

Does size matter? Pollination biology of *Aristolochia sessilifolia* (Aristolochiaceae)

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Abstract

Background and aims – The morphology of flowers with specialized pollination acts as a pollinator filter, resulting in compatibility between the flower and its visitors. These characteristics can influence plant diversity, and studying these interactions provides important models for understanding ecological and evolutionary processes. This is the case with *Aristolochia* flowers, which act as a trap, temporarily imprisoning their pollinators. This study aimed to investigate the reproductive biology of *Aristolochia sessilifolia*, a grassland species from South America, by addressing the following questions: (i) What is the duration of anthesis, and how is dichogamy expressed throughout this period? (ii) Which floral visitors are present, and which ones act as effective pollinators? (iii) Does fly body size influence pollen-carrying capacity? (iv) Is there a relationship between perianth dimensions and the presence of pollen on flies? (v) What is the natural fruit set rate?

Material and methods – We collected 50 flowers (10 for each anthesis stage), we defined the anthesis period, identified the floral visitors and measured the height of all flies found, the smallest diameter of the tube, and the distance from the utricle wall and the gynostemium. We evaluated whether these measures influence the fly's ability to transport pollen. In addition, we evaluated natural fruiting by marking flower buds.

Key results – Flowers exhibited a prolonged anthesis (up to five days) and clearly defined protogynous dichogamy. The main pollinators identified were flies from the family Chloropidae. The presence of pollen on fly bodies was associated with body height, the internal diameter of the floral tube, and the distance between the utricle wall and the gynostemium. Natural fruit set reached 50%, a relatively high value compared to other species of the genus.

Conclusion – These results support the hypothesis that compatibility between floral size and pollinator body size is necessary for effective pollination, emphasizing the specificity and complexity of *Aristolochia* flowers.

Keywords

Chloropidae, ecology, floral morphology, pollinator size, South America, trap flowers

INTRODUCTION

The interaction between flowers and pollinators shapes floral traits that maximize reproductive success, making it essential to distinguish floral visitors from effective pollinators (Neëman et al. 2010). Natural selection, as an ecological process, is strongly influenced by interactions among species, including the role of pollinators in the evolution of angiosperms (Opedal 2021). Understanding the function of floral attributes

in specific contexts, such as pollination, allows for the elucidation of phenotypic selection mechanisms and enhances the predictability of evolutionary processes (Opedal 2021). Specialized pollination systems reveal exclusive channels of communication between plants and pollinators (Chen et al. 2009; Schäffler et al. 2015), based on adaptations associated with the offering or simulation of resources (Ackerman et al. 2023). In this context, trap flowers represent valuable models for investigating evolutionary patterns, exhibiting elaborate morphological

mechanisms for the temporary retention and release of pollinating flies (Kidyoo et al. 2022). These flowers also constitute a remarkable example of convergent evolution, bringing together species from distinct families that have independently evolved trap-flower morphologies (Ollerton et al. 2017). Several attractive traits of specialized plants shape their reproductive performance, but non-attractive traits remain poorly explored, even though they may determine which animals effectively transport pollen (Matallana-Puerto et al. 2024a). Despite their ecological and evolutionary relevance, interactions between trap-flowers and flies remain understudied due to the methodological and taxonomic complexity associated with the group (Diptera) (Matallana-Puerto et al. 2025).

The genus *Aristolochia* L. (Aristolochiaceae) represents the earliest clade of angiosperms to evolve a highly elaborate pollination mechanism involving trap flowers (Oelschlägel et al. 2009). The pollination system of these species relies on temporary traps that capture small flies (Diptera). Flowers have a jug-shaped perianth, ranging in colour from purple to brown, with attractive appendages (Oelschlägel et al. 2016). The perianth consists of three main parts: limb, tube, and utricle (Fig. 1A–B). During the early days of anthesis, the gynostemium is in the stigmatic phase, and insects pass through the floral tube, becoming trapped in the inflated utricle (Fig. 1C). Subsequently, the staminate phase begins with anther dehiscence (Fig. 1D). At this stage, trichomes wither or the tube bends, releasing insects now covered in pollen (Fig. 1F–G). Upon visiting another flower in the stigmatic phase, these insects facilitate cross-pollination (Hipólito et al. 2012). However, studies exploring how floral morphology influences pollination mechanisms and how it is shaped by this interaction remain scarce (Park and Kim 2023). Thus, if most pollination systems in *Aristolochia* are specialized for particular groups of flies, it is likely that these Dipteras exert selective pressure on floral traits. These plants possess a set of features that make them ideal models for investigating the relationship between floral morphology and the fit between flowers and pollinators, such as the production of volatile compounds that attract specific pollinators (Oelschlägel et al. 2015; Rupp et al. 2021; Alpuente et al. 2023) and the presence of functional structures, such as trichomes that capture, retain, and subsequently release live pollinators, ensuring effective fruit formation (Oelschlägel et al. 2009; Matallana-Puerto et al. 2024a). Some *Aristolochia* species exhibit preferences for pollinators of particular body sizes, which may limit fruit set. In such cases, the perianth functions as a morphological filter, selecting which insects are capable of passing through the floral tube and reaching the gynostemium (Nakonechnaya et al. 2021). During the stigmatic phase, downward-oriented trichomes facilitate insect entry into the utricle but hinder their exit, acting as a unidirectional barrier (Oelschlägel et al. 2009). Furthermore, the size of specific floral structures, such as the tube and utricle, may determine

which insects can access the floral chamber and interact with the gynostemium. To act as effective pollinators, insects must meet two size-related criteria: they must be small enough to pass through the narrow floral tube but large enough for their bodies to contact the reproductive structures and efficiently transport pollen grains (Burgess et al. 2004; Rulik et al. 2008; Oelschlägel et al. 2009; Park and Kim 2023).

The functional pollinator group of *Aristolochia* consists of flies (Diptera). However, the level of floral specialization varies among species: some rely on exclusive pollinators, whereas others exhibit more generalist systems involving Diptera from different families (Sakai 2002; Burgess et al. 2004; Rulik et al. 2008; Alpuente et al. 2023). This degree of specialization may be reflected in distinct floral traits, such as flower longevity, attraction, and trapping mechanisms, and the extent of pollinator dependence and sharing (Berjano et al. 2009). The diversity of Diptera families associated with *Aristolochia* represents about 35% of all families, with a predominance of Phoridae, Drosophilidae, and Sciaridae (Matallana-Puerto et al. 2025). This wide range of potential pollinators suggests that *Aristolochia* species exploit attraction strategies based on different ecological behaviours of flies, encompassing groups of various body sizes (Burgess et al. 2004; Hipólito et al. 2012; Matallana-Puerto et al. 2025). For example, the main groups attracted to these flowers display diverse foraging and oviposition sources, including decaying organic matter, parasitism, or feeding on living animal and plant tissues, as well as nectar (Skevington and Dang 2002). In several *Aristolochia* species, the flowers produce compounds that mimic odours of decaying animals, injured insects (attracting kleptoparasitic flies), or oviposition sites, all of which are effective in attracting visitors (Oelschlägel et al. 2015; Rupp et al. 2021; Alpuente et al. 2023). These examples illustrate the ability of these flowers to exploit distinct ecological habits and develop specific communication channels that, together with their trapping, retention, and release structures, select for particular pollinator lineages (Matallana-Puerto et al. 2025).

Considering the highly specialized floral morphology of *Aristolochia*, the attraction of functional groups of pollinators with diverse ecological habits, and the set of mechanical structures involved in the capture, retention, and release of pollinators, these flowers represent a remarkable model for investigating how floral morphology influences pollination efficiency (Oelschlägel et al. 2009; Matallana-Puerto et al. 2024a, 2025). Furthermore, as a striking example of convergent evolution, they can shed light on general patterns of adaptation between flowers and their pollinators (Ollerton et al. 2017). We hypothesize that floral traits associated with flower–pollinator matching determine the potential pollinators and pollen transport efficiency in *Aristolochia sessilifolia* (Koltzsch) Duch. To test this hypothesis, we addressed the following questions: (i) What is the duration of anthesis, and how is dichogamy expressed throughout

this period? (ii) Which insects visit the flowers, and which act as effective pollinators? (iii) Does fly body size influence their ability to transport pollen? (iv) Is there a relationship between perianth size and the ability of flies to carry pollen? (v) What is the natural fruit set rate? To answer these questions, we conducted field experiments, morphological analyses, and statistical tests.

MATERIAL AND METHODS

Study species and site

Aristolochia comprises approximately 550 species, primarily distributed across tropical, subtropical, Mediterranean, and temperate regions (Nath et al. 2022). Brazil is one of the centres of diversity for the genus, with around 93 recorded species (Rebouças et al. 2020). Among them is *A. sessilifolia*, a prostrate herb with sessile leaves (Fig. 1A; Freitas et al. 2025), native to South America and occurring in Argentina, Paraguay, Uruguay, and southern Brazil (Zuloaga et al. 2008). The species is found in grassland areas, typically sunny and with dry soils, forming patches of individuals that undergo defoliation during colder periods, surviving through perennial rhizomes (Mega et al. 2015). The fruits are dehiscent capsules that open longitudinally along six sutures (Fig. 1K). The seeds are triangular in shape, with a rounded, non-winged base and a wrinkled, shiny surface. On the dorsal surface, a prominent aril forms a straight line extending from the base to the apex of the seed (Fig. 1L). Sampling of *A. sessilifolia* (J.M. Brandalise 14821, RSPF) individuals was conducted in the municipality of Passo Fundo, Rio Grande do Sul, Brazil (28°13'40.4"S, 52°22'51.1"W). This region is located on a plateau between the Atlantic Forest and Pampa biomes, with a predominance of Mixed Ombrophilous Forest (IBGE 2019). The population studied occurs in a small fragment of grassland vegetation, surrounded by planted woodland and urban areas.

Flower biology

To determine the flowering anthesis period, 50 floral buds were monitored daily. The beginning of anthesis was defined as the moment the flower opened, while its end was determined when the flower entered senescence, characterized by wilting and abscission. During each day of the anthesis period, 10 flowers were collected (totalling 50 flowers). All flowers were dissected to expose the gynostemium and the trichomes of the tube and utricle (Fig. 1C). To determine the pattern of dichogamy for each flower, the following characteristics were recorded: trichome turgidity, gynostemium maturation stage (colouration and anther dehiscence), and perianth curvature. The pistillate phase was characterized by a rigid and sticky stigmatic surface, closed anthers, rigid trichomes, and an erect flower (Fig. 1C). The staminate phase was identified by the onset of anther dehiscence,

with pollen grain exposure, flaccid trichomes, and a slightly wilted flower (Fig. 1D; Rulik et al. 2008). All flowers were analysed and categorized based on these morphological traits and the day of anthesis, in order to determine the dichogamy pattern and the duration of each phase.

Floral visitors and pollinators

All 50 collected flowers with the utricle chamber opened (Fig. 1C–D) had their floral visitors collected, preserved, and identified. Dipteran specimens were identified with the assistance of entomological specialists. Floral visitors were identified to the family level and subsequently classified into two categories: those carrying pollen (effective pollinators) and those without pollen (non-pollinating visitors).

Flower-pollinator fit

To measure the perianth structures, the same 50 flowers were assessed for: (i) the minimum diameter of the floral tube and (ii) the distance between the utricle wall and the base of the anthers on the gynostemium (Fig. 1C). These measurements were selected because they theoretically (i) filter the size of visitors capable of entering and exiting the flower and (ii) determine which pollinators can come into contact with the gynostemium. Measurements were taken during the anthesis period, corresponding to the day on which each set of flowers was collected (10 flowers per day of anthesis). All visitors and pollinators found within these flowers were measured individually by recording their maximum body height (from the thorax to the tibia), as these structures define the insect's body height and influence its ability to move within the floral chamber.

Natural reproductive success

Natural fruit set was determined by marking floral buds prior to anthesis and monitoring them until fruit formation, without any manipulation or interference (Brandalise et al. 2025). A total of 20 floral buds were used in this experiment.

Data analysis

All measurements of floral structures and fly body size were recorded. All data were normally distributed, according to the Shapiro-Wilk normality test ($p > 0.05$). To evaluate significant differences among groups, an analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparison test to identify statistically significant pairwise differences ($p < 0.05$). Flies were classified into three categories: "large", "small with pollen", and "small without pollen". This classification was based on the largest body size observed among individuals carrying pollen. Flies with a body size equal to or smaller than this value were classified as "small", while those with a larger body size were considered "large". The distribution of measurements across groups and the

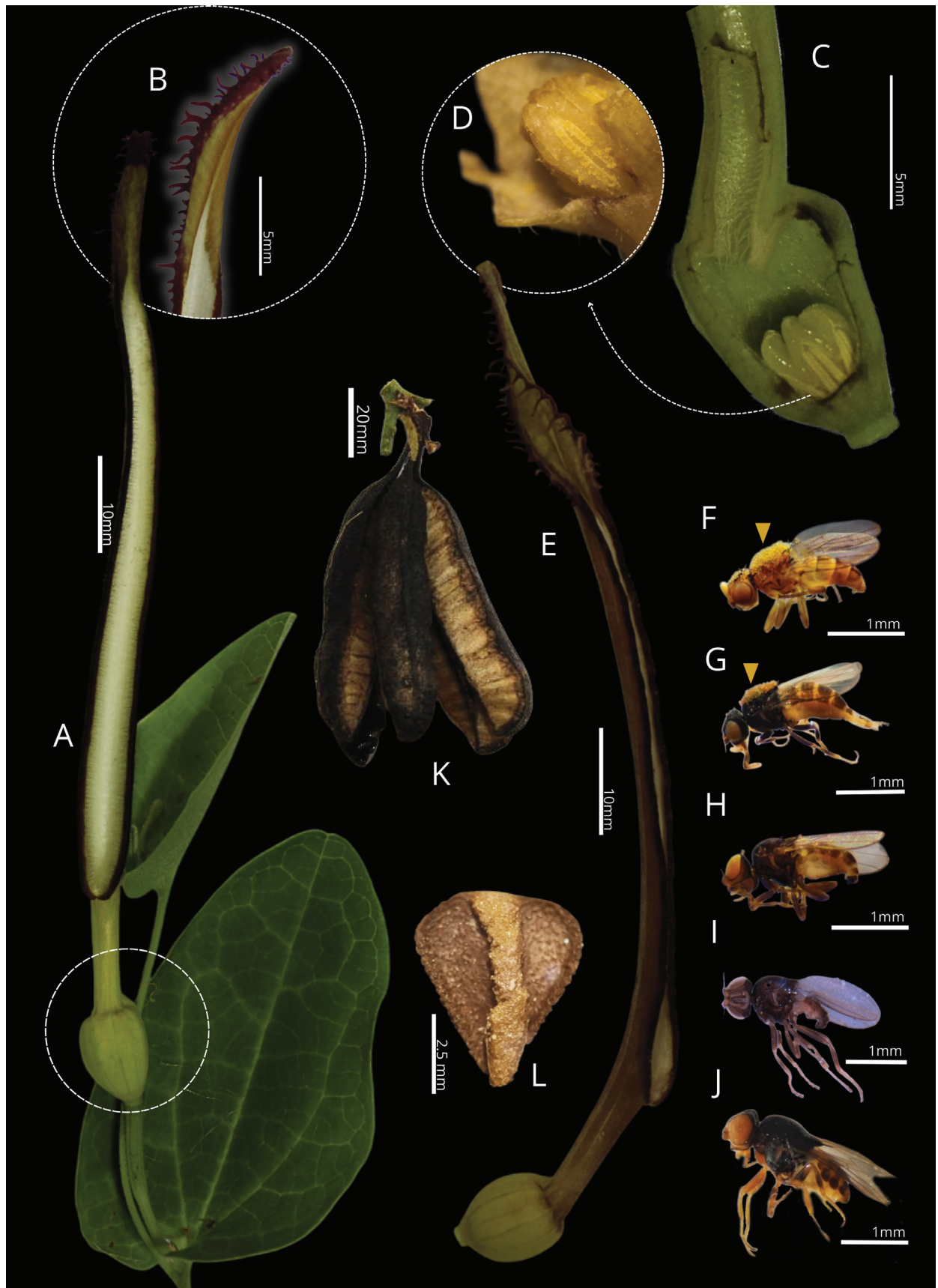


Figure 1. *Aristolochia sessilifolia*, pollinators and flower visitors (Chloropidae). **A.** Flower in stigmatic phase and leaves. **B.** Details of the limb. **C.** Longitudinal section of the utricule and tube, gynostemium in stigmatic phase. **D.** Gynostemium in staminate phase, with open anthers exposing the pollen grains. **E.** Flower in staminate phase. **F–G.** Pollinator carrying pollen (yellow arrowheads). **H.** Small fly without pollen. **I–J.** Large flies without pollen. **K.** Open fruit. **L.** Seed.

Table 1. Size categories of flies found on *Aristolochia sessilifolia* flowers, total number in each category, mean, and standard deviation of each group.

Fly category	n	Mean (min–max)	Standard deviation
Large	32	1.87 (1.50–2.50)	0.26
Small without pollen	105	1.24 (0.90–1.49)	0.15
Small with pollen	21	1.23 (0.90–1.50)	0.17

statistical differences were graphically represented using boxplots, with different letters indicating statistically distinct groups according to Tukey's test.

To assess patterns of morphological variation between groups of flies with and without pollen, we conducted a Principal Coordinates Analysis (PCoA) based on Gower distance as a dissimilarity measure (Gower 1971; Legendre and Legendre 2012). This metric is appropriate for datasets composed of variables of different types (continuous and categorical), such as the morphological data used in this study (floral and fly measurements, and the presence or absence of pollen adhered to the body). The distance matrix was constructed using fly body height and two floral traits: the smallest diameter of the floral tube and the distance between the utricle wall and the gynostemium. Samples were grouped based on the presence or absence of pollen on individuals, with groupings visualized through 95% confidence ellipses on the ordination plot. To statistically test whether morphological composition differed between groups, we applied a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations (Anderson 2001). The relationship between morphological variables and sample distribution along the PCoA axes was assessed using environmental vector fitting (envfit) onto the main ordination axes, based on vector correlation (Oksanen et al. 2024). Vectors of variables that were significant ($p < 0.05$) were plotted on the graph, indicating the direction and gradient of greatest variation associated with each. All analyses were performed in RStudio v.2024.09.0+375 using the packages *vegan* v.2.7-1, *cluster* v.2.1.8.1, *ellipse* v.0.5.0, and *ggplot2* v.3.5.2 (Wickham 2016; Murdoch and Chow 2023; Maechler et al. 2024; Oksanen et al. 2024; Posit Team 2024).

RESULTS

Flower biology

The flowers remained open for five days and exhibited protogynous dichogamy, in which the reproductive structures of the gynoecium mature before those of the androecium. Two distinct reproductive phases were observed throughout the five-day anthesis period. During the stigmatic phase, which occurs over the first two days of anthesis, the flowers display rigid trichomes, closed anthers, and a receptive stigma (Fig. 1A–C). From the third day onward, the anthers begin to dehisce, the

trichomes gradually lose rigidity, and both the tube and the limb become flaccid until the fifth day, when the flower completely withers (Fig. 1D–E). No changes in perianth curvature were observed between anthesis phases. Based on the recorded dichogamy characteristics, no evident overlap between phases was detected. If overlap occurs, the only possible window is very short, at the beginning of the third day, when the anthers start dehiscing and the stigma is already slightly wilted but may still retain some residual receptivity.

Floral visitors and pollinators

A total of 158 Diptera individuals were found inside the flowers (Table 1). Only 17 out of the 50 collected flowers contained insects trapped within the utricle chamber, representing 34% of the sampled flowers. Of these, 16 were in the stigmatic phase and only one in the staminate phase. Among the captured Diptera, only 21 individuals had pollen grains adhered to their thorax (Fig. 1F–G; Table 1). All flies belonged to the family Chloropidae (Diptera) (Fig. 1F–J). The insects were categorized into two groups based on body size: large and small (0.90–1.49 mm) ($p < 0.05$; Table 1). None of the large insects carried pollen grains (Fig. 1I–J). Small insects were further subdivided into “with pollen” and “without pollen”, according to the presence or absence of pollen grains (Fig. 1F–H). Among the 158 Diptera individuals found, 105 were small without pollen, 21 were small with pollen, and 32 were large (Table 1). There were statistically significant differences in body size between “small” and “large” flies (Fig. 2; $p < 0.05$).

Flower-pollinator fit and natural reproductive success

According to Tukey's test, no significant differences were observed between the body size of small flies and the distance from the gynostemium to the utricle wall, whereas large flies were significantly larger than this floral measurement (Fig. 2). The minimum tube diameter differed significantly from all other measurements, being larger than each of them (Fig. 2; $p < 0.05$). Principal Coordinates Analysis (PCoA), based on Gower distance, revealed a clear separation between samples of flies with pollen and those without, forming two distinct, non-overlapping ellipses (Fig. 3). The first two ordination axes together explained 92.3% of the total variation in the data (PCoA 1 = 63.7%; PCoA 2 = 28.6%). PERMANOVA analysis confirmed that this separation between groups was statistically significant ($R^2 = 0.55$; $p = 0.001$). The

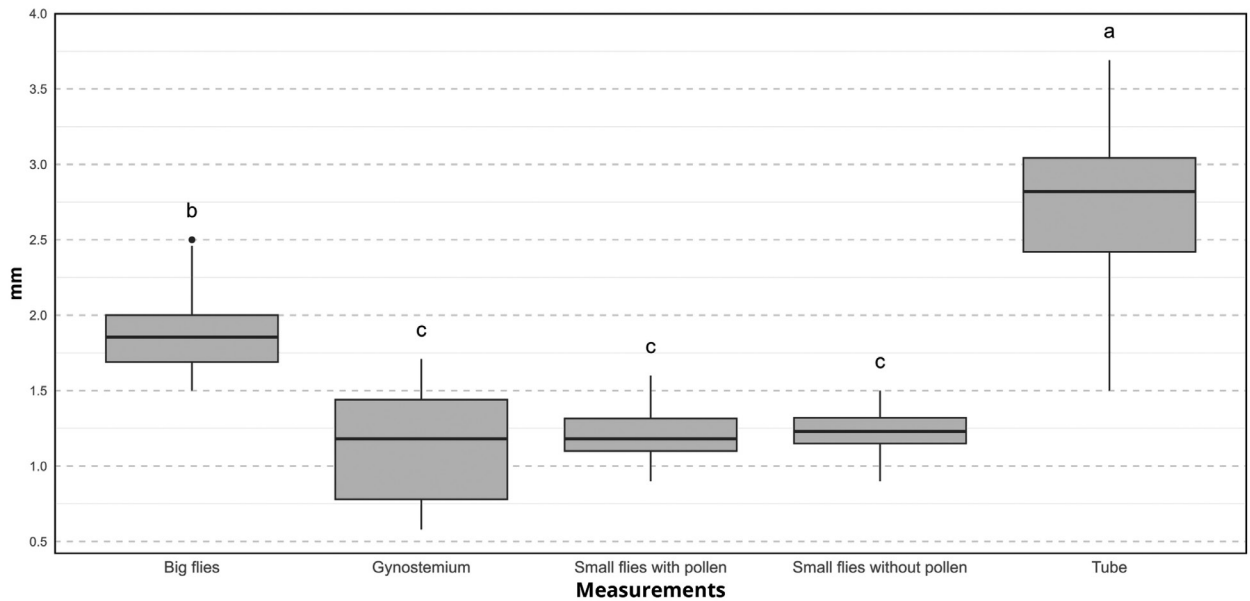


Figure 2. Boxplots showing the distribution of morphological measurements for each category: flies (large, small without pollen, and small with pollen), minimum floral tube diameter, and distance between the utricle wall and the gynostemium. Statistical differences between groups were assessed using Tukey's test. Different letters indicate statistically significant differences ($p < 0.05$).

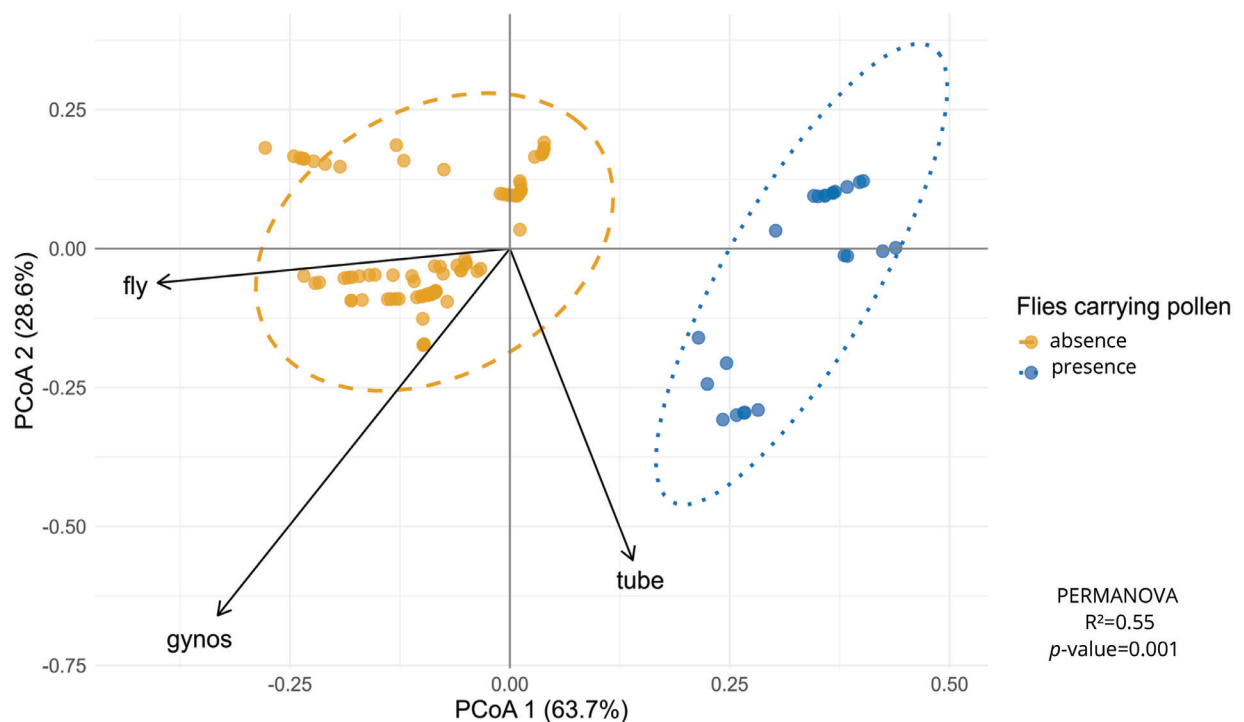


Figure 3. Principal Coordinates Analysis (PCoA) based on Gower distance, showing morphological separation between flies with (blue) and without (orange) pollen. Ellipses represent 95% confidence intervals for each group. Black vectors indicate morphological variables significantly correlated with the ordination ($p < 0.05$), with vector length and direction representing the strength and direction of the association with the main axes. Tube = minimum floral tube diameter; Gynos = distance between the gynostemium and the utricle wall; Fly = fly body height. PCoA axes 1 and 2 explain 63.7% and 28.6% of the total morphological variation, respectively. Group separation was statistically confirmed by PERMANOVA ($R^2 = 0.55$; $p = 0.001$).

projection of vectors from the envfit function indicated that floral traits, such as the minimum diameter of the floral tube and the distance from the gynostemium to the utricle wall, as well as fly body size, contributed significantly to the ordination structure (Fig. 3). Tube width was more strongly associated with the presence of pollen, while fly body size was correlated with the absence of pollen (Fig. 3). Reproductive success under natural conditions was assessed in 20 floral buds, of which ten developed into fruits, resulting in a 50% fruit set.

DISCUSSION

To date, it is known that most *Aristolochia* species with tropical distributions exhibit a maximum anthesis duration of two days (Costa and Hime 1981; Wolda and Sabrosky 1986; Sakai 2002; Hipólito et al. 2012; Matallana-Puerto et al. 2024b), whereas species from temperate regions display longer anthesis periods, as observed in the species studied here and in others (Berjano et al. 2009; Stotz and Gianoli 2013; Cunha et al. 2022; Song et al. 2022; Alpuente et al. 2023). The flowers of *Aristolochia sessilifolia* exhibit a floral longevity of five days, which is longer than that reported for most South American species of the genus. For example, *A. argentina* Griseb., native to Argentina, and *A. grandiflora* Salisb., from Mexico, have flowers that last approximately two days (Burgess et al. 2004; Trujillo and Sérsic 2006). In contrast, *A. chilensis* Bridges ex Lindl., native to Chile, has the longest floral lifespan in the genus, with flowers remaining open for up to eight days (Stotz and Gianoli 2013). Floral longevity has been shown to be directly influenced by environmental stressors such as aridity (Stotz and Gianoli 2013). In *A. rotunda* L., from the Mediterranean region, floral anthesis duration is flexible, with the stigmatic phase extending in populations where pollinator density is low (Oelschlägel et al. 2016). Similarly, *A. baetica* L. and *A. paucinervis* Pomel, also from the Mediterranean, exhibit floral longevity ranging from five to seven days, possibly related to their low fruit set rates (1–14%) (Berjano et al. 2009). These findings suggest that both environmental factors and pollinator availability play a key role in regulating flower longevity, enhancing reproductive success and opportunities for cross-pollination, albeit at a higher energetic cost (Stotz and Gianoli 2013; Oelschlägel et al. 2016).

As observed in *A. argentina*, the anthesis stages in *A. sessilifolia* are well-defined, with noticeable changes in the gynostemium, perianth, and trichomes throughout the different floral phases (Trujillo and Sérsic 2006). It is possible that, in some cases, incomplete protogyny occurs, in which the anthers begin dehiscence during a short period when the stigma still retains some degree of receptivity. However, geitonogamy may be compensated for by late-acting self-incompatibility mechanisms or by the absence thereof (Alpuente et al. 2023; Matallana-Puerto et al. 2024b). The staminate phase represents a critical point in the pollination process, as it is during this period that insects remain trapped inside the utricle and

must survive until their release (Stotz and Gianoli 2013). In some species, the presence of nectaries within the utricle provides nutritional resources to pollinators during entrapment (Erbar et al. 2017). However, we did not test for the presence of nectaries in *A. sessilifolia*; therefore, no evidence is currently available. The staminate and trapping phase in *A. sessilifolia* lasts approximately two days, representing an intermediate duration compared to other species in the genus (Stotz and Gianoli 2013). In species with short floral longevity, such as *A. argentina*, *A. maxima* Jacq., *A. inflata* Kunth, *A. grandifolia*, and *A. esperanzae* Kuntze, the trapping period usually lasts only one day (Sakai 2002; Burgess et al. 2004; Trujillo and Sérsic 2006; Matallana-Puerto et al. 2024b). In contrast, species with longer floral longevity, such as *A. chilensis*, may exhibit entrapment periods of two to three days, while in Mediterranean species like *A. paucinervis* and *A. baetica*, this phase can extend from three to five days (Berjano et al. 2009; Stotz and Gianoli 2013). In general, protogyny is one of the most effective mechanisms for preventing self-pollination when compared to protandry (Guo et al. 2013). Its effectiveness lies mainly in the ability to receive cross-pollen before the release of its own pollen (Griffin et al. 2000). In protogynous *Aristolochia* flowers, the stigmatic phase is typically longer than the staminate phase, increasing the likelihood of attracting visitors, receiving cross-pollen, and achieving fruit set (Matallana-Puerto et al. 2024b). These findings suggest a pattern in which the duration of insect entrapment is associated with floral longevity. In this context, it would be relevant to investigate the existence of mechanisms that support pollinator survival in long-lived flowers, such as in *A. sessilifolia*, particularly the presence of nectaries or other nutritional resources.

All 158 Diptera individuals found in *A. sessilifolia* belonged to the family Chloropidae (Fig. 1F–J), a group of small flies previously reported as pollinators of several *Aristolochia* species, including *A. arcuata* Mast., *A. rotunda*, *A. bianorii* Sennen & Pau, and *A. pilosa* Kunth (Wolda and Sabrosky 1986; Berjano et al. 2009; Oelschlägel et al. 2015; Alpuente et al. 2023). Among these individuals, only 21 had pollen grains attached to the dorsal region of the thorax (Fig. 1F–G), the typical site of pollen deposition in flies, as they walk along the inner wall of the utricle with their dorsum facing the gynostemium (Burgess et al. 2004). Additionally, the flies were concentrated in just 17 (34%) flowers, and most of them, including those carrying pollen, were found during the stigmatic phase. This suggests that these individuals had previously entered flowers in the staminate phase, acquired pollen, and successfully escaped the trap mechanism (Rulik et al. 2008). The aggregation of multiple flies in a limited number of flowers has also been observed in *A. contorta* Bunge and *A. bianorii*, indicating that certain flowers act as focal points of attraction, potentially reducing the overall effectiveness of the pollination system (Alpuente et al. 2023; Park and Kim 2023). This may be related to the attraction capacity of each flower or to the efficiency

of floral morphology in capturing visitors (Park and Kim 2023). The relationship between *Aristolochia* flowers and pollinating flies of the family Chloropidae is well established and has been documented in several species (Berjano et al. 2009; Oelschlägel et al. 2016; Alpuente et al. 2023). In such cases, the flowers attract almost exclusively Chloropidae flies, indicating a close flower–pollinator relationship (Oelschlägel et al. 2016; Alpuente et al. 2023). The flowers may mimic either oviposition sites or food sources, exploiting the behaviour of flies with kleptoparasitic habits (Oelschlägel et al. 2016; Alpuente et al. 2023). Although all flies belonged to the Chloropidae family, they were divided into two distinct groups based on body size: “large” and “small”, with statistically significant differences between them ($p < 0.05$; Fig. 2). Only the small flies carried pollen grains on their bodies (Fig. 1F–G), suggesting a potentially exclusive role in the pollination of *A. sessilifolia*. Body size has already been identified as a key factor influencing pollination efficiency in several *Aristolochia* species. In *A. manshuriensis* Kom., for instance, only individuals of a specific size range are capable of transporting larger amounts of pollen, reducing the need for multiple floral visits to ensure successful pollination (Nakonechnaya et al. 2021). Therefore, in addition to attracting a specific group of flies, floral morphology appears to impose a further filter on effective pollination, namely, the pollinator’s body size.

In the case of *A. sessilifolia*, both small and large flies had body sizes significantly smaller than the floral tube diameter ($p > 0.05$), which explains their ability to pass through this narrow structure (Fig. 2). However, when comparing body size to the distance between the utricle wall and the gynostemium, large flies were significantly larger than this spatial gap ($p < 0.05$), preventing direct contact with the gynostemium. As a result, these flies tend to move only along the upper wall of the utricle, without reaching the reproductive structures (Fig. 1). In contrast, small flies did not differ significantly from this measurement ($p > 0.05$), allowing them to interact with the gynostemium and, in some cases, carry pollen (Figs 1F–G, 2). These results demonstrate that body size is a key determinant of the pollination role of flies in *Aristolochia*. For a fly to be considered an effective pollinator, it must be able to: (i) pass through the floral tube, (ii) reach the gynostemium, (iii) acquire pollen, (iv) exit the flower alive, and (v) deposit the pollen onto a receptive flower (Rulik et al. 2008). Principal Coordinates Analysis (PCoA) revealed that flies carrying pollen on their bodies formed distinct clusters from those without pollen, with statistically significant separation as indicated by PERMANOVA (Fig. 3). The presence of pollen was associated with morphological variation in both floral traits (such as tube diameter and the distance between the gynostemium and the utricle wall) and fly body traits (height). Together, these factors explained 92% of the observed variation. Relationships between floral traits and pollinator body size have been reported for other *Aristolochia* species

(Burgess et al. 2004; Trujillo and Sérsic 2006; Rulik et al. 2008; Park and Kim 2023), and the results presented here reinforce this pattern in *A. sessilifolia*. In summary, only flies that are able to pass through the floral tube and interact with the reproductive structures are potential pollinators. This condition is reflected in the direction of the vectors for floral traits associated with the presence of pollen on fly bodies in the multivariate analysis (Fig. 3).

The natural fruit set rate of *A. sessilifolia* was 50%, a relatively high value compared to other species within the genus. Reproductive systems in *Aristolochia* are diverse, ranging from self-compatible to self-incompatible species. For example, *A. gigantea* shows approximately 40% natural fruit set, while *A. maxima* is self-incompatible and entirely dependent on pollinators for fruit production (Sakai 2002; Hipólito et al. 2012). In contrast, species such as *A. bracteolata*, *A. tagala*, *A. inflata*, and *A. manshuriensis*, which present only 2% natural fruit set, are self-compatible but still rely on pollinator visits for pollen transfer (Razzak et al. 1992; Sakai 2002; Murugan et al. 2006; Nakonechnaya et al. 2021). An exception is *A. bianorii*, which is capable of autonomous self-pollination, overcoming barriers of protogyny and herkogamy, and thus does not rely on pollinators for reproductive success (Alpuente et al. 2023). In self-compatible species, geitonogamy often results in higher reproductive success than pollination by natural pollinators, suggesting limitations in pollinator efficiency (Murugan et al. 2006). In *A. argentina*, for example, manual self-pollination resulted in 88% fruit set, while natural pollination by pollinators led to only 14.8% (Trujillo and Sérsic 2006). These limitations may be related to low pollinator abundance or efficiency, low pollen viability, high specificity in plant-pollinator interactions, or a short duration of the stigmatic phase (Oelschlägel et al. 2016; Nakonechnaya et al. 2021). In this context, self-compatibility may function as a compensatory mechanism, ensuring fruit production even under conditions of pollinator scarcity (Alpuente et al. 2023). Furthermore, our results are similar to those reported for *A. esperanzae* in a Brazilian population, which showed 40% natural fruit set (Matallana-Puerto et al. 2024b). This species was the first to be reported with late-acting self-incompatibility mechanisms, indicating that its reproductive success results from cross-pollination rather than self-pollination (Matallana-Puerto et al. 2024b). It is possible that a similar pattern occurs in *A. sessilifolia*, involving some degree of self-incompatibility or pollinator dependence. In our study, only fruit set under natural pollination was evaluated, suggesting that the reproductive success observed in *A. sessilifolia* may reflect both the effectiveness of pollinators and the possible occurrence of some degree of self-compatibility. To better understand the reproductive system of *A. sessilifolia*, additional experiments involving manual pollination are needed to isolate the effects of self-compatibility and pollinator dependence.

CONCLUSION

Our results support the initial hypothesis that compatibility between floral size and pollinator body size is necessary for effective pollination, highlighting the specificity and complexity of *Aristolochia* flowers. We found that this species exhibits prolonged floral longevity and that its flowers attract and are pollinated by specific flies belonging to the family Chloropidae. We demonstrated that the body size of these flies, along with specific floral morphological dimensions, determines which individuals are capable of carrying pollen. Thus, the size of both the pollinators and the flower acts as a selective filter, distinguishing occasional visitors from effective pollinators. The high natural fruit set rate observed (50%) indicates pollination system efficiency and/or the possibility of some degree of reproductive compatibility. However, these hypotheses should be tested through controlled pollination experiments. Additionally, further studies investigating the mechanisms of floral attraction are necessary, since only flies from a single taxonomic group were recorded, suggesting the possible existence of a specific chemical attraction targeting these pollinators. Together, our results enhance the understanding of pollination mechanisms in trap flowers, a complex system resulting from convergent evolution across different angiosperm lineages. We emphasize that *Aristolochia* represents a particularly valuable model for ecological and evolutionary studies, as it constitutes the earliest angiosperm clade to develop this highly specialized pollination mechanism.

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