

Stamen dimorphism in bird-pollinated flowers – investigating alternative hypotheses on the evolution of heteranthery

Running title: Stamen dimorphism in bird-pollinated flowers

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Author Contributions

ASD conceived and planned the study, ASD, DMFF and SA carried out the fieldwork, ASD analysed the data, ASD wrote the manuscript, DMFF, SA and JS revised the manuscript.

Acknowledgements

We thank the Estación Científica San Francisco and the Finca Truchas Selva Madre for support with fieldwork in Ecuador and Costa Rica. We further thank Constantin Kopper for help with fieldwork, Ruth Quint for support with calorimetric analyse of stamen tissue and Katharina Kagerl for assessments of stamen viability. This project was funded through FWF-grant 30669-B29 to ASD and JS.

Conflict of Interest Statement

There are not conflicts of interest.

Data and Code Availability

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/evo.14260](https://doi.org/10.1111/evo.14260).

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All datasets used in this study (i.e. molecular phylogeny and trait data on reward type and heteranthery for 63 Merianieae species, field data for *Axinaea confusa* and *A. costaricensis*) and R-skripts are freely available through the online repository dryad (<https://doi.org/10.5061/dryad.gxd2547m1>).

Abstract

Heteranthery, the presence of distinct stamen types within a flower, is commonly explained as functional adaptation to alleviate the ‘pollen dilemma’, defined as the dual and conflicting function of pollen as pollinator food resource and male reproductive agent. A single primary hypothesis, ‘division of labour’, has been central in studies on heteranthery. This hypothesis postulates that one stamen type functions in rewarding pollen-collecting pollinators and the other in reproduction, thereby minimizing pollen loss. Only recently, alternative functions (i.e. staggered pollen release), were proposed, but comparative and experimental investigations are lagging behind.

Here, we use 63 species of the tribe Merianieae (Melastomataceae) to demonstrate that, against theory, heteranthery occurs in flowers offering rewards other than pollen, such as staminal food bodies or nectar. Although shifts in reward type released species from the ‘pollen dilemma’, heteranthery has evolved repeatedly *de novo* in food-body-rewarding, passerine-pollinated flowers. We use field investigations to show that foraging passerines discriminated between stamen types and removed large stamens more quickly than small stamens. Passerines removed small stamens on separate visits towards the end of flower anthesis. We propose that the staggered increase in nutritive content of small stamens functions to increase chances for outcross-pollen transfer.

Keywords

Pollen loss, pollen reward, stamen dimorphism, male fitness, floral trait function, cross-fertilization

Introduction

Pollen dispersal by animal vectors represents a key process in plant evolution, and diverse strategies have evolved to attract and reward pollinators and to optimize pollen transfer (Barrett 2010, Minnaar et al. 2018). Bee-pollinated flowers are particularly interesting in this context, since bees actively collect pollen as fodder for their brood (Cane 2016). Pollen hence carries a dual and conflicting function in bee-pollinated flowers as protein-rich pollinator food resource and as reproductive agent (Endress 1996). This conflict of interest is commonly referred to as ‘pollen dilemma’ and is regarded as evolutionary driver of various adaptive strategies hindering pollen collectability by bees, such as increases in pollen grain size and spiny ornamentation (but see Konzmann et al. 2019 for experimental testing and contrasting findings). While the ‘pollen dilemma’ applies to all bee-pollinated flowers, it is likely most pronounced in flowers rewarding pollinators only with pollen (pollen flowers, Endress 1996). One prominent morphological floral feature that is evolutionarily linked to pollen-rewarding is heteranthery (Vallejo-Marín et al. 2010). Heteranthery refers to a state where a single flower carries stamens of two (or more) distinct types, differing in colour, size, pollen content, shape or scent profile (or combinations of these traits), and, consequently, in attractiveness to pollinators (Vallejo-Marín et al. 2009, Solís-Montero et al. 2017).

Heteranthery is rare overall but has evolved repeatedly and occurs in at least 12 orders across angiosperms (Vallejo-Marín et al. 2010). Although subject to experimental and comparative research for more than 140 years, our current understanding of the evolution and adaptive significance of heteranthery builds on a small set of studies and tests of a single primary hypothesis: ‘division of labour’ (but see recent work by Kay et al. 2020, Konzmann et al. 2020). The ‘division of labour’ hypothesis, put forward by Müller and investigated and discussed intensively also by Darwin, postulates that the different stamen types found within a flower carry different functions during the pollination process (Müller 1881, Müller 1883, Darwin to Hooker 1862, 1881, Telles et al. 2020). Specifically, the visually more distinctive stamen type (‘feeding stamens’) is supposed to function in rewarding the pollen-collecting pollinator, since it will focus its foraging activity primarily on this stamen type (Luo et al. 2008, Vallejo-Marín et al. 2009). The less conspicuous stamen type (‘pollinating stamens’), on the other hand, is not actively manipulated by the pollinator and functions in placing pollen on areas of the pollinator’s body which it cannot groom (‘safe sites’, Konzmann et al. 2020). In support of the ‘division of labour’ hypothesis, infertile pollen and lower amounts of pollen grains of feeding stamens compared to pollinating stamens (Ferreira & Araùjo 2016, Velloso et al. 2018) and bees rejecting flowers lacking feeding stamens (Mesquita-Neto et al. 2017) have been reported. Recently, however, studies have also demonstrated that bees do not always distinguish between stamen types and may equally exploit all stamens, challenging the ‘division of labour’ hypothesis as ubiquitous explanation (Brito et al. 2020a, Telles et al. 2020).

Staggered pollen release (pollen dosing), i.e. through the sequential ripening and exploitation of the different stamen types, has been proposed as alternative (or additional) hypothesis for the function of heteranthery (Dellinger et al. 2019a, Kay et al. 2020, Telles et al. 2020; see Castellanos et al. 2006 for pollen dosing strategies in non-heterantherous

species). Kay et al. (2020) found, for example, that in *Clarkia* (Onagraceae), the showy stamen whorl is depleted before the inconspicuous one. Later in anthesis, however, pollinators will shift their foraging to the inconspicuous whorl. This finding has important implications when thinking about male fitness and siring success (Kay et al. 2020) as well as about female fitness. Generally, reproductive success should be optimized if a pollinator delivers purely conspecific pollen loads, originating from several genetically different pollen donors (Paschke et al. 2002, Kron & Husband 2006, Breed et al. 2012, Minnaar et al. 2018). Consequently, one may expect selection to act towards increasing the number of independent foraging trips to a flower, thereby maximizing chances of receiving different (ideally outcross) pollen loads (increasing both male and female fitness) and distributing pollen to many different vectors (increasing male fitness, Kay et al. 2020). If important, this strategy may occur both in pollen-rewarding systems and in systems offering alternative rewards.

Finally, heteranthery is significantly associated with a functionally highly specialized pollination strategy commonly occurring in pollen-rewarding flowers: buzz pollination by bees (Vallejo-Marín et al. 2010). In buzz-pollinated flowers, pollen is concealed within poricidal anthers, i.e. anthers which only open by a small pore (in contrast to longitudinally dehiscent anthers found in ca. 90% of angiosperms; Buchmann 1983, Endress 1996). Pollen can only be removed in small doses from these poricidal anthers when bees apply specific vibrations (buzzes) to the flowers (Harder & Wilson 1994, Dellinger et al. 2019a, Kemp & Vallejo-Marín 2020). Thus poricidal anthers and buzz-pollination are generally interpreted as strategies to alleviate the ‘pollen dilemma’ through pollen dosing. Within buzz-pollinated lineages, heteranthery may be labile and evolve repeatedly in non-autogamous species (Melo et al. 2021). Recent studies have shown, however, that poricidal anthers may also occur in species, which offer alternative rewards such as nectar or food bodies, and are pollinated by vertebrates, which do not actively forage for pollen (Dellinger et al. 2014, Dellinger et al.

2019b). Hence, if the ‘pollen dilemma’ is the ultimate driver of the evolution of heteranthery, heteranthery should not evolve in lineages that have shifted to non-pollen rewards and may even be lost in the evolutionary history of such lineages. Alternatively, if heteranthery is indeed (also) functional in staggered pollen release, thereby increasing chances of outcrossing, heteranthery may undergo a change of function and should prevail, or even evolve, also in lineages providing alternative pollinator rewards.

The Neotropical Melastomataceae tribe Merianieae offers an excellent opportunity for investigating evolutionary patterns and the functioning of heteranthery in association with shifts in reward type. Merianieae (302 species) are ancestrally buzz pollinated by bees, and buzz pollination occurs in ca. 74% of species (Dellinger et al. 2019b, in press). The tribe is characterized by repeated independent shifts to passerine pollination (at least three times, ca. 17% of species) and to a mixed vertebrate pollination strategy (at least three times, ca. 9% of species) (Dellinger et al. *in press*). These pollinator shifts are, according to our current understanding, always correlated with shifts in reward type: from pollen rewards in bee-buzz-pollinated species to sugary staminal food body rewards in passerine-pollinated species, or to nectar rewards in mixed-vertebrate-pollinated species (Dellinger et al. 2019b). Further, the shifts in reward type go hand in hand with changes in pollen expulsion mechanisms (Dellinger et al. 2019b). The bee-pollinated Merianieae are classical buzz pollinated flowers where the application of specific vibrations by bees causes pollen release (Dellinger et al. 2019a). In the passerine-pollinated species, inflated bulbous stamen appendages (which also constitute the above-mentioned food body rewards Fig. 2) function as a “bellows” organ for pollen release (Dellinger et al. 2014). The flower-visiting passerine birds grasp the bulbous appendages with their bills and rip out entire stamens one by one to consume them. When they compress the bulbous appendage with their bill, they trigger the “bellows” mechanism, i.e. the compression of the inflated appendage forces the contained air into the anther and

blows out the pollen grains (Dellinger et al. 2014). Importantly, the passerines rarely remove all stamens in a single visit but return later on the same or on another day to remove the remaining stamens (Dellinger et al. 2014). Finally, in the mixed-vertebrate-pollinated species, flowers are pendant and stamens are arranged in a way that when foraging vertebrates insert their mouthparts into the flowers to take up nectar, they push against the stamens and trigger pollen release ('salt shaker' mechanism, Dellinger et al. 2019c).

Here, we use 63 species of Merianieae to test whether heteranthery is significantly associated with bee buzz pollination and pollen rewards, as expected if the 'pollen dilemma' is the primary driver of the evolution of heteranthery, or, alternatively whether it has also evolved *de novo* in species offering alternative rewards. We demonstrate that strong heteranthery is overall scarce in Merianieae, but moderate to strong heteranthery has evolved repeatedly and, against expectations, is commonly found in food-body-rewarding passerine-pollinated species. In a second step, we use field surveys to investigate the potential function of heteranthery in two of these passerine-pollinated species. We observe that passerines consistently remove large stamens early at anthesis and before small stamens. When assessing the nutritive value of the stamen appendages (food body reward), we find that large stamens are twice as nutritious as small stamens early at anthesis and that small stamens double in nutritive value towards the end of anthesis, when they finally get removed. We hence propose that heteranthery functions as pollen dosing strategy to assure multiple flower visits and increase chances for outcross pollen transfer in passerine-pollinated Merianieae. Overall, our results highlight the need to reconsider possible drivers of the evolution of heteranthery (Kay et al. 2020) and expand investigations to testing multiple, likely non-exclusive hypotheses.

Methods

Coding for heteranthery and reward type

In all Merianieae, an outer whorl of usually five antesepalous stamens can be distinguished from an inner whorl of usually five antepetalous stamens. The stamens of the two whorls may differ in size, shape, colour, and orientation of their filaments, pollen-containing anthers and/or stamen appendages (Fig. 1), and the outer whorl often is showier than the inner whorl. Here, we extracted information on heteranthery from Dellinger et al. (2019b), who had investigated ethanol-preserved floral material and field images, and coded for general differences in size (i.e. anther length, appendage dimensions), shape (i.e. appendage shape), structure (i.e. thecal wall structure) and colour (as perceived by humans) between stamen whorls (Table S1). We considered species as “strongly dimorphic” when stamen whorls differed in all three parameters; when whorls differed in one or two parameters only (e.g. size), we considered them as “weakly dimorphic”; and when stamen whorls presented no clear differences in any of these parameters, we coded them as “isomorphic”. Please note that none of the investigated species differed in colour only; we may hence rule out erroneous classification as strongly or weakly dimorphic based on human colour vision.

We also extracted information on reward types from Dellinger et al. (2019b), who had performed detailed studies on stamens to reliably determine the reward type. In nectar-rewarding species, nectar is secreted through distinct ruptures on the filaments, visible by eye or using Scanning Electron Microscopy, and nectar droplets are visible when fresh flowers are observed in the field (Dellinger et al. 2019c). In food-body-rewarding species, the foraging passerine birds rip the bulbously inflated (food body reward) stamens out of the flower (Dellinger et al. 2014), so that anthetic flowers are often missing one or several entire

stamens. This reward type is hence easily identified when assessing photos, herbarium vouchers or ethanol-preserved material. Finally, pollen-rewarding species are characterized by all stamens being present throughout anthesis, non-bulbous stamen appendages and smooth filaments, lacking conspicuous ruptures (Dellinger et al. 2019b). As a side note, empirical pollinator observations are available for 25 of the 63 Meranieae species studied here (Table S1).

Testing for correlated evolution between heteranthery and reward type

To assess relations between heteranthery and reward type in a phylogenetic framework, we used the 7-marker Bayesian molecular phylogeny for Meranieae of Dellinger et al. 2019d. We pruned the original phylogeny with 186 tips to only include the 63 taxa included in the present study using *drop.tips* (phytools, Revell 2012). We then performed ancestral character estimations for both traits. We first determined the best-fit model (equal rates ER or all rates different ARD) of character evolution using the *ace*-function (phytools 0.6-99, Revell 2012). The ER model was preferred for both traits (reward type: ER *AIC* 66.3, ARD *AIC* 71.8, *log-likelihood* – 29.9, *df* 6, *p* = 0.48; heteranthery: ER *AIC* 83.2, ARD *AIC* 86.3, *log-likelihood* – 37.2, *df* 6, *p* = 0.23). We hence proceeded to stochastic character mapping under the ER model using *make.simmap* with 1000 simulations (phytools, Revell 2012).

We then tested for correlated evolution between reward type and heteranthery using Pagel's lambda under the ER model (*fitPagel*, phytools, Revell 2012). We compared a model of independent evolution of the two traits against a model of correlated evolution using likelihood ratio tests. Since this method allows for binary traits only, we binarized reward type to 0 – pollen, 1 – alternative reward (nectar, food bodies), and heteranthery to 0 – no heteranthery, 1 – (weak or strong) heteranthery. As traits of likely adaptive importance,

changes in reward type and heteranthery may have affected the diversification process of Merianieae. This may, in turn, affect ancestral state reconstructions and tests on correlated evolution between reward type and heteranthery. We hence also fit 13 different MuSSE models to evaluate whether character states and joint transitions between trait states may have affected diversification, comparing models using weighted AICs (*make.musse.multitrait*, diversitree, Fitzjohn 2012). A model allowing for additive effects of reward type and heteranthery on speciation was recovered as best fit (for details see Tables S2, S3), but no interaction between the two traits. We hence consider our results on correlated character evolution as robust and report MuSSE models only in the SI.

Empirical investigation of the function of heteranthery in non-pollen rewarding flowers

Given the frequent occurrence of heteranthery in food-body-rewarding flowers (Fig. 1A), we aimed to better resolve the possible function of heteranthery in passerine-pollinated flowers. From previous work, we knew that passerines usually do not remove all ten stamens available per flower during the first visit (Dellinger et al. 2014). We had not investigated, however, whether passerines discriminate between the two stamen whorls and were more likely to remove stamens of one whorl first. Documenting foraging behaviour, however, is crucial to determine possible functional adaptations of heteranthery in non-pollen-rewarding flowers.

We selected two passerine-pollinated species (*Axinaea confusa* E. Cotton, *A. costaricensis* Cogn.), to study patterns of stamen removal. We conducted fieldwork in Ecuador (*A. confusa*, forests around Estación Científica San Francisco, Loja province, September 2016) and Costa Rica (*A. costaricensis*, forests around Finca Truchas Selva Madre, Alajuela province, March 2020). In both populations, we had monitored pollinators previously, and found different species of passerines (tanagers) as the only pollinators (i.e.

capable of activating the bellows mechanism and touching stigmas while foraging, Dellinger et al., *in press*). From these previous investigations, we know that in *A. confusa*, 6 (+4) stamens get removed on the first day of anthesis, 8 (+2) are removed by the end of the second day or later; in *A. costaricensis*, 3 (+3) stamens get removed on the first day of anthesis, 5 (+3) are removed by the end of the second day or later (Dellinger et al., *in press*). For the present study, we randomly collected 50 anthetic flowers in the population of *A. confusa* and 50 anthetic flowers in the population of *A. costaricensis*. We chose a broad sample of different flower ages, including both young (urceolate corolla) and older (more opened corolla) flowers, and without a priori checking the number of stamens present. For each flower, we then counted how many stamens had been removed per whorl. We visualize relative stamen removal of both stamen types per flower using the R-packages ggplot2 (Wickham 2016) and ggExtra (Attali & Baker 2019). Since we found strong indication for small stamen (inner whorl) removal occurring when large stamens (outer whorl) had been removed (Fig. 2), we used GLMs with a quasi-poisson distribution to test whether removal of large stamens significantly influenced removal of small stamens. We visualized the models by plotting marginal effects and the raw data using the sjPlot package (Lüdcke 2021).

Finally, we selected ten flowers per species preserved in 70% ethanol for an assessment of pollen viability. We chose one large and one small stamen per flower, placed them in separate 1.5ml Eppendorf tubes filled with 100µl 1% acetocarmine and macerated stamens to release all pollen grains. Acetocarmine is commonly used for chromosome staining and may be used as rough estimate of pollen viability, with likely viable pollen grains staining red and unviable (i.e. shrivelled grains) remaining white (Kearns & Inouye 1993). We pipetted a drop of the solution on a microscope slide and counted the first 110 pollen grains encountered under a light microscope at 20x magnification, classifying them as stained or not stained (Maia et al. 2016). We then calculated the percentage of stained versus

not stained grains per stamen type and used paired Wilcoxon tests to test for significant differences in viable pollen grains between stamen types.

Calorimetric analyses

The bulbous stamen appendages of passerine-pollinated Merianieae serve as food body reward. To determine whether differences in the caloric content of stamen appendages may cause differences in removal patterns, we collected single stamens of both *Axinaea* species. Further, we wanted to test whether the caloric content changes across anthesis, and hence separated stamens from young flowers (first day of anthesis) from old flowers (second day or later). From closely monitoring the duration of anthesis (ca. four days) in these species (Dellinger et al., *in press*), we could easily distinguish young flowers from old flowers by their strongly urceolate corolla with a small opening only, while corollas openings are considerably wider in old flowers. We aimed to collect at least 0.1 g dry weight per stamen whorl. For *A. confusa*, we could separate stamens into the two distinct stamen whorls, for *A. costaricensis*, we pooled both whorls and just separated young from old stamens since dry weight of young small stamens was below 0.05g. We microwave-shock-dried stamens (2.5 minutes at 600 watt) to conserve sugars, then slowly dried them in a drying oven overnight at 40° and stored them in silica gel (Dellinger et al. 2014). We then removed the filaments and anthers from the stamens, pulverized the appendages using a mortar and compressed the pulverized appendage material into a pellet. We analysed the six samples in an IKA Calorimeter C 2000 basic version 1 (IKA-Werke GmbH & Co. KG, Germany).

Results

Does heteranthery correlate with pollen rewards in Merianieae?

Heteranthery has evolved repeatedly across Merianieae and, against expectations, is not restricted to species offering pollen rewards (Fig. 1A). Indeed, moderate or strong heteranthery is found in the majority of food-body-rewarding (passerine-pollinated) species (Fig. 1C, E, F), and in one group of nectar-secreting (mixed-vertebrate-pollinated) species (Fig. 1B). Strong heteranthery occurs mainly in bee-pollinated species (Fig. D, G).

Reconstructing the evolutionary history of heteranthery and reward type, flowers with pollen rewards and isomorphic androecium are supported as ancestral in Merianieae (Fig. 1A). Heteranthery appears as more labile trait than reward type (13.98 state transitions compared to 8.11 for reward type). We estimated 7.29 shifts from isomorphic stamens to moderate or strong heteranthery, and 6.15 shifts from pollen to alternative rewards (Table 1). Reversals from heteranthery to isomorphic stamens were relatively common (4.05 shifts on average) and more common than reversals to pollen rewards (Table 1). Heteranthery has likely evolved *de novo* three times in non-pollen-rewarding lineages (Figure 1). There was no correlated evolution between reward type and heteranthery (independent: *log-likelihood* -61.9, *AIC* 127.7, dependent: *log-likelihood* -59.4, *AIC* 126.8; *likelihood-ratio* 4.9, $p = 0.09$, Supplementary Figures 1, 2).

How is heteranthery achieved in flowers with different rewards?

Across Merianieae, heteranthery is achieved through the differentiation of various stamen parts, and includes differentiation in size, shape, structure, or colour (Fig. 1). In bee-pollinated species such as *Meriania calophylla* (Cham.) Triana or *M. nobilis* Triana, for example, stamen whorls are mostly differentiated by anther length (short versus long), anther structure (smooth versus corrugated anther walls), and anther orientation, with the pore of the outer stamens positioned close to the floral center and the pore of the inner stamens at a

distance from the floral center (Fig. 1D). In *M. nobilis*, stamen whorls additionally differ in appendage size and colour, rendering outer stamens visually more attractive (Fig. 1G).

As in pollen-rewarding flowers, heteranthery also is expressed through a generally more attractive outer whorl and a less conspicuous inner whorl in food-body rewarding species (Fig. 1C, E, F, Fig. 2). The most prominent differences between stamen whorls lie in the size and colour of the stamen appendages, most pronounced in species such as *Meriania peltata* L.Uribe (Fig. 1C). In nectar-rewarding species, heteranthery involves differences in anther colour, anther length, anther width and pore number (Fig. 1B).

Pattern of stamen removal

Passerine birds removed large stamens before small stamens in both *Axinaea* species (Fig. 2). A high proportion of flowers had all five large stamens removed (*A. confusa*: 22 flowers, 44%; *A. costaricensis*: 37 flowers, 74%), while only one flower in *A. confusa* and none in *A. costaricensis* had all five small stamens removed. The number of large stamens removed was significantly correlated to the number of small stamens removed in both species (*A. confusa*: GLM estimate 0.34, *t*-value 2.74, *df* = 48, *p* = 0.009; *A. costaricensis*: GLM estimate 0.49, *t*-value 3.19, *df* = 48, *p* = 0.002, Fig. 2).

We did not find differences in pollen grain viability (assessed through aceto-carmin stains) between the two stamen types (*A. confusa*: *V* = 22, *p* = 0.625, *A. costaricensis*: *V* = 29, *p* = 0.921).

Calorimetric analyses

In both species, the caloric content of stamen appendages doubled or even tripled across flower age (Table 2). The caloric content of small stamens of *A. confusa* was half that of large stamens both in young and old flowers (Table 2). With stamens becoming sweeter

during the course of anthesis, old small stamens of *A. confusa* reached approximately the same caloric content (21.9 Joule/appendage) that large stamens had in young flowers (23.2 Joule/appendage).

Discussion

Our results challenge the ‘division of labour’ hypothesis as functional explanation of heteranthery since we show that the evolution of heteranthery is not restricted to species underlying the ‘pollen dilemma’, but is indeed also present in species providing alternative rewards to their pollinators. As we show here for vertebrate-pollinated Merianieae lineages with nectar or food body rewards, heteranthery has not merely been maintained during shifts in reward type but, in most cases, has actually evolved *de novo* from pollen-rewarding, bee-pollinated ancestors with isomorphic stamens (Fig. 1A). Consequently, the dual function of pollen as reward and as male reproductive agent is not the ubiquitous or only driver of the evolution of heteranthery. Instead, our field results on patterns of stamen removal in food-body-rewarding, passerine-pollinated species support the hypothesis that heteranthery serves in staggered pollen release. Overall, these findings accentuate the need for investigating alternative, mutually non-exclusive hypotheses on the function of heteranthery also in other lineages.

This being said, the stamen differences of bee-pollinated Merianieae closely reflect functional adaptations as expected under the classical ‘division of labour’ hypothesis. The short, showy outer stamens represent ‘feeding stamens’ since bees actively manipulate them when buzzing flowers (Dellinger et al. 2019a, c). The stamens are arranged in a way that when bees crouch above the showy outer stamens and grasp them for buzzing, pollen released simultaneously by the long inner stamens may indeed be deposited on ‘safe sites’ on

their back (Dellinger, pers. obsv.). Performing artificial vibration experiments in the bee-pollinated Merianieae species *Adelobotrys adscendens* (Sw.) Triana, we found that feeding stamens scatter pollen more broadly than pollinating stamens, but whether this is a functional adaptation to place pollen in safe sites remains unknown (Dellinger et al. 2019a). Importantly, there is evidence both from Merianieae and from Microlicieae (also Melastomataceae) that feeding anthers release higher proportions of pollen upon a single buzz (Dellinger et al. 2019a, Telles et al. 2020). Increased investment in feeding stamens may be regarded as a strategy to assure high numbers of bee visits (Telles et al. 2020) and, thereby, to increase chances of outcross pollen transfer (Castellanos et al. 2006, Kay et al. 2020).

Despite the overall close resemblance to the ‘division of labour’ hypothesis, other factors may have contributed to the evolution of heteranthery also in bee-pollinated Merianieae. Studying the Melastomataceae species *Rhynchanthera grandiflora* (Aubl.) DC, for example, Konzmann et al. (2020) proposed that heteranthery may be seen as adaptive strategy to increase pollen transfer through a broader niche of differently sized bee pollinators. In buzz pollination, pollen is released in clouds and scattered broadly across the pollinator (Minnaar et al. 2018). Such inaccurate pollen placement is generally regarded as maladaptive (Armbruster et al. 2009). It may, however, be adaptive (*‘adaptive inaccuracy’*) particularly in heterantherous flowers: resulting from the different orientation of the two stamen types, both ventral and dorsal sides of pollinators of different sizes may be covered in pollen (Konzmann et al. 2020). This may, in return, increase chances of successful pollen transfer since stigma contact by any body part of the pollinator will result in pollen transfer. Similar adaptive hypotheses have been proposed in wild radish flowers (Brassicaceae), which have one set of long stamens exerted from the pseudotubular corolla and one short set at the entrance to the pseudotubular proximal part of the corolla (Conner et al. 2009). While short

stamens (or decreased dimorphism) may be adaptive to pollen transfer by small bees, large stamens may be adaptive under large bee visitation (Conner et al. 2009). Further, such stamen dimetrisism may significantly affect pollinator attraction, and lead to different patterns of fly and bee pollinator attraction in wild radish (Sapir et al. 2017). In connection with this, it is important to note that across Melastomataceae, but also in other systems, heteranthery may be achieved solely through colour and shape differences of stamens or even through shape differences alone (Brito et al. 2020). Detailed comparative studies quantifying the attraction function of stamens (i.e. colour-modelling in bee visual space, scent, reward quantity) as well as pollen placement and dosing are required to better resolve the potentially different, multifarious adaptive functions of heteranthery even among closely related taxa. Finally, we observed also structural differences between stamen whorls (e.g. smooth versus corrugated thecae). Recent investigations on the biomechanical properties of flowers have shown that tissue sturdiness critically influences motion responses to vibration buzzes (Brito et al. 2020b). How such structural differences between stamen whorls play out in pollen-release dynamics remains to be investigated.

Our results on the faster removal of large stamens than small stamens in passerine-pollinated Merianieae are in line with the hypothesis that heteranthery (also) functions to increase pollen delivery, and, potentially, cross-fertilization (Kay et al. 2020). Indeed, previous investigations in *Axinaea confusa* have shown that 54% of flowers are visited on at least two different days (Dellinger et al. 2014). Even when all ten stamens are removed within the same day, however, this usually occurs on separate visits to the flower, with large stamens removed first (Dellinger, pers. obs.). The flower visiting tanagers usually forage in small flocks and move frequently between trees (Dellinger et al. 2014). Hence, a different bird may return to the same flower to remove the remaining (small) stamens, potentially carrying a very different pollen load compared to its first visit. Importantly, this staggered

pattern of stamen removal is likely triggered by the gradual increase of caloric content of the bulbous appendages during anthesis (Table 2). Behavioural assays performed with different tanager species have shown that tanagers may discriminate between fruits with just a 1% difference in sugar concentration, and generally prefer the more nutritive diet (Schaefer et al. 2003). Since the two *Axinaea* species investigated here usually bear hundreds of flowers at once, we suggest that the generalist tanagers (also feeding on insects and fruits) may simply be choosy and select the most nutritive stamens at each visit. We are confident that the increase in caloric content influences stamen choice in these birds, and, importantly, has evolved as adaptive pollen dosing strategy to prevent complete exploitation in one visit.

The staggered stamen removal in passerine-pollinated Merianieae bears similarities to pollen dosing in pollen-rewarding flowers underlying the pollen dilemma, but is achieved through a different mechanism (staggered maturation of food bodies). Further, passerine-pollinated Merianieae flowers differ in traits commonly expected under the division of labour hypothesis, such as differential pollen placement by the two stamen types in safe and un-safe sites (Konzmann et al. 2020). Instead, pollen expulsion through the bellows mechanism is highly imprecise, with pollen from both stamen types scattered broadly across the bill and front head of the foraging tanagers. We did not observe any indication for differential pollen placement during observations. Differences in pollen viability form another important component of the division of labour concept, with the assumption that feeding stamens produce infertile pollen while pollination stamens produce fertile pollen. Comparing stained pollen grains under the light microscope, we did not find any differences in the proportion of stained (viable) and unstained (non-viable) pollen grains. Since this method can, at best, only serve as a rough estimate, however, pollen germination experiments are required to clarify whether there are, indeed, no differences in viability between stamen types (Kearns & Inouye 1993).

While heteranthery has evolved repeatedly and is common in some families with a high percentage of pollen flowers such as Melastomataceae and Fabaceae, it is relatively rare at the level of the angiosperms as a whole (Vallejo-Marín et al. 2010, Melo et al. 2021). Several hypotheses have been proposed to explain this rarity, including the overall rarity of pollen-flowers, the idea that most pollen-flowers may actually not suffer from excessive pollen consumption by bees (Harder & Wilson 1998), or that pollen-deposition in safe versus un-safe sites does not come along with a significant fitness gain (Vallejo-Marín et al. 2010, Tong & Huang 2018). Developmental constraints in the differentiation of two distinct stamen whorls have also been proposed as possible explanation for the rarity of heteranthery (Endress 2010, Vallejo-Marín et al. 2010). With the repeated evolution and frequent occurrence of heteranthery in Merianieae (Fig. 1), developmental constraints are likely not hindering its evolution both in this group and the family as a whole (Melo et al. 2021). We propose that heteranthery is particularly likely to evolve only when stamen dimorphism itself increases the probability of receiving multiple independent pollinator visits, and hence higher chances for cross-pollination. However, this condition is neither met in pollen-rewarding nor in nectar-rewarding flowers. With pollen concealed in poricidal anthers in buzz-pollinated pollen-rewarding flowers, bees cannot a-priori tell whether anthers have already been exploited. With this type of dishonest signalling (Knauer & Schiestl 2014), flowers may receive multiple independent visits regardless of how much pollen is left. Likewise, when nectar rewards are offered, the availability and quantity of nectar determines visitation frequency, not the presence of dimorphic stamens. Here, we want to note that the possible functional role of heteranthery in nectar-rewarding Merianieae (Fig. 1B) remains unclear given the lack of empirical field data. The two nectar-rewarding species included here belong to a small clade, which has radiated in the Greater Antilles (Michelangeli et al. 2015). It is possible that heteranthery in these species is not functional and has been merely retained from

bee-pollinated ancestors not included in the current sample. In the passerine-pollinated Merianieae where birds are rewarded directly with the stamens, however, signalling is honest since birds can always tell a priori whether a flower still contains rewards. Losing all ten stamens during the first pollinator visit, however, would confine the flower to a single pollen vector. Avoiding this danger is apparently a powerful evolutionary driver towards heteranthery.

In conclusion, our study provides intriguing ground for a new macroevolutionary and functional perspective on the adaptive role of heteranthery as a mechanism to optimise outcross-pollen transfer (Papaj et al. 2017, Kay et al. 2020, Konzmann et al. 2020). Our results are in line with recent work by Kay et al. (2020), challenging the long-standing view that the ‘pollen dilemma’ drove the evolution of heteranthery. While we are convinced that ‘division of labour’ is one important functional aspect of heteranthery in pollen rewarding flowers, we call for rigorous testing of alternative functional and evolutionary hypotheses, especially in lineages with non-pollen-rewarding heterantherous flowers, to arrive at a more inclusive evolutionary perspective on this fascinating aspect of floral diversity and evolution.

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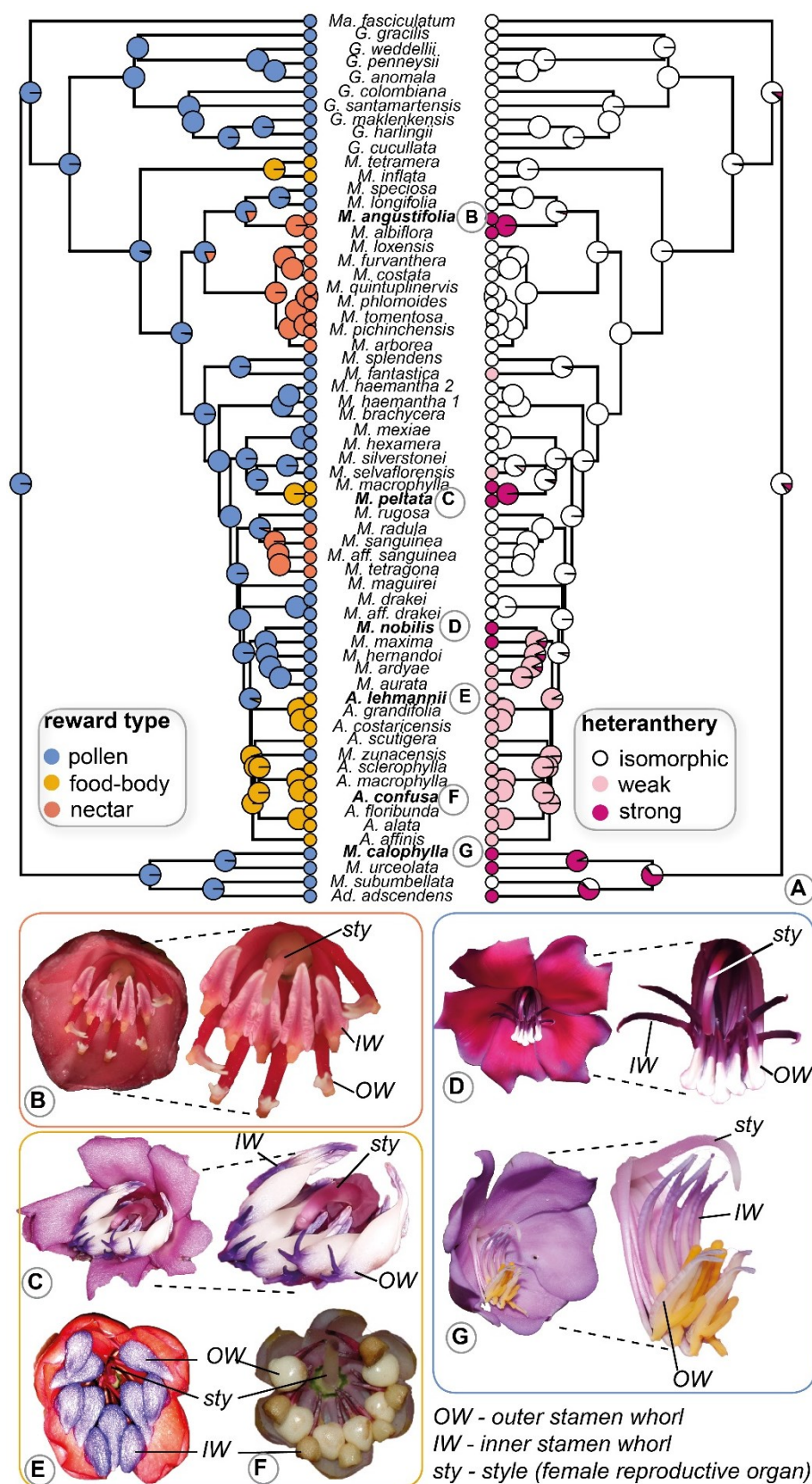


Figure 1. Trait distribution of floral reward type and heteranthery in 63 Merianieae species shows that heteranthery has evolved repeatedly and is not correlated with pollen rewards. A) Weak to strong heteranthery occurs in most food-body-rewarding, passerine-pollinated species (12 out of 14

spp.) as well as in some mixed-vertebrate pollinated, nectar offering species (two out of 14 spp.). Note that in their current taxonomic circumscription, the genera *Axinaea* and *Meriania* are non-monophyletic and in need of taxonomic revision. Examples of heterantherous Meranieae: B) Nectar-rewarding *Meriania angustifolia*, strongly dimorphic with outer stamens having long filaments and short bifurcate anthers, while inner stamens have short filaments and longer anthers; C) Food-body-rewarding *M. peltata*, strongly dimorphic with prominent bulbously inflated connectives in outer stamens and strongly reduced, non-inflated connectives in inner stamens; D) Pollen-rewarding *M. nobilis*, strongly dimorphic, outer stamens with visually attractive whitish appendages and short anthers, inner stamens with small inconspicuous appendages but long, curved anthers extending beyond the floral centre; E) Food-body-rewarding *Axinaea lehmannii*, weakly dimorphic, with stamens primarily differing in appendage volume; F) Food-body-rewarding *A. confusa*, weakly dimorphic, with stamens differing in appendage volume and colour; G) Pollen-rewarding *M. calophylla*, strongly dimorphic, outer stamens with yellow anther appendages and short anthers, inner stamens with yellow appendages and long anthers extending beyond floral centre. Abbreviations of genera: A. – *Axinaea*, Ad. – *Adelobotrys*, G. – *Graffenrieda*, M. – *Meriania*, Ma. – *Macrocentrum*. © B, G – Fabián Michelangeli.

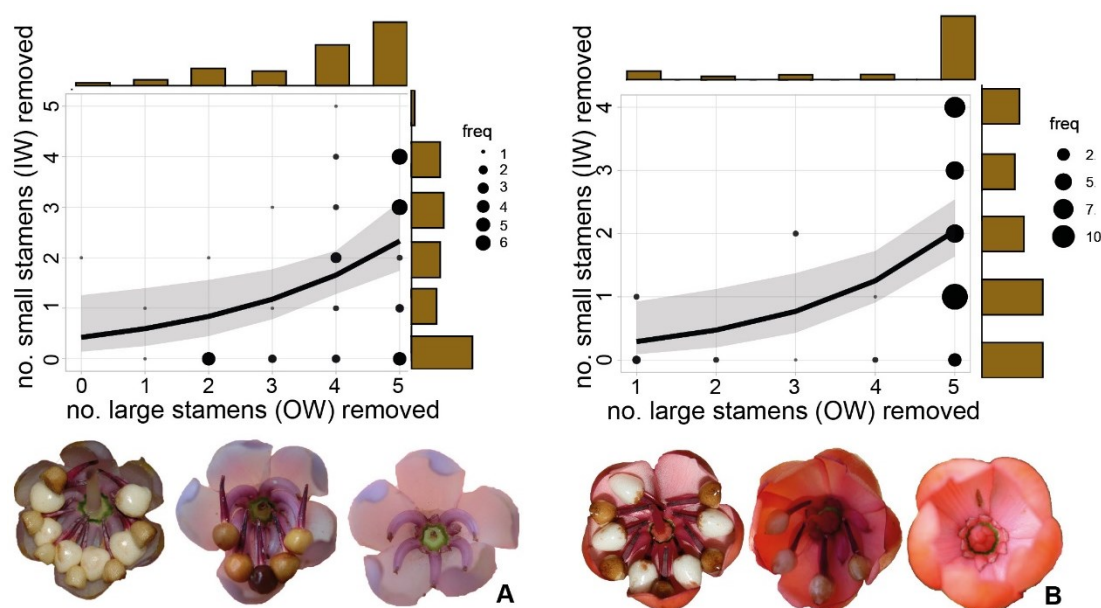


Figure 2. Passerine bird pollinators removed stamens with large bulbous appendages (OW – outer whorl, x-axis) before stamens with small appendages (IW – inner whorl, y-axis). A) Stamen removal in *Axinaea confusa*. B) Stamen removal in *A. costaricensis*. The raw data is shown (dots, sized by the frequency of the respective number of large and small stamens removed) as well as the predicted relationship (marginal effects) from GLMs (line with 95% confidence intervals); histograms at the side of the x-axis and y-axis indicate the frequency of flowers with the respective number of stamens removed. Note that there was a high frequency of flowers with the five large (outer) stamens removed in both species, while small (inner) stamens were generally removed only when large stamens had already been removed earlier.

Table 1. Estimated number of shifts between reward types and isomorphic or heterantherous flowers across Merianieae.

trait	ancestral	derived	no. of shifts
reward	pollen	nectar	3.00
	pollen	food body	3.15
	nectar	pollen	0.48
	food body	pollen	1.24
	nectar	food body	0.13
	food body	nectar	0.11
heteranthery	absent	weak	3.54
	absent	strong	3.75
	weak	absent	2.61
	strong	absent	1.44
	weak	strong	2.2
	strong	weak	0.44

Table 2. The caloric content of stamen appendages increases with flower age in *A. confusa* and *A. costaricensis* and is positively correlated with appendage size, and hence stamen type.

sample	flower age	stamen type	total dry weight	weight/stamen [g]	Joule/g	Joule/appendage
<i>A. costaricensis</i>	young	all	0.216	0.001	16112	16.805
<i>A. costaricensis</i>	old	all	0.505	0.004	15402	58.374
<i>A. confusa</i>	young	small	0.108	0.001	16454	10.531
<i>A. confusa</i>	young	large	0.247	0.002	15989	23.184
<i>A. confusa</i>	old	small	0.536	0.001	15670	21.938
<i>A. confusa</i>	old	large	0.479	0.003	15340	42.032