

Original Research

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. This study demonstrated the flowering phenology, pollinators, and breeding system of *Hemiboea ovalifolia* in karst regions. Findings revealed that the anthesis of *H. ovalifolia* often occurred late, during sunset, or early morning, with duration of 2-4 days; there was a certain level of temporal overlap between pollen viability and stigma receptivity; the most effective pollinators were *Bombus ignitus* and *Anthophora zonata*. Controlled pollination indicated that these plants were pollen limited and exhibited late-acting inbreeding depression resulting from the seed sets; there were significant differences in fruit sets between open-pollination and self-pollination or cross pollination, and in seed sets between self-pollination and cross-pollination or open-pollination. 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 establishing first-year seedlings in natural populations suggests that the species does not easily recover from damage.

Keywords: endangered species, breeding system, *Hemiboea ovalifolia*, late-acting inbreeding depression

Introduction

Information on the biology of rare species and those with restricted geographical ranges is crucial for predicting their reproductive success, understanding their population dynamics, and suggesting appropriate conservation measures [1-3]. As one of the key

contributing factors of a species becoming endangered, the plant breeding system has received the most widespread attention and can vary according to species distribution [4]. In general, both the direct and indirect changes in habitat fragmentation can threaten the survival of endangered plants by reducing potential habitat and population viability, or disrupting plant pollinator interactions [1, 5-6]. However, small and isolated plant populations have been one of the problems that scientists have been arguing about for a long time [7-8].

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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 [9]. 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 [1, 10-11]. Previous studies indicate that the breeding system of many rare plants may combine selfing with crossing — sometimes in a form of vegetative propagation [10, 12-13]. Meanwhile, many rare plants, characterized by small and isolated plant populations, may be less conspicuous for pollinators, which leads to fewer visits, changes in pollinator behavior, disruption of plant-pollinator interactions, and reduced reproductive success [4, 8]. 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 [14].

Gesneriaceae is a highly abundant and diverse plant family in China; it contains a reported 59 (27 endemic) genera and 460 (375 endemic) species [15]. Flowers in this family are highly diversified, reflecting adaptation to a wide range of pollinators including bees, birds, and bats [15-18]. Although widespread research has been conducted on the reproductive biology of Gesneriaceae [17-20], 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 [21]. It is therefore important to understand the reproductive biology of these species. *Hemiboea ovalifolia* (W.T. Wang) A. Weber and Mich. Möller. [22], a perennial herb that can grow up to 200 cm in height, is endemic solely to southwestern China [22-24], and is one of the four endemic species of the family listed in the China Red Book [23]. Based on molecular data and morphology, the genus *Metabriggsia* is reduced to synonymy with *Hemiboea*, and two species have been transferred to this genus [22]. The chromosome number ($2n=24$), geographic distribution, and tissue culture of *H. ovalifolia* have been previously assessed [22-27]. 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.

Hence, 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.

Materials and Methods

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 the Plant Protection Station of the 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) Burtt and Hill, *Itoa oreintalis* Hemsl, *Schefflera octophylla* (Lour) Harms, *Begonia cavaleriei* LévI, and *Elatostema sublineare* Wang. *H. ovalifolia* commonly established as a uniform patch of plants covering an area of up to 25 m² and comprising up to 40 individual plants. It flowers in autumn, from October to November; flowers are bisexual and occur in axillary cymes.

Flowering Process 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 on this site between October 16 and November 15, 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 H₂O₂-catalase method of Dafni [28]. 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, and then each pollinator was captured for identification.

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.

Statistical Analysis

Means (±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). Statistically significant differences were set at $P < 0.05$ unless otherwise stated.

Results

Flowering Phenology, Pollen Viability, and Stigma Receptivity

H. ovalifolia flowers are shown in Fig. 1(a-n). The mean (±standard error [SE]) number of inflorescences the plant produces is 7.36 ± 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 days 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 (Fig. 1g). The fruits matured approximately 1 month later.

Mean pollen viability (±SE) was $19.4 \pm 2\%$, when flowers were in anthesis (Fig. 2). Ten hours after flowering, stigmas were white and had lower receptivity. Twenty hours after flowering, both pollen viability and stigma receptivity were high during the flowering

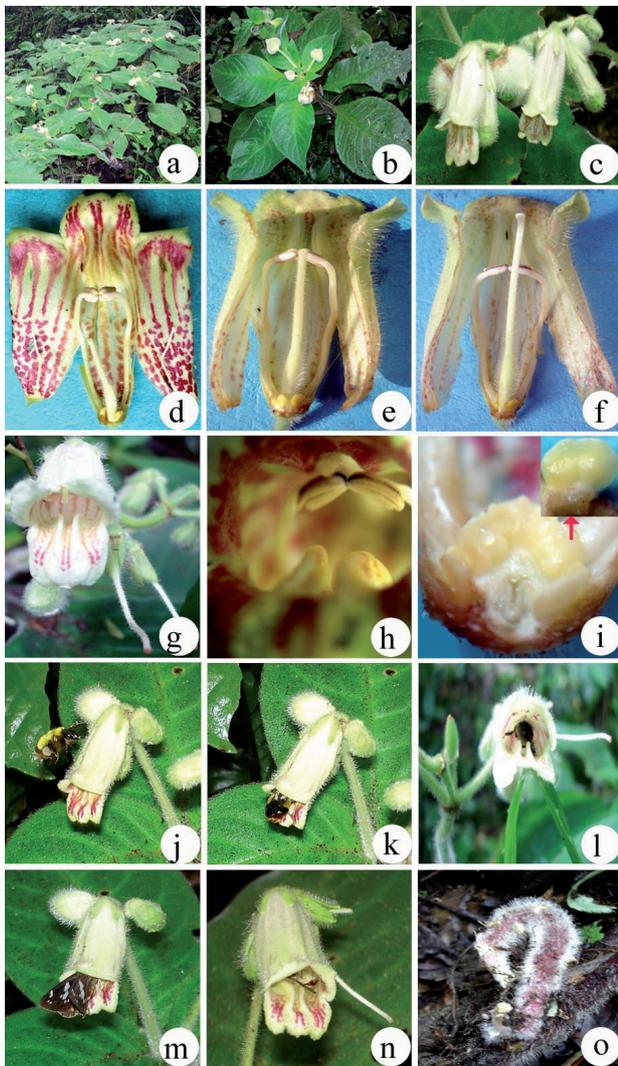


Fig. 1. Floral syndrome and pollinators of *H. ovalifolia*. a-c, Flowering of *H. ovalifolia*; d-f, the process of development and curve of style after flowering; g-h, flower; i, nectary; j-l, *Bombus speciosus* and *Anthophora zonata* visiting *H. ovalifolia*; m, *Celoenorhinus asperses* Leech visiting *H. ovalifolia*; n, *Ophion* sp. visiting *H. ovalifolia*; and o, radical bud.

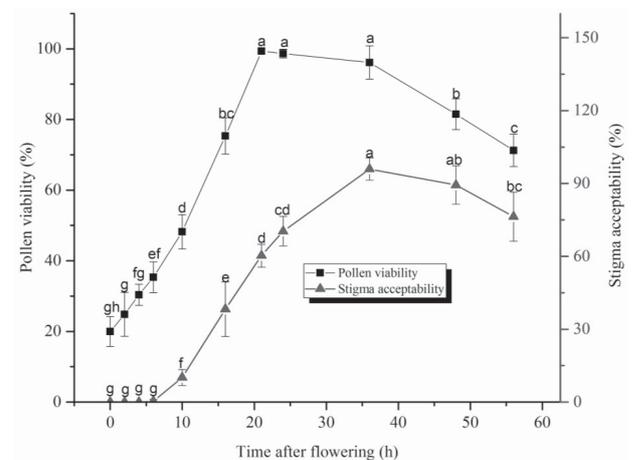


Fig. 2. Changes of pollen viability and stigma receptivity in *H. ovalifolia*; different lowercase letters on the top of the bars denote significant differences ($P < 0.05$).

period, while 40 hours after the initiation of flowering, both pollen viability and stigma receptivity decreased (Fig. 2).

Flower Visitors

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* L. 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 ± 0.013 flower⁻¹.hour⁻¹ and 0.583 ± 0.017 flower⁻¹.hour⁻¹, respectively. These two pollinators visited flowers primarily during the morning, with peak visits occurring at 10:00-12:00 and 14:00-16:00 (Fig. 3). *Celoenorhinus asperses* Leech was also an effective pollinator, with frequently recorded visits to the flowers (0.167 ± 0.007 flower⁻¹.hour⁻¹), and body contact was made with the stigma and anthers (Fig. 1m). *Ophion* sp. was only considered an occasional visitor since behaviors such as pollen collection, nectar consumption, stigma touching, and grooming were not observed (Fig. 1n).

Breeding System

Hemiboea 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 set numbers between these two types of pollination (Fig. 4), while there

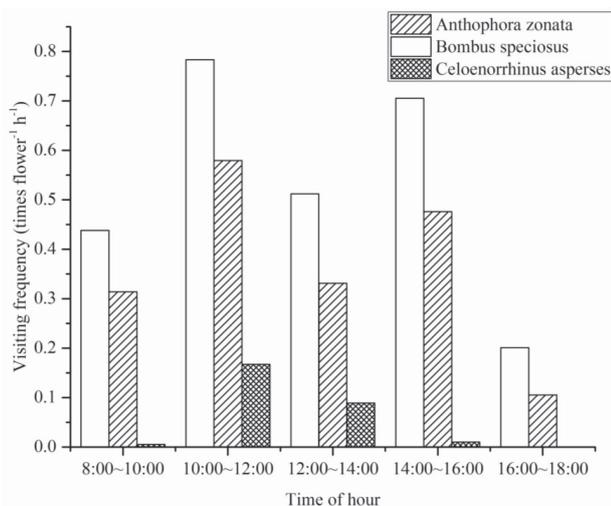


Fig. 3. Frequency of visits by *Bombus speciosus*, *Anthophora zonata*, and *Celoenorhinus asperses* Leech to flowers of *H. ovalifolia* during 80 h of observation of 20 plants.

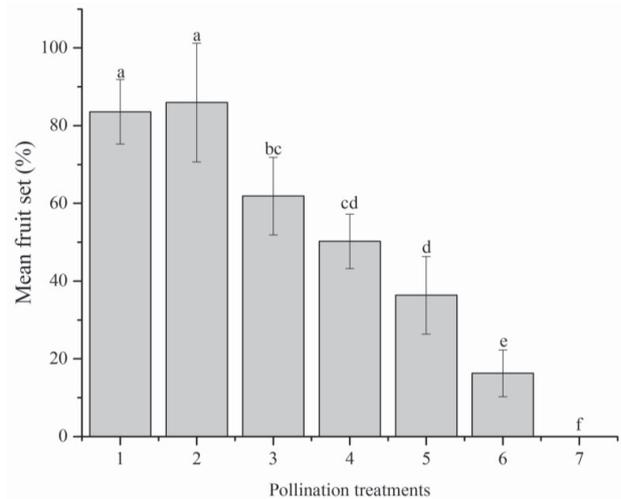


Fig. 4. Mean (\pm standard error) fruit set of *H. ovalifolia* inflorescences subjected to seven pollination treatments: 1) cross-pollination, 2) self-pollination, 3) open-pollination, 4) pollen-stigma interference, 5) pollinator exclusion, 6) perianth-removed, and 7) agamospermy; different lowercase letters on the top of the bars denote significant differences ($n = 6$, $P < 0.05$).

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. 4). Furthermore, open-pollinated flowers produced significantly fewer seeds than cross-pollination, open-pollination, pollen-stigma interference, and perianth

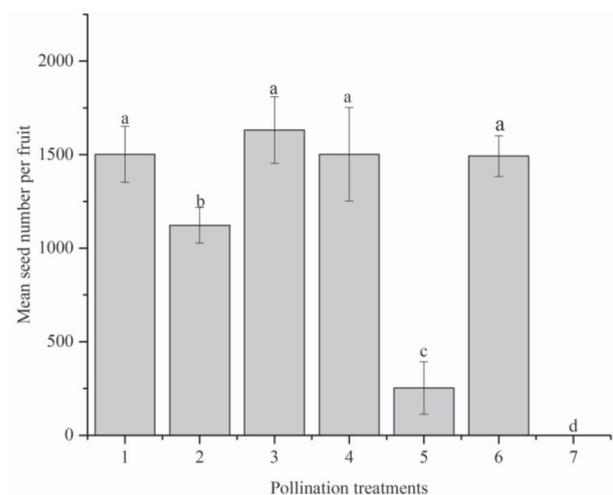


Fig. 5. Mean (\pm standard error) seed number per *H. ovalifolia* fruit resulting from seven pollination treatments: 1) cross-pollination, 2) self-pollination, 3) open-pollination, 4) pollen-stigma interference, 5) pollinator-exclusion, 6) perianth-removed, and 7) agamospermy. different lowercase letters on the top of the bars denote significant differences ($n = 6$, $P < 0.05$).

removal treatments (Fig. 5). In agamospermy treatment, no fruit and seed sets were observed (Figs 4-5).

In regard to vegetative propagation, *H. ovalifolia* has evolved a survival strategy by means of a thick perennial rhizome-like root stock (Fig. 1o). In this field investigation, the species was recorded as population with a narrow geographic distribution comprising a mean (\pm SE) of only 50 ± 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 ± 4 buds vs. 4 ± 2 seedlings every 100 m^2). Furthermore, the establishment of flowering *H. ovalifolia* from seedlings or root sprouts took approximately 3-4 years.

Discussion of Results

Previous studies have indicated that pollen limitation was common in angiosperm species [29-31] and in some *Gesnerioideae* species found in karst regions with restricted geographical distribution [15, 17, 19-20, 32]. 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 [33], 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 not affect seed sets.

The coexistence of dichogamy and herkogamy is generally considered to promote cross pollination and avoid sexual conflict [34]. 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 been reported in other geographically restricted *Gesnerioideae* species in karst regions, such as *Dayaoshania cotinifolia* W. T. Wang [17]. 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, suggesting a failure in seed germination in natural *H. ovalifolia* populations. One possible reason may be due to the occurrence of late-acting inbreeding depression. 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 [33, 35-36]. In addition, although the field investigation revealed that the number of buds from the root stock of *H. ovalifolia* was 2.5-fold greater than 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.

Conclusions

H. ovalifolia was self-compatible and the population investigated experienced a degree of pollen limitation, which may contribute to the low pollination quality since there were same fruit sets in both cross-pollination and self-pollination treatments but low seed sets only in self-pollination treatments. 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). 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 recover once damaged. Therefore, the preservation of its habitats is the most critical aspect in any strategies for the conservation of *H. ovalifolia*.

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Conflict of Interest

The authors declare no conflict of interest.

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