. 2), we consider the significant differences detected in experiment 2 as meaningful. Also, anthers of short stamens are only about half the length (ca. 3.8 mm) of anthers of long stamens (ca. 7.6 mm) and have smooth thecal walls as opposed to corrugated thecal walls in long stamens. Thus, the distance that pollen has to travel through short anthers is not only much shorter, but the smooth thecal walls may allow for relatively unhindered passage and the bigger pore for less restricted release (Fig. 2D, E). This incongruence in results, together with the fact that some buzzed stamens contained more pollen than virgin reference stamens, point towards methodological shortcomings of our study. We cross-checked pollen counts from the particle counter by inspecting single samples under the light microscope to rule out effects of pollen clumping possibly confounding total pollen counts. We could rule out problems related to clumping as our treatment with the ultra-sonication bath effectively broke up possible pollen clumps. Single pollen grains were present in all samples inspected with the light microscope. Other reasons for the observed differences could be gradual pollen maturation with flower age. We made reference counts for virgin flowers of *S. reticulata* and *C. subcrustulata* collected at 7 am and at 12 pm. The mean number of pollen grains did not differ from the pollen counts obtained from virgin flowers collected at earlier stages. Also, we removed one stamen type in experiment 2 before buzzing. This alteration in the floral architecture may affect how vibrations are transmitted through the tissue and hence influence pollen dispensing. Finally, we cannot rule out that the application of buzzes itself has an effect on pollen grains, possibly affecting their electric charge. Further experiments are, however, required to clarify these issues. For future studies, one possible solution to the apparent variability in pollen numbers across individual flowers would be to collect a single virgin stamen from each experimental flower before the experiment, as a reference.

Kay & Jogesh (2017) have shown that dimorphic stamens in *Clarkia* function in staggered pollen dosing, with conspicuous stamens depleted first by bees and cryptic stamens only depleted at later stages. In contrast to *Senna* or *Clarkia*, bees visiting *A. adscendens* do not buzz only one stamen type but crouch above the entire androecium and buzz all stamens simultaneously. We argue that stamens in *Adelobotrys* carry mixed dosing and 'division-of-labour' functions. In our assessment of differential pollen placement on microscope slides (Fig. 4), we did not find support for differential pollen placement between the two stamen types. However, the fact that the two stamen types of *A. adscendens* differ in how densely they deposit pollen (Fig. 4B) supports the idea that pollen may indeed be deposited differently on the bee's body. Furthermore, pores differ in their orientation: pores of the long stamens are dorsally inclined and likely fire pollen at the bee's head and back (safe sites, Mesquita-Neto et al. 2017), while pores of short stamens are apically inclined and shoot pollen directly at the bee's face and belly (Fig. 1 J, K). It is possible that our approach of assessing pollen deposition on the flat surface of a microscope slide could

not pick up these small scale differences in the direction of pollen expulsion between the two stamen types. Pollen expulsion movement has been shown to be highly complex (e.g. pollen reflected by petals in *Senna*, Amorim et al. 2017) and clear differential pollen placement between *Adelobotrys'* stamen types may only be picked up on surrogate or real three-dimensional bees and in completely intact flowers.

Traditional 'division-of-labour' into pollination and feeding functions of stamens has been experimentally shown for some buzz-pollinated species of *Senna, Solanum rostratum* (Solanaceae) and the melastome species *Melastoma malabathricum* (Luo et al. 2008, Vallejo-Marín et al. 2009, Mesquito-Neto et al. 2017). Taking the idea of Kay & Jogesh (2017) one step further, we believe that stamens in *Adelobotrys,* and possibly many other buzz-pollinated flowers, show even more complex 'division-of-labour' between stamen types by combining differential dosing ('shift-working') with differential pollen placement. Bees likely direct their initial collection activity to conspicuous, short stamens which deposit pollen conveniently and abundantly on their bellies (Luo et al. 2008). We hypothesize that once these (feeding/early-shift) stamens are depleted, bees may start to buzz (often less showy) longer (pollination/late-shift) stamens. In this context, it would be interesting to assess pollen viability of the two stamen types in *A. adscendens*, as studies on other heteranthérous species have found mixed support for reduced viability of pollen from feeding stamens (Gross & Kukuk 2001, Luo et al. 2008, Mesquita-Neto et al. 2017). We agree with Luo et al. (2008) that viability of pollen from both stamen types may be maintained as a safeguard as long as pollen from either stamen type may be deposited on stigmas. Indeed, continued viability of pollen from both stamen types may be particularly important if stamens perform 'shift-working'. In case bees start actively buzzing pollination/late-shift stamens, they may have to re-adjust their position in the flower (e.g. move closer towards the pores in the flower centre) and thereby possibly transfer pollen deposited on different body parts (Mesquita-Neto et al. 2017). Experimental studies have shown that the size of the bee and fit with the flower has a major influence on how much pollen is transferred (Mesquita-Neto et al. 2017, Solís-Montero & Vallejo-Marín 2017). More detailed experiments are required to understand if and how mechanical fit between bees and flowers may affected the transfer of pollen of different stamen types. Furthermore, comparative studies may help to understand under which conditions selection may lead to the evolution of heteranthery and in which cases sufficient pollen dosing may be achieved by poricidal anthers only.

Taken together, our results underline the complex nature of stamen functioning in buzz-pollinated flowers. While we found some support for adjustments of pollen dosing strategies over anthesis, differential dosing between stamen types was apparent. Heteranthery may thus represent an elaborate combination of strategies maximizing reproductive success, including differential pollen placement ('division-of-labour') as well as differential dosing ('shift-working').

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Addresses:

Agnes S. Dellinger, Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Wiena. E-Mail: agnes.dellinger@univie.ac.at

Léa Pöllabauer, Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Wien. E-Mail: lea.poellabauer@hotmail.fr

Milan Loreti, Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Wien. E-Mail: loreti.milan@gmail.com

Janis Czurda, Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Wien. E-Mail: j.czurda@gmail.com

Jürg Schönenberger, Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Wien. E-Mail: juerg.schoenenberger@univie.ac.at

ZOBODAT - www.zobodat.at

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