The functional roles of 3D heterostyly and floral visitors in the reproductive biology of *Turnera subulata* (Turneroideae: Passifloraceae)

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ABSTRACT

Heterostyly is a floral polymorphism that reduces conflicts between sexual functions (sexual interference), such as self-pollination in self-incompatible flowers and loss of pollen to incompatible stigmas. In many distylous pollination systems, there is a remarkable structural fit between the stigmas of each morph and the part of the pollinator body where compatible (disassortative) pollen is deposited. Nevertheless, inter-morph pollen transfer is often asymmetrical, with short-styled flowers (S-morph) receiving less compatible pollen than long-styled flowers (L-morph). One way to reduce the problem of sexual interference in short-styled flowers is to place stigmas outside of the flower centre, as seen in three-dimensionally (3D) heterostylous flowers. Heterostyly in *Turnera* has been extensively studied; however, 3D heterostyly has not been previously reported in this genus. The aims of this study were: 1) to elucidate the pollination system of *T. subulata;* and 2) to assess whether 3D heterostyly promotes disassortative pollination and reduces self-pollination in short-styled flowers in this taxon. The study population of *Turnera subulata* exhibited an isoplethic (1:1) ratio, with two well-defined morphs, and a high floral accuracy index for both morphs. Results from an experiment in which we manipulated the 3D orientation of the style showed that compatible crosses were favoured by the 3D flower morphology, consistent with a high floral accuracy. Our results also demonstrate the functional importance of 3D heterostyly in reducing intramorph pollination, as it increases the amount of cross pollen reaching the S-morph flowers. Our results provide the first observation of the presence of 3D heterostyly in this well-studied species and demonstrate the importance of this kind of morphological specialization in ensuring efficiency in an ecologically generalized pollination system.

1. Introduction

The reciprocal correspondence of anther and stigma positions in the vertical dimension (heterostyly) was the phenomenon that perhaps most amazed Darwin in his studies of floral morphology and pollination (Darwin, 1905, p. 74). He discussed the evolution and function of heterostyly in great detail and established that only inter-morph pollinations produce viable seeds (Cohen, 2010; Darwin, 1877). Since then, heterostyly has been shown to reduce sexual interference and self-pollination, and promote disassortative (inter-morph) mating (Barrett et al., 2000; Barrett and Shore, 2008). Indeed, reciprocal differences in the position of reproductive organs between morphs makes inter-morph cross-pollen deposition more likely than intra-morph deposition, at

least in some systems (Barrett et al., 2000; Keller et al., 2014; Wu et al., 2018). Nevertheless, breakdown of intra-morph compatibility, has also been shown, especially in compatible monomorphic populations colonizing new areas (Barrett, 2002; Castro et al., 2013).

In many distylous pollination systems, there is remarkable structural fit between the stigmas of each morph and the part of the pollinator body where compatible (disassortative) pollen is deposited (Costa et al., 2017). However, inter-morph pollen transfer may not always be "on target", with short-styled flowers (S-morph) receiving fewer compatible pollen grains than long-styled flowers (L-morph), the occurrence of which is sometimes related to pollinator behaviour (Keller et al., 2014). Another deviation from perfect disassortative pollen transfer happens when the deposition of self-pollen precedes, and
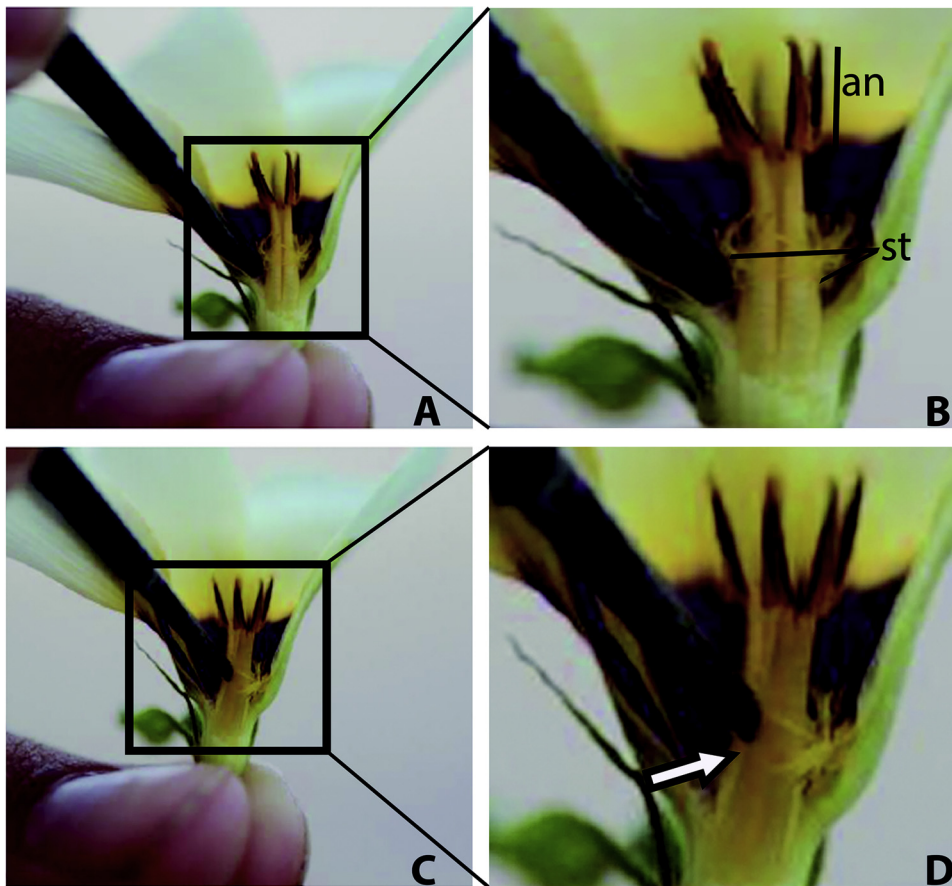
Fig. 1. Cancellation of the 3D conformation, moving one of the stigmas to the central position of the flower (among the stamens) of *Turnera subulata*. A. Natural 3D stigmas. B. Close up of the stigmas projected out of the androecium whorl. C. One stigma in natural 3D position and another one moved to the central part of the flower (cancellation of the three-dimensionality of the stigma). D. Close up of the moved stigma. an: anthers, st: stigmas, arrow: point of the stigma moved inside the central part of the flower.

therefore limits or prevents, cross-pollination. In *Lythrum salicaria* the S-morph had more interference from self-pollination than the other floral morphs, compromising seed set of this floral morph (Waites and Ågren, 2006).

One peculiar variation of the classical distylous systems happens when gynoecia and androecia vary not only in height but also occur in reciprocal positions laterally creating a three dimensional variation. Previously described 3D heterostyly involved short-styled flowers with stigmas projected through the basal portion of the stamens, and therefore projecting away from the centre, functionally creating a second whorl (Armbruster et al., 2006). One expected advantage of the 3D heterostylous flowers is an increased rate of compatible pollen receipt and a reduction in self-pollination, because stigmas are no longer positioned right below the anthers (Armbruster et al., 2006). Even though 3D heterostyly has been described (see also Turketti et al., 2012), to the best of our knowledge there is no experimental evidence testing the hypothesis that the 3D morphological inversion of the position of fertile organs increases the receipt of compatible pollen or reduces the adherence of self-pollen to the stigmatic surfaces of short-styled flowers.

Three-dimensional heterostyly was first described in *Linum suffruticosum* L., a species that has positional reciprocity between L-morph and S-morph flowers in three dimensions (Armbruster et al., 2006). The inter-morph reciprocity is achieved with both anthers of long stamens (S-morph) and stigmas of long styles (L-morph) contacting the dorsal region of the bodies of nectar-seeking pollinators, while the anthers of short stamens (L-morph) and the stigmas of short styles (S-morph) contact their ventral surface. Therefore, the three dimensional position of stigmas should insure pollen deposition with the same or at least similar efficiency for both morphs of *L. suffruticosum*. However, Armbruster et al. (2006) did not present experimental evidence that 3D heterostyly effectively increases disassortative mating in *L.*

suffruticosum. Turketti et al. (2012) reported a complex case of three-dimensional reciprocity in tristylous populations of *Oxalis* L. section *Sagittatae* in South Africa. This species is self-compatible, making the system more reliant on reciprocity, and requiring consistent and precise floral visitation by pollinators, to achieve out-crossing. No data on pollination efficiency were presented, however.

Heterostyly in *Turnera* L. has been extensively studied (Baker and Shore, 1995; Barrett, 1978, 1990; Barrett and Shore, 1987; Belaoussoff and Shore, 1995; Bentley, 1979; Medeiros and Schlindwein, 2003; Schlindwen and Medeiros, 2006; Shore, 1991; Shore and Barrett, 1984, 1985, 1987; Swamy and Bahadur, 1984). However, 3D heterostyly has not been reported previously in this genus. Baker and Shore (1995) studied the pollination of *T. ulmifolia* L. and suggested that pollination success was due to it being an ecological generalist in terms of pollination and thus adapted to a diverse range of pollinators (Ollerton et al., 2007). The flowers of this species were reported to attract a diverse group of insects that feed on pollen and/or nectar (Barrett, 1978). In this study we focus on *Turnera subulata* Sm. [syn = *T. ulmifolia* var. *elegans* (Otto ex Nees) Urb.]. This species also has an open floral morphology and wide diversity of floral visitors (Barrett, 1978, ARR unpublished observations) that are consistent with it having an ecologically generalized pollination system. Nonetheless, preliminary observations suggested that the flowers were phenotypically specialized (sensu Ollerton et al., 2007) and exhibited 3D heterostyly. We therefore addressed the following questions: 1) How does the pollination system of *T. subulata* work? 2) How accurate are the 3D flowers of *T. subulata* (cf. Armbruster et al., 2009, 2017)? 3) Is 3D heterostyly able to promote disassortative (compatible) pollination and, in the short-styled flowers of *T. subulata*, to reduce self-pollen deposition?

Fig. 2. Flower visitors of *Turnera subulata* in the Campinas – São Paulo population. A) *Exomalopsis* sp. B) Phthiriinae sp. C) Halictidae sp. D) *Trigona spinipes*. E) *Apis mellifera*. F) *Tetragonisca angustula*.

2. Materials and methods

2.1. Study species

Turnera subulata was chosen as an experimental species for this study because of its unusual floral morphology with presumed 3D heterostyly. It is a rhizomatous shrub with heterostylous flowers whose corolla is made of yellow petals with lighter apices and a purplish spot at the base; the androecium is composed of flattened-subulate stamens with yellow filaments, which are tightly merged to form a staminal tube. The anthers are orange with basi-dorsal filament insertion, bearing yellow pollen. The gynoecium comprises an ovoid or conical tricarpellar, trichomatous ovary, three yellow styles (measuring 6.5–9 mm in L-morph, and 3 – 5 mm in S-morph), and yellow stigmas. The fruits are subglobose, and the seeds are claviform with unilateral arils (Arbo, 2005; Woodson et al., 1967). The flowers of *T. subulata* have three separate styles, each with a three-branched stigma oriented in a dimension perpendicular to the floral axis in the S-morph flowers. The species is ruderal, occurring in clearings in woods and disturbed areas, including roadsides and urban centres.

2.2. Study population

Experiments and observations were performed with a population of more than 300 individuals of *T. subulata* in the city of Campinas, at the Ecological Park "Prof. Hermógenes de Freitas Leitão Filho" (22°48′44″ / 47°04′26″), in an area of semi-natural vegetation (135,000 m²). The study population flowers year round, but with a flowering peak from July to February. In December 2015, July 2016, and January 2017 we collected data for three weeks, with daily observations from pre-anthesis to the end of insect visitation or until flowers closed. For comparison of flower visitors and results of incompatibility tests, we used data collected in January 2015 from a population in the city of Presidente Figueiredo, Amazonas (02°73′17″ / 60°01′29″).

2.3. Isoplethy and morphometric analysis

Members of both plant morphs were counted in the population to determine if the population was isoplethic (equal morph frequencies). The nature of floral heterostyly was evaluated morphometrically by measuring 40 flowers of each morph, from which two petals had been removed so that the reproductive organs could be photographed from a fixed distance. To avoid damage or change in functional distances, measurements of stamens (height) and style (length) were taken from
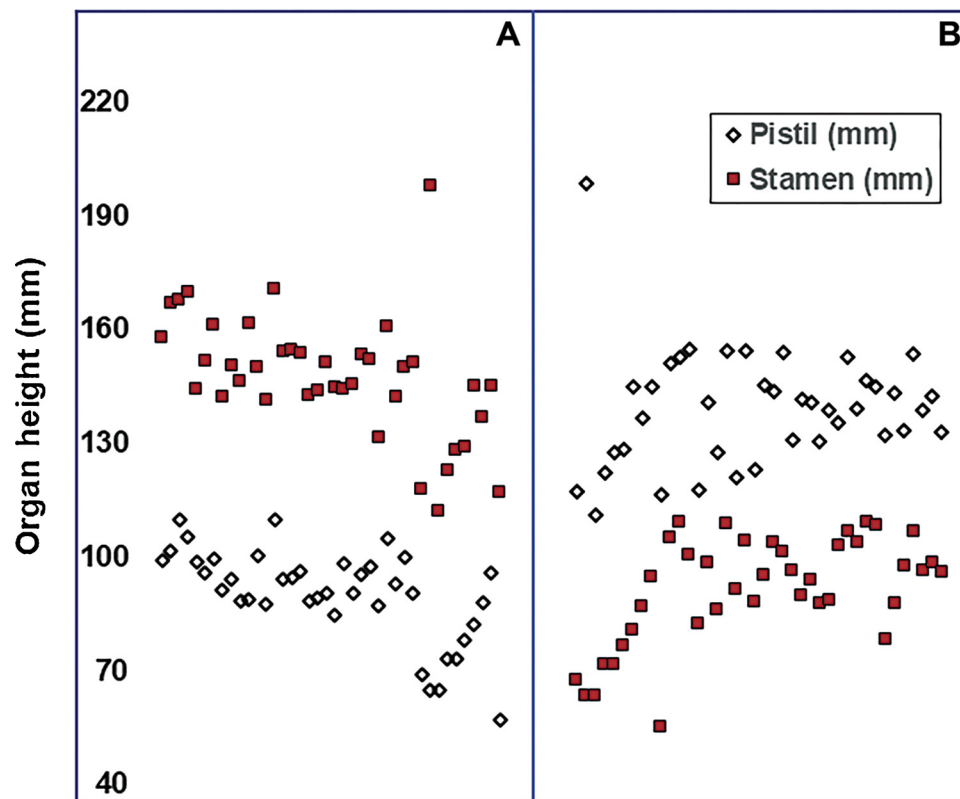
Fig. 3. Distyly in *Turnera subulate*. Range of variation in the pistil and stamen heights, in a total of 80 flowers. A. 40 S-Morph. B. 40 L-Morph. For each flower the length of the style (from the ovary base to the tip of the stigma), and the length of the stamen (from the ovary base to the tip of the anthers) was measured.

Table 1

Model selection evaluating the factors explaining pollen deposition in *T. subulata*. dAIC represents the delta AICs (difference between difference values of the Delta AIC by the Akaike information criteria (Akaike, 1974) for sequential models. and df represents the degrees of freedom for each model. "Donor" refers to donor morph, and "receiver" refers to recipient morph.

Model	dAIC	df
Pollen donor	**0.0**	**5**
Pollen donor + receiver	**0.2**	**6**
Pollen Receiver + flower visitor	6.4	17
All factors added	5.2	12
Pollen donor + flower visitor	16.7	18
Pollen receiver	15.2	5
Flower visitor	22.3	10
Null	45.1	3

photographs using a caliper-ruler as a scale to maintain floral functional dimensions. Afterwards, measurements were recorded using the Image J software, and from these parameters the floral reciprocal accuracy was calculated using the following formulas (Armbruster et al., 2009; see also Armbruster et al., 2017):

Tall Organ Inaccuracy

$$TOI = (\overline{LS} - \overline{SP})^2 + V_{LS} + V_{SP}$$

Short Organ inaccuracy

$$SOI = (\overline{SS} - \overline{LP})^2 + V_{SS} + V_{LP}$$

Where LS means Mean Anther Height of L-morph; SP means Mean Stigma Height of S-morph; SS means Mean Anther Height of S-morph; and LP means Mean Stigma Height of L-morph. The V's represent the respective variances.

To compare the inaccuracy values proportionally to the organ size, the final values were scaled according to the following formula:

Mean-Squared-Scaled Inaccuracy of Tall Organs

$$MSTOI = (TOI * 100 / ((LS + LP)/2)^2)*100$$

Mean-Squared Inaccuracy of Short Organs

$$MSSOI = (SOI * 100 / ((SS + SP)/2)^2)*100$$

2.4. Frequencies and efficiencies of floral visitors

In order to measure the efficiency of each floral visitor as a potential pollinator we used tests of single-visit pollen deposition onto stigmas of virgin flowers, based on Freitas (2013). To ensure all flowers analyzed were virgin prior to observed visitation, floral buds were bagged, preventing visitor interference. Flowers were individually uncovered, and one of the three stigmas was immediately collected as an unvisited control group. The flower was then observed until it received its first visit; immediately after the visit, one of the two remaining stigma was collected and stored for pollen quantification. We counted pollen under a light microscope, using slides coated with a medium made of gelatin-glycerin and basic fuchsine to enhance contrast of the pollen grain relative to stigma tissue (Beattie, 1971).

To determine visitation frequency, we observed flowers for periods of 10 min per morph per day, for a total of 80 min for each morph (S-morph and L-morph). Visitors were considered pollinators when the difference between the number of pollen grains on the visited stigma and the virgin stigma was greater than zero (King et al., 2013). To ensure that we correctly inferred the morph of the pollen-donor plant we considered differences in pollen size. We tested and confirmed that pollen grains of the L- and S-morphs differ in size (Mean L: 54.43 versus Mean S: 74.04; t: -8.9873 p < 0.0001 - see also Fig. 3 from Schlindwen and Medeiros, 2006). Because Barrett (1978) recorded that there may be some overlap in pollen size from S- and L-morph flowers, we ignored pollen grains between 60 and 70 μm in size. Fig. S1 (supplementary
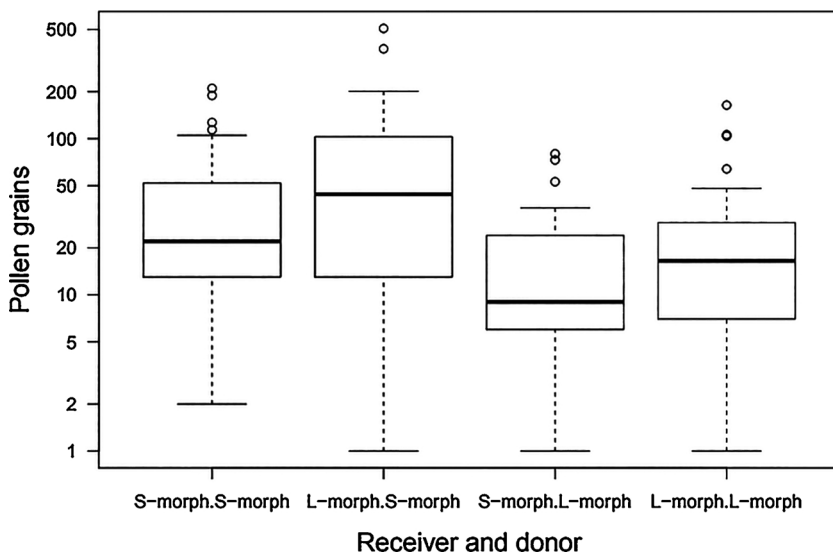
Fig. 4. Pollen-deposition on stigmas of *Turnera subulata* after a single visit by a pollinator, grouped according to the floral morph pollen donor and the floral morph pollen-receiver. The boxes represent the median and 1 st and 3rd quartiles, and the whiskers represent the extreme values up to 1.5 times the interquartile range. Values beyond this limit are represented as dots above the whiskers.

Table 2
Fruit production in the Incompatibility Tests of *Turnera subulata* flowers. Treatments: inter-morph (L-morph vs. S-morph), intra-morph (L-morph vs L-morph; and S-morph vs. S-morph), self-pollination, and natural fruit-production (n = 154).

Treatments		Results	Rate (%)
Intermorphs	L-morph	18/19	95 %
	S-morph	17/18	94 %
Intramorphs	L-morph	0/18	0 %
	S-morph	0/19	0 %
Self-pollination	L-morph	0/18	0 %
	S-morph	2/17	12 %
Natural fruit-production	L-morph	17/21	81 %
	S-morph	22/24	81 %

Fig. 5. Total L-morph pollen-deposition on the S-morph stigmas of *T. subulata*. Treatments tested were as follows: Virgin refers to stigmas collected before any visit of pollinators; Natural refers to stigmas in their original arrangement; Experiment refers to the manipulated stigmas for which the 3D position was cancelled. Natural and experimental stigmas were collected at the end of anthesis. The boxes represent the median and 1 st and 3rd quartiles, and the whiskers the extreme values up to 1.5 times the interquartile range. Values above this limit are represented as dots above the whiskers (post-hoc comparisons, all p < 0.001).

material) shows the range of sizes of pollen from S- and L-morph flowers of our experimental species.

2.5. Incompatibility tests

We determined the fruit-production rates for each possible cross within the population: inter-morph (L-morph vs. S-morph), intra-morph (L-morph vs L-morph; and S-morph vs. S-morph), and self-pollination. In total, we bagged 154 floral buds from 76 L-morph and 78 S-morph plants (Table 2). After anthesis started we made all crosses manually, making sure stigmas were well covered with pollen. Pollen used for crosses was a mixture from at least five different anthers (one per flower) from five different individuals of the same morphs. We performed 40 inter-morph crosses (20 with S-morph pollen deposited on L-morph stigmas and 20 with L-morph pollen deposited on S-morph stigmas); 40 intra-morph crossings (20 with L-morph pollen deposited on L-morph stigmas and 20 with S-morph pollen deposited on S-morph stigmas); and 40 self-pollinations (20 S-morph and 20 L-morph flowers). Flowers were marked and identified according to the treatment they were subjected to and then re-bagged until the end of anthesis. After seven days, we evaluated the fruit set.

2.6. Three-dimensional (3D) heterostyly experiment

Thirty S-morph floral buds in upright positions were bagged until anthesis. When virgin flowers were uncovered, we collected one of the stigmas and prepared it on a slide as the control for pollen counts
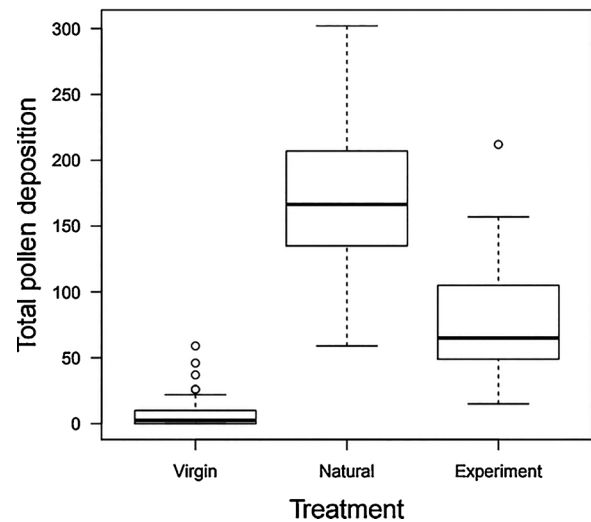
(hereafter called virgin). We manipulated a second stigma ("experimental stigma") to move its position to the centre of the flower (simulating standard unidimensional reciprocity, with the gynoecium in the centre), while the third stigma (called "natural") was kept in the original/natural position (3D) (Fig. 1A–D). In order to move one stigma to the flower centre (inside stamens ring) we used forceps to gently press the style in between the filaments (Fig. 1C–D). Since the filaments are positioned very close to each other at the base of the flower, the manipulated style remained in the manipulated position by itself during the course of the experiment.

Flowers of *T. subulata* last for a single day, therefore after the visitation period when flowers started to close, we collected the remaining two stigmas (the manipulated and the unmanipulated 3D one) for pollen counts. Slides were prepared and pollen was counted using the same method described in the previous section.
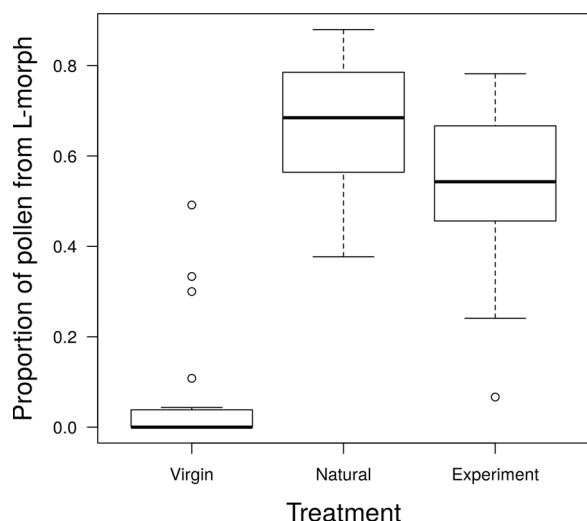
Fig. 6. Proportion of pollen-grains received from L-morph flowers in relation to all the pollen-grains deposited on the stigma of the S-morph flowers during the 3D cancellation experiment. The boxes represent the median and 1 st and 3rd quartiles, and the whiskers the extreme values up to 1.5 times the interquartile range. Values above this limit are represented as dots above the whiskers (post-hoc comparisons, all p < 0.001).

2.7. Data analysis

We used linear mixed models to evaluate the effect of pollen source (intra- vs. inter-morph pollen transfer) on pollen deposition rates in the experiment testing the efficiency of different pollinators. To include the blocked design of our sampling, in which stigmas from the same flower were used for different treatments, individual flower was treated as a random variable in all models. Starting with a null model using only the random factor and an intercept, we compared models with different sets of fixed effects to test for a treatment effect (virgin flower vs. visited flower; included in all models except the null), such as morph of the pollen donor, morph of the pollen recipient, and the taxonomic group of the floral visitor. To compare the models and to identify which best explains pollen deposition on stigmas, we used the Akaike information criterion (AIC – Akaike, 1974; Burnham and Anderson, 2002), treating models with AIC differences (delta-AICs) less than 2 as equally well-supported (Burnham and Anderson, 2002).

We used linear and generalized mixed models to test if there were differences in the total number and proportion of pollen grains from L-morph flowers deposited on the stigma surface in S-morph flowers subjected to different treatments (Fig. 4). The response variables were 1) the total number of pollen grains from L-morph flowers deposited on the stigma (legitimate pollen) and 2) the proportion of legitimate pollen relative to the total. Total number was modelled as a linear variable with gaussian error distribution, while the proportion was modelled as a logistic variable with a binomial error distribution. Predictor variables were single fixed factors representing the three treatments used: virgin stigma, 3D stigma and manipulated stigma (positioned centrally). Individual flower was considered as a random factor to account for the blocked design of our experiment. We used the AIC (Akaike, 1974; Burnham and Anderson, 2002) to determine whether this model fit the data better than a null model with only the random factor. Subsequently, we used multiple comparisons through one-way ANOVA, followed by a Tukey test to verify whether the differences were significant between every pair of treatments. The analyses were conducted in R (R Development Core Team, 2006), using the lme4 package for mixed models (Bates et al., 2015). TGgraphics were produced from data analysis with R software (R Development Core Team, 2006) and GraphPad Prism 7.00 (GraphPad Software, La Jolla California USA www.graphpad.com).

3. Results

3.1. Isoplety and morphometric analysis

The sample population of *Turnera subulata* in Campinas comprised 76 L-morph and 77 S-morph plants and was considered isoplethic (1:1 ratio). The morphometric characterization of heterostyly exhibited two-well defined morphotypes (p,0.0001; Fig. 3). The inaccuracy of reciprocal organs was 0.04 for tall organs, and 0.05 for short organs, indicating relatively high levels of reciprocal accuracy in both. Short organs had a higher inaccuracy (lower accuracy) when not scaled by mean-squaring, but tall organs were proportionately more accurate (MSTOI 4.03 % versus MSSOI 2.28 %).

3.2. Frequencies and efficiencies of floral visitors

Flowers were visited most frequently by the non-native bee species *Apis mellifera*, followed by the native bees *Trigona* spp. in both locations where pollinator frequencies were recorded. In Campinas we recorded bees and, very infrequently, a bee fly species (Phthiriinae) visiting the flowers (Fig. 2). In Presidente Figueiredo (Amazonas) Hesperidae butterflies were also recorded as flower visitors. Bees mostly visited a single flower per individual and collected nectar, although pollen collection was also common. Bees frequently landed onto the stigma as they approached the L-morph flowers. To collect nectar, bees needed to crawl down towards the flower base touching reproductive organs on the way. There was no difference in the position (dorsal/ventral) of the pollen deposition onto the bee body. S-morph flowers deposited pollen predominantly on the visitor abdomen while L-morph pollen were concentrated on the bee head. Butterflies always collected nectar and mostly touched only the tall organs.

Experiments evaluating the efficiency of floral visitors showed that the model with donor morph only, and the model with the donor morph associated with the pollen receiver morph were the most highly ranked and equally likely (Table 1, dAICc 0.2). Since the donor-morph model is the simpler model, it was considered the more appropriate to explain factors influencing pollen deposition. Adding pollinators to the model, either as an additive factor or as an interaction factor, did not improve the explanatory power, suggesting that different pollinator species did not substantially influence the amount of pollen transferred. S-morph flowers received more self-pollen on their stigmas and more total pollen after a single visit than L-morph flowers (Fig. 4). Additionally, pollen from S-morph flowers was more frequently found on the stigma surfaces of both S and L-morph flowers (Fig. 4).

3.3. Incompatibility tests

The cross pollination between different morphs (L-morph vs. S-morph) showed high rates of fruit production (above 94 %). Cross pollination within the same morphs, but different flowers, (L-morph vs. L-morph and S-morph vs. S-morphs), did not lead to fruit set. Curiously, the self-pollinated flowers had a non-zero (but very low: 12 %) rate of fruit production, but only in the self-pollinated S-morph (Table 2).

3.4. Three-dimensional (3D) heterostyly experiment

The experiment involving stigma repositioning in the S-morph flowers (Fig. 5) revealed an effect of the three treatment classes (virgin, natural and experiment). The natural (control) treatment (original 3D arrangement) received the most pollen grains (P < 0.0001). Additionally, the stigmas in 3D arrangement (natural in S-morph) received more pollen grains than L-morph flowers, both in absolute terms and as a proportion relative to S-morph pollen, compared to the manipulated stigmas (with the 3D arrangement removed, Fig. 6).

4. Discussion

This study reports the third plant family with evidence of 3D heterostyly in nature. It is also the first time that an important functional role of this mechanism in improving disassortative mating (compatible pollination) and reducing self-pollination in S-morph flowers is shown for isoplethic populations of *Turnera subulata*. The almost three-fold increase in total number of compatible pollen grains deposited in the 3D arrangement compared to the experimental flowers (116.4 vs. 43.5) is a good index of the gain in reproductive fitness by this type of heterostyly.

Both tall and short organs presented a high level of reciprocal accuracy, and two well-defined morphs exist (also recorded by Barrett, 1978 and Schlindwen and Medeiros, 2006). The tall organs are proportionally more accurate, which is a common phenomenon (Armbruster et al., 2009). High reciprocal accuracy is consistent with the results from the 3D cancellation experiment, which shows that compatible crossings (disassortative) were favoured by natural 3D flower morphology. High floral accuracy is not a rule for heterostyly, and some genera such as *Pulmonaria* show extensive variation (up to 20 %) in the scaled Inaccuracy Index (Jacquemyn et al., 2018). In fact, the existence of intra-morph pollination in non-3D-heterostylous species has raised recurrent questions about the functional and evolutionary significance of heterostyly (discussed in Wu et al., 2018).

Although 3D heterostylous flowers may be considered to be phenotypically specialized, due to the elaborate system of reciprocal herkogamy, they also show features which indicate ecologically generalised pollination (Ollerton et al., 2007). Generalized pollination was indicated by the fact that several kinds of floral visitors deposited similar amounts of pollen grains after a single visit. Although the population at Campinas was visited almost entirely by bees, these bees had diverse morphologies and behaviours, arguably belonging to several functional groups (Fenster et al., 2004). In other 3D heterostylous species, *Linum suffruticosum*, pollen was consistently deposited onto either the ventral or dorsal regions of visitors' bodies, therefore flower phenotypic specialization resulted in a specialized use of pollinator body and behaviour (Armbruster et al., 2006). Instead, *T. subulata* seems to rather resemble species of *Oxalis* section *Sagittatae*, where the 3D phenotypic specialization results in a more even distribution of pollen on the animal's body, which allows for a larger set of pollinator sizes and behaviour to be utilized (Turketti et al., 2012).

In line with a relatively generalist pollination system, butterflies of the Hesperiidae family were recorded visiting flowers in the pilot study conducted in Presidente Figueiredo (Amazonas). Furthermore, Barrett (1978) reported butterflies visiting five varieties of *T. ulmifolia* [including *T. ulmifolia* var. *elegans,* synonym of *T. subulata*] in Central America (Costa Rica, Nicaragua, Panamá) and South America (Venezuela and Northern Brazil - Amazon region). Schlindwen and Medeiros (2006) also recorded visits by three species of Hesperiidae, besides one beetle and over 20 species of Hymenoptera (mostly bees) in the seminatural vegetation of seasonal semideciduous forest of coastal plateaus ("Tabuleiro Nordestino" in João Pessoa city Northeastern Brazil). Therefore, it seems possible that lepidopterans may also be effective pollinators despite their absence in the Campinas population. More research is required to fully understand the reproductive biology of *T. subulata*. For example, a report by Medeiros and Schlindwein (2003) found an intimate relationship between *T. subulata* and the oligolectic bee species *Protomeliturga turnerae*, which appears both to mate in the flowers and to collect its pollen.

The well recorded asymmetry in pollen transfer of heterostylous flowers was corroborated in our study, with S-morph flowers exporting more pollen (see Keller et al., 2014; Piper and Charlesworth, 1986; Schlindwen and Medeiros, 2006; Waites and Ågren, 2006). The number of pollen grains of S-morph origin deposited on the stigmatic surfaces was higher in both S-morph and L-morph flowers. This pattern makes sense because S-morph flowers have longer and more exposed anthers,

thus pollen is more accessible to visitors, and pollen export is therefore likely more efficient (Piper and Charlesworth, 1986). Our results are consistent with those of Swamy and Bahadur (1984), who also recorded S-morph flowers as more efficient pollen donors in the reproductive system of *T. subulata*.

Since we could differentiate morph origin by their pollen grain size, we were able to evaluate whether the 3D arrangement enhances disassortative pollen deposition. Indeed, we observed that legitimate pollen flow between morphs was favored by 3D arrangement, as expected for distylous populations (Barrett et al., 2000; Sánchez et al., 2013). Our experiment also revealed that stigmas with a 3D arrangement (S-morph) received significantly higher amounts of pollen grains, and proportionally higher amounts of compatible L-morph morph pollen grains when compared to stigmas with a cancelled 3D arrangement. Therefore, the 3D arrangement increases the amount of legitimate pollen grains received in relation to illegitimate pollen grains (disassortative mating). Thus, 3D heterostyly in *T. subulata* may have evolved for the promotion of legitimate pollen receipt on S-morph stigmas. To distinguish the most likely evolutionary driver, future comparative studies should consider separating intrafloral from intramorph pollen deposition.

5. Conclusions

The export of pollen grains from S-morph flowers was significantly higher than export from L-morph flowers, and the 3D arrangement of stigmas in S-morph flowers increased the proportion of legitimate pollen-grains deposited. In addition, we conclude that the identity of floral visitor species was not a significant factor in explaining differences in pollen deposition. Furthermore, experiments revealed that 3D heterostyly improves pollination by reducing reproductive interference from illegitimate crossings and self-pollination, even in a pollination system that can be considered ecologically generalized.

CRediT authorship contribution statement

André Rodrigo Rech: Conceptualization, Methodology, Project administration, Supervision, Writing - review & editing. **Marina Teixeira Achkar:** Data curation, Investigation, Methodology, Validation, Writing - original draft. **Leonardo Ré Jorge:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - review & editing. **W. Scott Armbruster:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Odair J.G. Almeida:** Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.flora.2020.151559.

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