

# Pollination biology of *Hemiboea ovalifolia* (Gesneriaceae), an endangered herb from Guangxi, China

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**Abstract** Although information on the reproductive biology of the endangered plant family *Gesneriaceae* is well known, the pollination mechanisms of these plants in karst regions are poorly understood. To determine the pollination ecology of *Hemiboea ovalifolia*, a rare and highly geographically restricted endemic species in karst regions, flowering phenology, pollinator observations, and pollination manipulations were conducted in situ. Findings revealed that the anthesis of *H. ovalifolia* often occurred late, during sunset, or early morning, with duration of 2–4 days. The most effective pollinators were *Bombus ignitus* and *Anthophora zonata*. The co-existence of spontaneous self-pollination, protandry and herkogamy in *H. ovalifolia* suggested that this species outcrosses with partial self-compatibility, and exhibits late-acting inbreeding depression in seed sets. Controlled pollination indicated that these plants were pollen limited resulting from the seed set. However, despite the co-existence of large numbers of fruit and seed sets, and vegetative propagation in *H. ovalifolia*, a failure in seedling survival and long duration to establishment of first-year seedlings in natural populations suggests that the species does not easily recover from damage.

**Keywords:** *Hemiboea ovalifolia*; pollination biology; late-acting inbreeding depression.

## 1. Introduction

Information on the biology of rare species and those with restricted geographical ranges is crucial for predicting their reproductive success and suggesting appropriate conservation measures (Rodríguez-Perez 2005, Clivati *et al.* 2014). As one of the key contributing factors of a species becoming endangered, the plant breeding system has received the most widespread attention (Eckert *et al.* 2010; Ling *et al.* 2017). In general, small and isolated plant populations may be less conspicuous for pollinators, leading to fewer visits, changes in pollinator behavior, and reduced reproductive success (Meeus *et al.* 2013). Furthermore, habitat fragmentation caused by human disturbance can further threaten the survival of endangered plants (Rodríguez-Perez 2005).

A host of plants maintain mixed mating systems that permit both self- and cross-fertilization; each mode of sexual reproduction has associated advantages and

disadvantages (Van Drunen and Dorken 2012). In general, rare plants may be limited by reduced reproduction and exhibit slightly higher levels of self-compatibility compared with more common plants. Due to selective advantages, selfing provides reproductive assurance during colonization processes, bottlenecks, times of scarce or unreliable pollinators, and instances of two-fold gene transmission (Rodríguez-Perez 2005; Minuto *et al.* 2013). Previous studies indicate that the breeding system of many rare plants may combine selfing with crossing, sometimes in a form of vegetative propagation (Minuto *et al.* 2013). Furthermore, disruption of plant-pollinator interactions may lead to inbreeding that generates different selective forces on plant mating systems (Eckert *et al.* 2010). In addition, reductions in pollinators may directly affect trade-offs by influencing reproductive output, reducing the quantity and/or quality of fruit and seed sets, and promoting selfing in self-compatible species (Ling *et al.* 2017).

*Gesneriaceae* is a highly abundant and diverse plant family in China; it contains a reported 56 (25 endemic) genera and 442 (354 endemic) species (Wang *et al.* 1998). Flowers in this family are highly diversified, reflecting adaptation to a wide range of pollinators including bees, birds, and bats (Gao *et al.* 2006; Martén-Rodríguez and Fenster 2010; Wang *et al.* 2011). Although widespread research has been conducted on the reproductive biology of *Gesneriaceae* (Pu *et al.* 2009; Martén-Rodríguez and Fenster 2010; Wang *et al.* 2011; Ling *et al.* 2017), little is known about the pollination biology of endangered species in this family from China, where more than half the species exist in a restricted geographical area. Furthermore, a host of *Gesneriaceae* species have significant ornamental or medicinal values; consequently, these species have high conservation and scientific value (Ma *et al.* 2011). It is therefore important to understand the reproductive biology of these species.

*Hemiboea ovalifolia* (W.T. Wang) A. Weber & Mich. Möller. (Weber *et al.* 2011), a perennial herb that can grow up to 200 cm in height, is endemic solely to Southwest of China (Wang *et al.* 1998; Weber *et al.* 2011), and is one of the four endemic species of the family listed in the China Red Book (Wang and Xie 2004). Based on molecular data and morphology, the genus *Metabriggsia* is reduced to synonymy with *Hemiboea* and two species have been

transferred to this genus (Weber *et al.* 2011). The chromosome number ( $2n=24$ ), geographic distribution, and tissue culture of *H. ovalifolia* have been previously assessed (Wang 1998; Cao *et al.* 2003; Weber *et al.* 2011; Ma *et al.* 2011). However, despite its conservation status, rarity, and occurrence in a protected area, to the best of our knowledge, no information is available on its reproductive biology. The present study aimed to clarify the pollination ecology of *H. ovalifolia*. Our specific objectives were to: (a) describe its floral morphology and phenology, (b) identify candidate pollinators and ascertain any pollinator limitation, (c) describe its breeding system, and (d) examine the implications of these characteristics for its conservation.

## 2. Materials and Methods

### 2.1. Species and sites

Plants of *H. ovalifolia*, were studied in montane rainforests on the limestone hills of Mulun National Nature Reserve (25°07'01" to 25°12'22" N, 107°54'01" to 108°05'51" E), over an altitude range of 625–1000 m in Huanjiang county, Guangxi, China. Plants of *H. ovalifolia* across the whole altitude range were observed and the numbers of buds of old plants were counted. Data from Plant Protection Station of Agricultural Bureau in Huanjiang County of Guangxi Zhuang Autonomous Region, the nearest weather station, revealed that the location has an annual mean temperature of 17–19.3°C, with August being the warmest (28 °C) and February the coldest (-5.3°C). The location has an annual rainfall of 1750 mm, with June being the wettest (260 mm) and December the driest (70 mm). In the study area, *H. ovalifolia* is found growing on rocky outcrops, with a sparse cover over trees and shrubs such as *Choerospondias axillaris* (Roxb) Burt and Hill, *Itoa oreintalis* Hemsl, *Schefflera octophylla* (Lour) Harms, *Begonia cavaleriei* Lévl, and *Elatostema sublineare* Wang. *H. ovalifolia* commonly establishes as a uniform patch of plants, covering an area of up to 25 m<sup>2</sup> and comprising up to 40 individual plants. It flowers in autumn, from October to November; flowers are bisexual and occur in axillary cymes.

### 2.2. Flowering process, measurements of stigma receptivity and pollen viability and flower visitation

To observe the flowering process, three flowers on each study plant ( $n=20$ ) were marked as buds at each location. From anthesis, observations were made at approximately 2-hour intervals during daylight hours between 06:00 and 18:00 on the first day, and between 09:00 and 18:00 over the next 2 days. For each flower, corolla color was recorded and the spatial distribution of floral organs was observed under a stereomicroscope. Additionally, five flowers were collected at each observation time to provide material for pollen viability and stigma receptivity testing. Observations and tests were conducted between October 10–26, 2006, and repeated between October 21 and November 20, 2008. Pollen grains were stained with dimethylthiazol diphenyl tetrazolium bromide, to test for the presence of dehydrogenase as an indicator of pollen viability. Stigma receptivity was determined by the H<sub>2</sub>O<sub>2</sub>-

catalase method of Pu *et al.* (2009). Between October 11–16, 2006 and October 25–29, 2008, the identity and number of pollinating insects were recorded on up to 20 flowers per observation between 09:00 and 18:30 each day. A total of 80 hours of observations were made each year. All pollinators and their behaviors were recorded, then each pollinator was captured for identification.

### 2.3. Breeding system

In both years, several pollination treatments were applied to individual plants in two populations of *H. ovalifolia* as follows: (1) cross-pollination, 35 flowers of 11 plants were hand pollinated with pollen from another individual; (2) self-pollination, a total of 35 flowers of 10 plants were self-pollinated by placing flower pollinia onto the stigmas of the same plant; (3) open-pollination, 30 untreated control flowers from ten plants; (4) pollen-stigma interference, stamens from 30 flowers of 10 plants were removed; (5) pollinator-exclusion, 30 flowers of 10 plants were bagged for spontaneous self-pollination testing; (6) perianth removal, the perianths were removed from buds just before anthesis, and the buds were then left exposed; and (7) agamospermy, stamens or stigma were removed from buds just before anthesis, and the buds were then enclosed in bags. The number of seedlings and root sprouts per plant were counted at five 10×10 m sites in 2009.

### 2.4. Statistical analysis

Means ( $\pm$ SE) were calculated for all measurements using Excel 2010 (Microsoft). Pollen viability, stigma receptivity, and fruit set and seed number were compared between different pollination treatments using one-way analysis of variance in SPSS (version 18.0).

## 3. Results

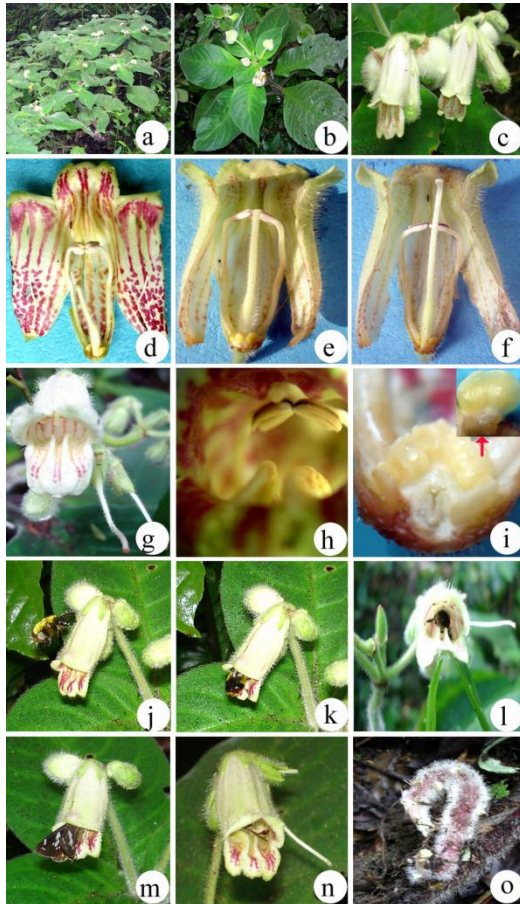
### 3.1. Flowering phenology

*H. ovalifolia* flowers are shown in Figure 1a-c. The mean number of inflorescences the plant produces is  $7.36\pm 0.24$  (range 3-15,  $n=20$ ). Flowering of the whole population occurred between early October and late November. Flowers opened during early morning (04:00–05:00) or late afternoon (18:00–19:00), and remained open for 2-4 day depending on environmental conditions. Once a flower was pollinated, the corolla and attached anthers fell off the following day, while un-pollinated flowers remained open for up to 4 days before the corollas wilted and closed. The fruits matured approximately 1 month later.

### 3.2. Pollen viability, stigma receptivity and flower visitors

Mean pollen viability was  $19.4\pm 2\%$  when flowers were in anthesis (Fig. 2). Ten hours after flowering, stigmas were white and had a very lower receptivity. Twenty hours after flowering, both pollen viability and stigma receptivity were high during the flowering period, while 40 hours after the initiation of flowering, both pollen viability and stigma receptivity decreased (Fig. 2). During a total of 80 observation hours, four pollinators, including three bee species (Fig. 1j-l, n) and one butterfly species (Fig. 1m),

were recorded visiting *H. ovalifolia* flowers. The two most effective pollinators were *Bombus ignitus* Smith and *Anthophora zonata* Linnaeus. This is because, when visiting *H. ovalifolia* flowers, their head and back made contact with the stigma first, followed second by the anthers; this caused pollen grains to stream out of the anthers. Mean ( $\pm$ SE) visiting frequency of *B. speciosus* and *A. zonata* was  $0.783 \pm 0.013$  flower<sup>-1</sup>.hour<sup>-1</sup> and  $0.583 \pm 0.017$  flower<sup>-1</sup>.hour<sup>-1</sup>, respectively. These two pollinators visited flowers primarily during the morning,



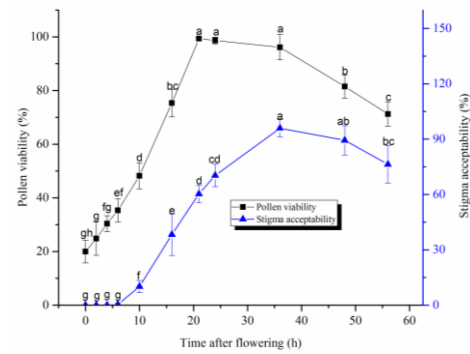
**Figure 1.** Floral syndrome and pollinators of *H. ovalifolia*. a–c, Flowering of *H. ovalifolia*. d–f, The process of development and curve of style after flowered. g–h, Flower. i, nectary. j–l, *Bombus speciosus* and *Anthophora zonata* visiting *H. ovalifolia*. m, *Celoenorrhinus asperses* Leech visiting *H. ovalifolia*. n, *Ophion* sp. visiting *H. ovalifolia*. o, radical bud.

with peak visits occurring at 10:00–12:00 and 14:00–16:00. *Celoenorrhinus asperses* Leech was also an effective pollinator with frequently recorded visits to the flowers ( $0.167 \pm 0.007$  flower<sup>-1</sup>.hour<sup>-1</sup>) and body contact was made with the stigma and anthers (Fig. 1m). *Ophion* sp. was only considered occasional visitors since behaviors such as pollen collecting, nectar consumption, stigma touching, and grooming were not observed (Fig. 1n).

### 3.3. Breeding system

*H. ovalifolia* combined sexual reproduction with vegetative propagation. In sexual reproduction, the pollination treatments revealed that *H. ovalifolia* was self-compatible. Both cross- and self-pollination produced a large number

of fruit sets and there were no significant differences in fruit sets number between these two types of pollination (Fig. 3a).



**Figure 2.** Changes of pollen viability and stigma receptivity in *H. ovalifolia*. Different lowercase letters on the top of the bars denote significant differences ( $P < 0.05$ ).

While, there were significant differences in fruit sets between open-pollination and self-pollination or cross-pollination ( $t = -4.24$ ,  $df = 10$ ,  $P = 0.005$  and  $t = -4.74$ ,  $df = 10$ ,  $P = 0.003$ , respectively). Pollinator-exclusion and perianth removal treatment significantly reduced the number of fruit sets and significant differences in the number of fruit sets were observed between these two treatments ( $t = -6.74$ ,  $df = 10$ ,  $P < 0.001$ ; Fig. 3a). Furthermore, open-pollinated flowers produced significantly fewer seeds than cross-pollination, open-pollination, pollen-stigma interference, and perianth removal treatments (Fig. 3b). In agamospermy treatment, no fruit and seed sets were observed (Fig. 3). In regard to vegetative propagation, *H. ovalifolia* has evolved a survival strategy by means of a thick perennial rhizome-like rootstock. In this field investigation, the species was recorded as population with a narrow geographic distribution comprising a mean of  $50 \pm 8$  individual plants ( $n = 5$ ). The number of buds of the root stock from the old stock was higher than the number of seedlings ( $10 \pm 4$  buds vs.  $4 \pm 2$  seedlings every  $100 \text{ m}^2$ ). Furthermore, the establishment of flowering *H. ovalifolia* from seedlings or root sprouts took approximately 3–4 years.

## 4. Discussion

Previous studies have indicated that pollen limitation is common in angiosperm species (Pearse *et al.* 2015) and in some Gesnerioideae species found in karst regions with restricted geographical distribution (Pu *et al.* 2009; Wang *et al.* 2011; Ling *et al.* 2017). In the present study, pollination experiments showed that there were significant differences in the number of fruit sets between open-pollinated samples and artificially fertilized flowers (both cross- and self-pollination) suggesting that the population investigated experiences a degree of pollen limitation. Since pollen limitation is a significant determinant of seed production (Hirayama *et al.* 2005), a potential explanation for this pollen limitation may be due to low pollination quantity and/or quality. There were no significant differences in fruit sets between cross-pollination and self-pollination but significant differences in seed sets, indicating that pollen quality may be a possible cause of pollen limitation in *H. ovalifolia*. Since there were significant differences in the number of fruit sets between



cross-pollination, open-pollination, and perianth removal, but no significant differences in seed sets, this suggests that insufficient pollen quantity may do not affect seed sets.

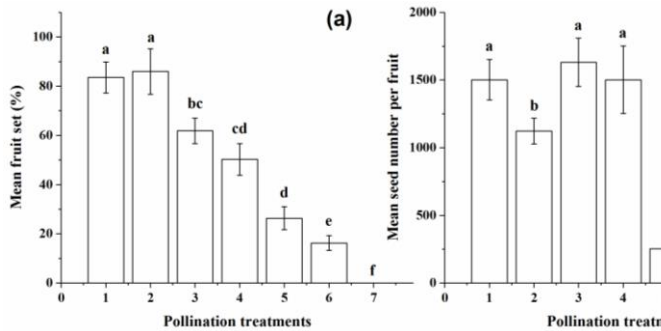


Figure 3. Fruit set of *H. ovalifolia* (a) and seed number per plant fruit (b) resulting from seven pollination treatments: 1, cross-pollination; 2, self-pollination; 3, open-pollination; 4, pollen-stigma interference; 5, pollinator-exclusion; 6, perianth-removed; 7, agamospermy. Different lowercase letters on the top of the bars denote significant differences ( $n=6$ ,  $P < 0.05$ ).

Coexistence of dichogamy and herkogamy is generally considered to promote cross-pollination and avoid sexual conflict (Lloyd and Webb 1986). The present study showed that there was spatial and temporal gender separation in the flowering process of *H. ovalifolia*, indicating strong temporal protandry (dichogamy) and complete spatial separation between male and female function within each flower (herkogamy). However, there was a certain level of temporal overlap between pollen viability and stigma receptivity; pollinator exclusion treatments sharply reduced the number of fruit and seed sets, suggesting a certain degree of spontaneous self-pollination in *H. ovalifolia*. This phenomenon has also reported in other geographically restricted Gesnerioideae species in karst regions, such as *Dayaoshania cotinifolia* W. T. Wang (Wang *et al.* 2011). Furthermore, the present pollination experiments showed that there were no significant differences in fruit sets between the cross- and self-pollination treatments, but there were significant differences in seed sets, and that the pollinator exclusion treatment produced the lowest number of fruit and seed sets (except when the stigma was removed) in *H. ovalifolia*. These results suggest that the breeding system of *H. ovalifolia* is predominantly cross-pollination with partly spontaneous self-pollination, but without apomixes (stigma removal treatment did not produce fruit or seed sets).

Although the maximum proportion of fruit sets following open pollination was recorded at 61.87%, the field investigation results showed that there were only a few seedlings growing around the old one, plants suggesting a failure in seed germination in natural *H. ovalifolia* populations. One possible reason may due to the occurrence of late-acting inbreeding depression (Hirayama *et al.* 2005). Our results showed that both self-pollination and pollinator exclusion treatments produced the lowest quality of seed sets despite the highest fruit sets. This suggests the existence of late-acting inbreeding depression

in *H. ovalifolia* since the low quality of the offspring could lead to low seed germination or increased seedling mortality due to the expression of deleterious alleles (Mena-Alí *et al.* 2005). In addition, although the field investigation revealed that the number of buds from the rootstock of *H. ovalifolia* was 2.5-fold greater the number of seedlings, the establishment of flowering *H. ovalifolia* from seedlings or buds took approximately 3–4 years. This indicates that this species does not easily recover once damaged.

In sum, despite the existence of spatial and temporal gender separation in the flowering process of *H. ovalifolia*, there was a certain level of temporal overlap between pollen viability and stigma receptivity. *H. ovalifolia* was self-compatible and the population investigated experienced a degree of pollen limitation. The existence of late-acting inbreeding depression in *H. ovalifolia* leads to low seed germination. Despite the existence of asexual reproduction in *H. ovalifolia*, a failure of seed germination and a narrow restricted population indicated that the species was not easy to recovery once being damaged. Therefore, the preservation of its habitats is the most critical aspects in any strategies for the conservation of *H. ovalifolia*.

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