Mechanical stimulation of the stigmas triggers switch from female to male phase in the protogynous trap flower of Aristolochia rotunda (Aristolochiaceae)

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Mechanical stimulation of the stigmas triggers switch from female to male phase in the protogynous trap flower of *Aristolochia rotunda* (Aristolochiaceae)

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Abstract. Floral longevity is a selected trait that shows plasticity, allowing plants to balance resource allocation and reproduction. In dichogamous flowers —in which female and male functions are decoupled in time— the duration of the female phase is expected to vary according to pollination status. We used *Aristolochia rotunda* as a model to test the hypothesis that the female phase should be shortened following pollen deposition on the stigma, and to identify the signal for phase switching. *Aristolochia* flowers are protogynous (female phase first) and trap pollinators for one to two days (trap flowers). The four experimental treatments we applied to flowers, i.e. hand pollination, presence of pollinators with or without pollen load in the flower, and deposition of a nylon thread on the stigma, shortened the female phase to a similar extent, demonstrating that the duration of the female phase depended on the presence of pollinators, independently of whether or not they carried pollen, and that mechanical stimulation of the stigmas was the signal for phase switching. This mechanism of post-anthesis floral changes is original because usually such changes are triggered by chemical interactions between pollen and stigmas. We interpret the mechanical signal used in *A. rotunda* for phase switching to be adaptive when pollinators are limiting, because switching to the male phase even if the trapped pollinator does not bring pollen would ensure fulfilling the flower's male function.

Keywords: pollination, floral longevity, dichogamy, pollinator limitation, Mediterranean region.

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Introduction

Floral longevity is part of the evolutionary strategy of plants, as it is under selective pressure acting on the balance between opportunities to ensure reproductive functions and the costs of maintaining flowers (Ashman & Schoen, 1994). As a consequence, floral longevity is highly variable across species, depending on factors such as environmental constraints, pollination strategies and breeding systems (Primack, 1985). Plasticity in floral longevity can also be adaptive. For instance, floral longevity can be extended in case of insufficient fertilization, until the costs of floral maintenance exceed the benefits of improved fertilization (Ishii & Sakai, 2000).

Dichogamy, the temporal separation of female and male functions within an individual plant or bisexual flower, is a common strategy in angiosperms that is considered to have evolved to avoid self-interference between female and male functions. Self-interference includes selfing, but not only, as many dichogamous plants also display self-incompatibility (Lloyd & Webb, 1986; Bertin & Newman, 1993; Endress, 1996). For animal-pollinated plants, dichogamy may be one way to solve the dilemma of touching a single spot on the pollinator with both stigmas and anthers (necessary to ensure effective pollen transfer between flowers) whilst avoiding deposition of self-pollen on stigmas, which would result in clogging of stigmas and loss of pollen load (negatively affecting both female and male functions) (Lloyd & Webb, 1986). Dichogamous flowers can be either protogynous (female phase first) or protandrous (male phase first), and the degree of overlap between female and male functions varies among species (Bertin &

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Newman, 1993, Alpuente *et al.*, 2023). Floral longevity is under the same selective pressures in both dichogamous and adichogamous species. For instance, increased longevity is found in species from environments where pollinator abundance is low or unpredictable (Stotz & Gianoli, 2013). However, selection for plasticity in floral longevity has slightly different outcomes in dichogamous and adichogamous species. As it seems more difficult for plants to detect pollen removal than pollen deposition status (but see Devlin & Stephenson, 1984), the duration of the male phase should be less plastic than the duration of the female phase (Ishii & Sakai, 2000). The female phase is expected to end as soon as sufficient fertilization is achieved, or even earlier, for instance as soon as pollen is deposited, in response to floral maintenance costs and the time needed to fulfill the male function. As expected, pollination has been shown to shorten floral longevity in dichogamous species (Mione *et al.*, 2017; but see Zhao *et al.*, 2020), and more specifically to shorten the duration of the female phase in both protogynous (Gottsberger *et al.*, 1980; Wells & Lloyd, 1991) and protandrous (Devlin & Stephenson, 1984; Richardson & Stephenson, 1989) species. Pollen removal and increased pollinator availability are also known to shorten the duration of the male phase in protandrous species (Devlin & Stephenson, 1984; Richardson & Stephenson, 1989; Sargent & Roitberg, 2000; Giblin, 2005). In addition, increased temperature was shown to shorten the duration of the female phase (Gottsberger *et al.*, 1980; Wells & Lloyd, 1991). Whereas response to the status of the sexual organs is most likely adaptive, response to environmental factors might be merely a physiological effect, not an adaptive response driven by selection.

Aristolochia L. (Aristolochiaceae) is a genus belonging to the magnoliids, a basal angiosperm clade (Soltis *et al.*, 2018). Like most ancestral lineages of angiosperms, *Aristolochia* species are protogynous (Endress, 2010). They stand out among basal angiosperms because they have trap flowers —flowers that retain insect pollinators for hours or days. Protogyny is a plesiomorphy that preceded and likely facilitated the evolution of the trap flower in *Aristolochia*. First, protogyny limits interference between pollen and stigmas, a condition that is particularly important when pollinators are detained in the flower. Second, protogyny allows cross-fertilization to occur first, and in the case of trap flowers with incomplete dichogamy, it also allows self-fertilization as a back-up strategy (Alpuente *et al.*, 2023) either through autonomous selfing or because there is a high probability that a trapped insect would transfer self-pollen to the stigmas if there is a slight overlap between the female and male phases. Trap flowers are unlikely to evolve in non-protogynous species because the combination of protandry or adichogamy and trap flowers would result inevitably in massive self-pollination, and indeed, such combinations appear not to occur in nature (Lloyd & Webb, 1986).

In *Aristolochia* flowers, the fused tepals form a pitcher called the utricle, which is surmounted with a narrower tube (Figure 1A). During the female phase, insects that enter the tube are prevented from escaping, usually by downward-pointing hairs (Knuth, 1909; Oelschlägel *et al.*, 2009) (Figures 2A, B). At this stage, the stigma is receptive. It is fully exposed and green (Figure 2E). Insects carrying pollen would deposit it on the stigmas and pollinate the flower. When the flower switches phase, pollen is released from the anthers and loaded on the insects (Figure 1B). Afterwards, the hairs wilt (Figures 2C, D), allowing the release of the insect now loaded with pollen. During the male phase, the stigmatic surface becomes brown and is concealed by the folding of the stigmatic lobes (Figures 2F-H).

As *Aristolochia* flowers are protogynous, we expect the female phase to be shortened when pollination occurs. Casual observations suggest that this is the case. For instance, in *A. beatica*, female phase was shorter in December, when pollinator availability was higher, and longer in March, when pollinator availability was lower (Berjano *et al.*, 2009). In *A. rotunda*, female phase was longer in a natural population without pollinators than in other populations with pollinators (Oelschlägel *et al.*, 2016). In these cases, however, the effect of weather conditions could not be dissociated from the effect of pollinators. In open dichogamous flowers, an individual pollinator can only contribute to one sexual function of a given flower. Protogyny combined with pollinator retention, as in *Aristolochia* flowers, allows an individual pollinator to fulfill both sexual functions as "visitation" covers both sexual phases. Besides, a putative pollinator entering the trap could contribute to the plant’s reproduction through the male function, even if it does not bring exogenous pollen. In addition, absence of pollen could indicate a shortage of pollen donors and thus a great advantage in investing more (time and other resources) in the male function. It could thus be advantageous for *Aristolochia* flowers to switch from female to male phase even if a visiting insect does not ensure deposition of cross-pollen on stigmas. Moreover, for a flower receiving the visit of a putative pollinator not loaded with pollen, waiting for a pollinator loaded with pollen before switching phase increases the risk of not fulfilling any of the two functions (female and male) because trapped insects would probably die after a

few days. We predicted that in this case the presence of pollinators in the flowers, without the need for pollen deposition, should be sufficient to initiate the transition from female to male phase.

In this study we used *A. rotunda* as a model to test the above predictions regarding plasticity of the female phase in protogynous trap flowers. In a first experiment (experiment A) we tested for the effect of hand pollination and of presence of pollinators with or without pollen load on the switching from female to male phase. As all experimental treatments, in particular the last one (pollinators without pollen load), shortened the female phase compared to the control, we tested for the effect of mechanical stimulation of the stigmas on the transition from female to male phase (experiment B).

Material and methods

Aristolochia rotunda is a mostly northern Mediterranean species. It occurs in various habitats, as distinct as moist loamy soil, and calcareous karstic soil, usually in open environments, but also under light forest cover. It has a single, relatively short, flowering season, peaking from April to June. Pollinators are small Diptera of the families Chloropidae and Ceratopogonidae (Oelschlägel *et al.*, 2015; 2016). Autonomous selfing does not seem to occur (Oelschlägel *et al.*, 2016). The experiments took place in the field (N 43.7514, E 3.72194, 244 m asl, Viols-en-Laval, France), on one patch of 150 m^2, where more than 220 individuals of *A. rotunda* (most with several stems) naturally occur.

Experiment A, designed to test the effect of pollination and pollinators on phase switching, was carried out between May 4-18, 2022. Forty-four fully developed floral buds were individually labeled (using printed paper tags loosely tied to the stems with a cotton thread) and bagged between May 4–6, 2022, in order to keep flowers free of uncontrolled visitors. Bags were made of mesh gauze fine enough to prevent passing of any invertebrate. Only terminal flowers were used, so that bags could be tightened around the main stem instead of around the pedicel of the flower, limiting the risk of damaging flowers. Floral buds were checked every day for opening (which took place between one and four days after bagging). One day after opening, one of the four following treatments was applied to the flower. "*Control*" (N = 11): The control treatment consisted in making a slit on the side of the utricle with a scalpel, taking care not to damage the gynostemium (Figure 1C). "*Hand pollination*" (N = 11): A slit was made on the side of the utricle as in the control treatment, and two pairs of anthers excised from flowers in the male phase were inserted through the slit and deposited on the stigmas. This treatment was meant to test the effect of pollen deposition, independently of pollinator presence. "*Pollinator P+*" (N = 11): A slit was made on the side of the utricle as in the control treatment, and two pollinators bearing pollen on their backs were inserted in the flower, one through the slit, and one through the natural entrance of the flower. This treatment meant to simulate natural pollination (stimulation of the natural entrance and pollen deposition) and ensure the presence of a healthy pollinator in the utricle (the one introduced through the slit) because some individuals struggled in the floral tube and got stuck or damaged before reaching the utricle. "*Pollinator P-*" (N = 11): A slit was made on the side of the utricle as in the control treatment, and two pollinators without pollen were inserted in the flower, as in the previous treatment. This treatment was meant to test the effect of stimulation by natural behaviour of pollinators independently of pollen deposition. Anthers and pollinators were taken from *A. rotunda* flowers from another patch, 300 m away. Pollinators were manipulated with a moist brush: wings got stuck onto hairs of the brush by surface tension of water. However, many individuals were strong enough to free themselves from the brush and took flight before being inserted into flowers. Although moist wings usually prevented pollinators from flying, inserted pollinators could freely walk in the flower utricle. Flowers were bagged again as soon as each treatment was applied. The phase (female or male) of the gynostemium was checked (bags removed temporarily) on the second day after treatment, and then on every following day. The two-day latency was determined arbitrarily to let the flowers recover from any treatment-induced disturbance before manipulating them. The male phase was recognized by distinguishing pollen grains on the open anthers and by the darkening and folding of the gynostemium on top of stigmatic lobes (Fig. 2), by visual inspection through the slit using a x10 magnification lens. The number of days between treatment application and detection of the male phase was recorded for each treated flower.

Experiment B, designed to test the effect of mechanical stimulation on phase switching, was carried out between May 11–19, 2022. Thirty-one fully developed floral buds were bagged on 11 May 2022. Floral buds were then checked every day for opening (which took place between one and two days after bagging). One day after opening, one of the three following treatments was applied to the flower. "*Control*" (N = 10) and "*Hand pollination*" (N = 10) followed the same procedure as in experiment A. "*Thread*" (N = 11): A slit

was made on the side of the utricle as in the control treatment, and a piece of nylon thread (2 mm in length, 0.3 mm in diameter) was introduced through the slit with fine forceps and deposited onto the stigmas. The thread was left permanently on the stigmas. Trapped insects move inside the utricle, walking on the utricle wall and on the stigmas, jumping around, thereby stimulating the stigmas recurrently during their stay in the flower. Although we were not able to reproduce the same stimulation, the permanent contact of the thread onto the stigmas was the most realistic way we found to mimic pollinator action. This treatment was meant to test the effect of mechanical stimulation of the stigmas independently of pollen deposition and pollinator presence. Although flowers of *A. rotunda* did not seem to produce stigmatic fluids, we used a non-absorbent material (nylon, instead of paper for instance) to make sure the putative effect of the treatment was not due to drying of the stigmas. Pollinators are known to consume stigmatic fluids (e.g. Gottsberger *et al.*, 1980; Wells & Lloyd, 1991) and we could not exclude *a priori* that stigma drying could be a trigger for phase change. Follow up of the experiment was similar to that in experiment A.

The identity of the pollinators used in our experiments depended on their availability in the neighbouring plant patch. All individuals used in our study were taken from inside flowers. They belonged to the following species: *Forcipomyia aristolochiae* (Ceratopogonidae), *Hapleginella laevifrons* (Chloropidae) and *Tricimba cincta* (Chloropidae). These species were not recorded as pollinators in previous investigations on *A. rotunda* (Oelschlägel *et al.*, 2015, 2016; in Croatia), but their status as pollinators was confirmed in our study area by finding them carrying *Aristolochia* pollen in female stage flowers.

As for each flower, treatment was applied one day after flower opening. Variations in the number of days between treatment application and detection of the male phase corresponded to variations in the duration of the female phase. In order to test for the effect of experimental treatments on the duration of the female phase, for each experiment, the number of days between treatment application and detection of the male phase was compared among treatments (including controls) using a contrast analysis on the result of a generalized linear model (GLM) with a Poisson link function. In order to test for a difference in the duration of the female phase of the control flowers between the two experiments, the number of days between treatment application and detection of the male phase was compared between the control groups of each experiment using a deviance analysis on the result of a GLM with a Poisson link function. Statistical analyses were performed with R 4.2.2 (R Core Team, 2022).

Results

Overall, treatments had a significant effect on the number of days between treatment application and detection of the male phase within both the experiment designed to test the effect of pollination and pollinators on phase switching (experiment A, GLM, $Chi^2 = 19$, $p = 5 \times 10^{-6}$) and the experiment designed to test the effect of mechanical stimulation on phase switching (experiment B, GLM, $Chi^2 = 5$, $p = 0.02$). Pairwise comparisons using contrast analysis showed that, in each experiment, the number of days between treatment application and detection of the male phase was significantly greater for control than for each experimental treatment (for all tests, $p < 3 \times 10^{-4}$), but that there was no significant difference among experimental treatments (for all tests, $p > 0.4$) (Figures 3A, B). As the first check for phase switch was performed two days after treatment application (see Methods), the minimum number of days recorded between treatment application and detection of the male phase was two. However, phase switch may have occurred in a shorter lapse of time.

Discussion

Our first experiment showed that all three experimental treatments—hand pollination and the presence of pollinators with or without pollen load—reduced the duration of the female phase to a similar extent. The fact that these three treatments reduced the duration of the female phase to a similar extent indicates that (1) pollen grains independent of pollinator behaviour (hand pollination) or (2) pollen grains combined with pollinator behaviour (P+) or (3) pollinator behaviour independent of pollen grains (P-) could all play a role as trigger for phase switching. All of these equally efficient triggers interact with the stigmatic surfaces in two ways: (i) mechanical stimulation of the stigmas and (ii) drying of the stigmas (through absorption of the stigmatic fluid, if any, by pollinators feeding on it or by contact with the anthers full of pollen). The second experiment was designed to test whether the first of these interactions functions as a triggering mechanism, independently of the second interaction. This experiment showed that both hand pollination and deposition

of a nylon thread on the stigmas reduced the duration of the female phase to a similar extent, demonstrating that mechanical stimulation of the stigmas was sufficient to trigger phase switching in *A. rotunda*.

Pollinator availability can exert selective pressure on two aspects of longevity of *Aristolochia* flowers. First, mean longevity is under selection. In species living in environments with low and/or unpredictable pollinator abundance, such as in Mediterranean and arid climates, selection would be expected to favor longer mean total longevity, because a longer-lived flower has a higher probability of being visited by pollinators (Stotz & Gianoli, 2013). Secondly, our results suggest that plasticity in floral longevity is also under selective pressure. We showed that a signal of pollinator presence reduces the duration of the female phase, which may have several benefits. First, it would save floral maintenance costs. Second, it would reduce the risk that a pollinator will die in the plant's trap flower before leaving it with a pollen load. Third, in our case where mechanical stimulation was sufficient to induce the switch from female to male phase, this signal of pollinator presence without a pollen load could indicate that pollen is limiting, indicating in turn that the plant is likely to have high success in the male function if it produces pollen now. Pollination limitation can result either from low availability of pollinators or from pollinators not bearing enough pollen for efficient fertilization. Studies reporting intraspecific phenoplastic variation in floral longevity suggest that *Aristolochia* flowers may respond in opposite directions to these two situations. The female phase in *A. rotunda* was longer in a site characterized by pollinator scarcity (Oelschlägel *et al.*, 2016) and the female phase in *A. beatica* was longer in a season characterized by pollinator scarcity (Berjano *et al.*, 2009). In contrast, we found that when pollinators of *A. rotunda* are present but do not bring pollen (stimulation of the stigmas but no pollination), the female phase is shortened. Further experimental studies are needed to measure and distinguish the effects of *pollinator* limitation and *pollen* limitation on pollination success and on plasticity of floral longevity in *Aristolochia*. Flowering phenology is a target of selection (Munguía-Rosas *et al.*, 2011; Ollerton & Lack, 1998) and is a factor affecting pollen limitation. In *A. rotunda*, predictable variation in pollen limitation may select for variation of the trigger to switch phase. For instance, pollen limitation is expected to be lower at the peak of the flowering season, when the highest number of flowers are present. We may thus expect a lower response to mechanical stimulation at peak of flowering because there is a higher chance of trapping a pollen-loaded pollinator, making it less risky to wait for actual pollination before switching phase. Moreover, pollinator and pollen limitation may follow different trends across the flowering season, depending on the phenology of the involved fly species and on the plant population's flowering phenology, allowing to disentangle their respective roles in the response to mechanical stimulation of the stigmas.

Using mechanical stimulation as a trigger for phase switching means that the flower would sacrifice female function if the stigmas were touched by anything other than a pollinator loaded with pollen. Although this strategy might appear risky, it must be remembered that *Aristolochia* flowers are trap flowers, imposing a physical filter to access their sexual organs. Although various insects can enter the trap flowers of *A. rotunda* (Berjano *et al.*, 2009; Oelschlägel *et al.*, 2016) —not all of them potential pollinators— the likelihood that the stigmas would be touched by a putative pollinator is still high compared to open flowers with a similar phenotypically specialized pollination system. However, to be effective, pollinators must have visited another flower before, to be loaded with pollen. In flowers of many plant species, post-anthesis modifications are induced by chemical interactions between pollen and stigma (O'Neill, 1997), indicating that signals more specific to pollination than mechanical stimulation are often used in angiosperms. In addition to such pollen-stigma interaction, mechanical stimulation also acts as a signal in *A. rotunda*, as proven in this study, suggesting that switching phase even in presence of a putative pollinator not bearing pollen—and thus sacrificing the female function, as *A. rotunda* is self-incompatible (Oelschlägel *et al.*, 2016)—is advantageous to the flower. This strategy would be selected in case of strong pollinator limitation. Indeed, if pollinator availability is low or unpredictable, the risk of sacrificing the female function is balanced by the benefit of ensuring the male function, as the putative pollinator present in the flower, even if not bearing exogenous pollen, will inevitably leave the flower loaded with pollen, as long as the insect is released quickly enough to have time to visit another flower before dying (small flies are usually short-lived). We thus predict that *A. rotunda* is pollinator-limited, as suggested by Oelschlägel *et al.* (2016), and as shown for other *Aristolochia* species (Berjano *et al.*, 2006; Murugan *et al.*, 2006; Trujillo & Sérsic, 2006). In addition, waiting for fertilization to have occurred before switching phase would be risky because the insect responsible for fertilization could die by the time fertilization status could be assessed by the plant, and thus could not contribute to the plant's male function.

Besides the shortening of the female phase by mechanical stimulation of the stigmas, there is a maximum duration of the female phase after which the flower will switch phase even in the absence of signal, to limit floral maintenance costs at least, and maybe also to benefit from ensuring the male function (if flowers in male phase are still attractive to pollinators, which is not known in *A. rotunda*). There must be a genetic component in the maximum duration of the female phase, as floral longevity is under selective pressure in *Aristolochia* (Stotz & Gianoli, 2013). Our results suggest that there is also plasticity in this trait. We saw high variance in the duration of the female phase among flowers within each control batch. Such variation could not result from environmental factors but could reflect the reproductive status of each individual plant. For instance, it may be advantageous for a plant already bearing the maximum number of developing fruits it can support (as *Aristolochia* may be expected to be resource-limited [Berjano *et al.*, 2011]) to systematically shorten the female phase of its flowers in order to invest in the much less costly male function.

Conclusion

Our study revealed that the presence of pollen on the stigma is not needed to induce reduction in floral longevity in *A. rotunda*, in contrast to other angiosperms that have been studied (O'Neill, 1997), and that a simple mechanical stimulation of the stigma is sufficient for the transition from female to male phase. This peculiarity could be easily interpreted in an evolutionary framework in light of the specific reproductive biology of *A. rotunda* (see above). All species in the genus *Aristolochia* are protogynous and have trap flowers. However, species vary in other aspects of pollination biology. For instance, floral longevity is highly variable in the genus, reflecting adaptations to different levels of resources and pollinator abundance (Stotz & Gianoli, 2013). Does the signal for phase switching vary across species depending on the level of pollinator abundance and predictability? We may expect that a species experiencing higher pollinator abundance than *A. rotunda* could afford to wait for actual pollen deposition before switching to the male phase. Potential for selfing is another component of reproductive biology that varies across *Aristolochia* species. Does the maximum duration of the female phase vary across species depending on the opportunity for selfing? We may expect the maximum duration of the female phase to be longer in species not able to self-fertilize because they have no other opportunity to fulfill the female function than waiting for a pollinator loaded with pollen. The genus *Aristolochia*, with more than 500 species, offers an opportunity to investigate issues in the evolution of floral longevity through comparative studies.

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Figure 1. A, Overall view of the trap flower of *A. rotunda*; B, The utricle of a male-phase flower has been cut vertically to show a ceratopogonid pollinator loaded with pollen; C, Each treatment included making a slit on the side of the utricle. For experimental treatments, the slit was used to insert anthers, pollinators, or the nylon thread.

Figure 2. Morphological and functional changes in the protogynous flower of *A. rotunda* with time. A-B, general view of a female (first) phase flower cut vertically, showing at the base of the tube the long hairs directed downward, allowing the insects to enter the utricle but not to exit; C-D, General view of a male (second) phase flower cut vertically, showing the hairs wilted, allowing the release of the trapped insects; E-H, changes in the gynostemium during the switch from the female to the male phase; E, stigmas are exposed and receptive; stamens are closed; the flower is functionally female; F, Stigmatic lobes become erect; the anthers dehisce; G, stigmatic lobes fold toward the center; stigmas are no longer receptive; pollen is expelled; the flower is functionally male; H, the stigmatic surface is entirely covered by the stigmatic lobes; the anthers have released their pollen.

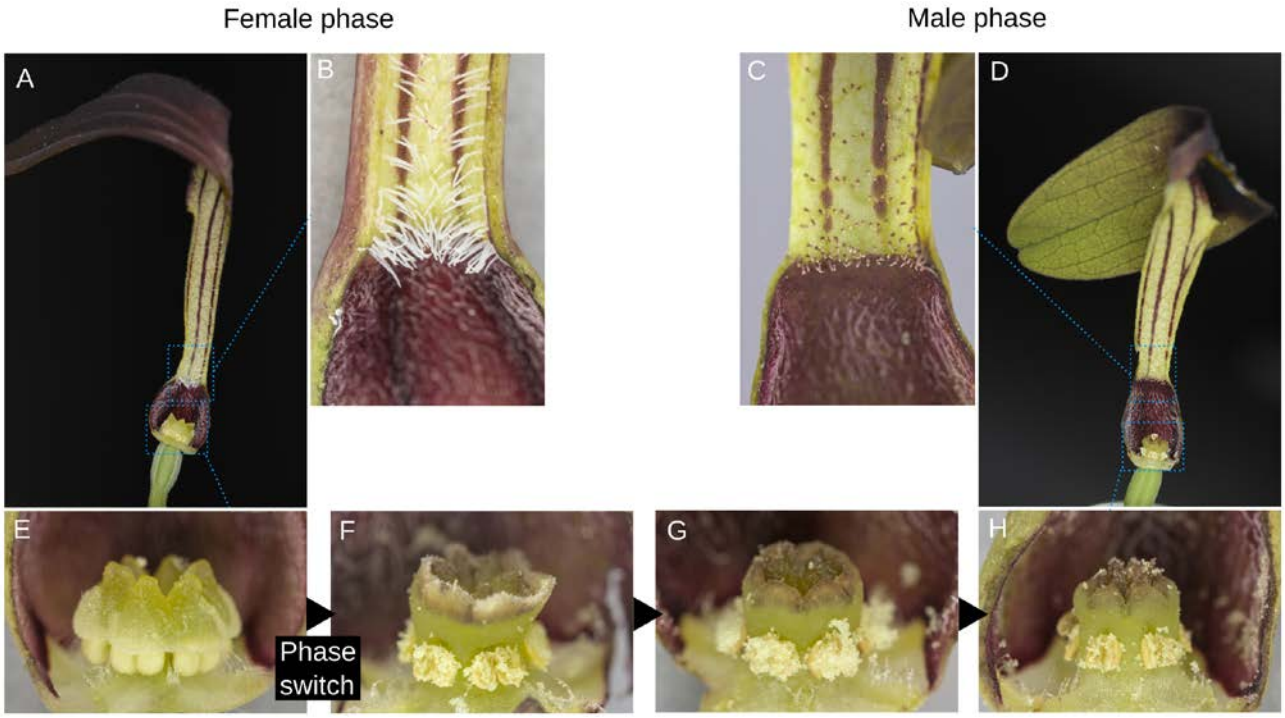

Figure 3. Variation in the duration of the female phase in the trap flowers of the protogynous *A. rotunda* according to various treatments performed on the flowers. Hand pollination: deposition of open anthers on the stigmas; Pollinator P-: insertion into the flower of pollinators not bearing pollen; Pollinator P+: insertion into the flower of pollinators bearing pollen; Thread: deposition of a nylon thread onto the stigmas. Experiments A and B were conducted successively. Different letters indicate significant differences. Note that we waited for two days between treatment application and the first check for phase switch, but that the exact lapse of time before phase switch may have been shorter.