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Which food-mimic floral traits and environmental factors influence fecundity in a rare orchid, *Calanthe yaoshanensis*?

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Orchid species that are food mimics produce fewer fruits than species offering rewards, but few studies have shown the impact of environmental factors (e.g. anthropogenic activity, frost and herbivores) on their reproductive success over several seasons. In this study, we focused on the sole population of the endangered *Calanthe yaoshanensis* as it secretes no nectar. We investigated its floral biology, fruit set rates and prevailing environmental factors over three seasons (2008–2010). Mechanical self-pollination did not occur in *C. yaoshanensis*, but hand-selfed and crossed flowers produced equal numbers of fruit. However, seed viability and embryo size were significantly higher in cross-pollinated fruits maximizing embryonic fitness. Large hoverflies (Syrphidae) and *Bombus patagiatus* (gynes) were the only pollinarium vectors, but they often failed to disperse pollinaria. We interpret the temporary retention of the anther cap over the pollinarium as an adaptation lowering self-pollination. Insect-mediated rates of pollinarium removal were always higher than rates of pollinia deposition on stigmas. Over 3 years, natural rates of pollinarium removal differed significantly, whereas natural rates of fruit set were not significantly different (< 22%). Climate, herbivory and anthropogenic collections also inhibited some fruit set and maturation. Both biotic and abiotic factors appear to lower the fecundity of this endangered population. *Botanical Journal of the Linnean Society*, 2014, **176**, 421–433.

ADDITIONAL KEYWORDS: conservation – embryo – fly pollination – food deception – fruit set – Orchidaceae – pollinarium removal – pollinators – pollinia deposition.

INTRODUCTION

The exhaustive review by Tremblay *et al*. (2005) offered convincing evidence that evolution of new species in the family Orchidaceae is a process driven by a combination of pollination novelties, highly vagile seeds and the establishment and isolation of small populations. Therefore, the protection of small, often fragmented populations of orchid species is essential to their conservation as anthropogenic activity reduces their natural distributions. As cross-pollination is most important to the evolution of the majority of orchid species, the protection of individual sites will be inadequate to maintain, restore and/or expand populations unless pollinators and conditions conducive to cross-pollination are also present (Dixon, 2009). Cross-pollination in the majority of orchid species appears to occur via several modes of pollination-by-deceit. Indeed, it is estimated that about one-third of all orchid species produce flowers lacking edible rewards, but persisting as food, sexual or brood site mimics (e.g. Ackerman, 1986; Dafni & Bernhardt, 1989; Nilsson, 1992; Cozzolino & Widmer, 2005; Jersáková, Johnson & Kindlmann, 2006).

Tremblay *et al*. (2005) noted that reproductive success (fruit set) in these mimetic flowers was lower than in orchid species offering nectar (see also Neiland & Wilcock, 1998). In particular, food-mimic

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species tended to show a lower conversion rate of flowers into fruits relative to sex-mimic (pseudocopulatory) flowers. However, although food mimicry pollination may be less efficient, it appears to dominate modes of pollination-by-deceit in some of the largest genera, e.g. *Cypripedium* L. (Bernhardt & Edens-Meier, 2010; Ren *et al.*, 2011a) and *Thelymitra* J.R.Forst. & G.Forst. (Edens-Meier & Bernhardt, 2014).

The genus *Calanthe* R.Br. (Epidendroideae) consists of about 150 species, and is distributed throughout the tropical–subtropical archipelagos of the southern Pacific basin and on every major continent with tropical–subtropical zones excluding North America (Chen, Cribb & Gale, 2009; Zhai *et al*., 2014). China is a centre of diversity with 51 species (Chen *et al*., 2009). None of the 13 species studied to date is known to secrete nectar (e.g. Catling, 1990; Juillet *et al*., 2010; Delle-Vedove *et al*., 2011; Sugiura, 2013). Eight species self-pollinate mechanically (Catling, 1990; Jacquemyn *et al*., 2005), and the remaining five species are entomophilous, but their floral presentation appeals to different insects, suggesting some degree of adaptive radiation of food mimicry (Sugiura & Miyanaga, 1996; Sugiura, Yoshida & Maeta, 1998; Zhang *et al*., 2010; Sugiura, 2013; Sakata, Sakaguchi & Yamasaki, 2014; Suetsugu & Fukushima, 2014; Zhai *et al*., 2014). A male *Redena similis* (Danaidae) carried the pollinarium of *C. triplicata* (Willem.) Aimes on its proboscis (Sugiura & Miyanaga, 1996), whereas common cabbage butterflies, *Pieris rapae* (Pieridae), pollinated *C. argenteo-striata* C.Z.Tang & S.J.Cheng (Zhang *et al*., 2010). Both of these species have long, narrow floral spurs, but lack nectar. More recent studies have shown that, in temperate–subtropical regions, some *Calanthe* spp. with short spurs or no spurs are pollinated by large-bodied bees in the family Apidae (e.g. the genera: *Apis*, *Bombus*, *Eucera* and *Xylocopa*; Sugiura *et al*., 1998; Sugiura, 2013; Sakata *et al*., 2014; Suetsugu & Fukushima, 2014). *Osmia cornifrons* (Megachilidae), *Apis cerana* ssp. *japonica* and *Eucera nipponensis* are effective pollinators of *C. discolor* Lindl. (Suetsugu & Fukushima, 2014).

What is not well understood in *Calanthe*, or in any other lineage of orchids pollinated by deceit, are the roles of different and varying environmental factors which may limit or increase fruit set *in situ* (Bernhardt & Edens-Meier, 2010). It is understood that food-mimic orchids are pollinator limited (*sensu* Committee on the Status of Pollinators in North America, National Research Council, 2007). Few species show pre-zygotic self-incompatibility and do not self-pollinate in the absence of their primary pollinators (Tremblay *et al*., 2005; Edens-Meier *et al*., 2010). However, we know nothing about the viability of pollinia after they are removed from the anther and attached to the body of the pollinator. If the pollinator carries the same pollinia for hours or days, will the pollen germinate and fertilize ovules on contact with a receptive stigma? Other biotic factors reducing orchid fecundity must include florivory, frugivory (Edens-Meier *et al*., 2011) and anthropogenic activity (Koopowitz & Kaye, 1990; Alcock, 2006). Climatic patterns may also destroy flowers before pollinators visit them (Edens-Meier *et al*., 2011) and could reduce stigmatic receptivity as in unrelated angiosperms (Mao & Huang, 2009). Small populations may be particularly vulnerable to biotic and abiotic disturbance (Phillips *et al*., 2014).

Small populations in *Calanthe* may therefore become useful models to unite the study of food mimesis in orchids and the impact of environmental factors on fecundity, because the single anther in all flowers of *Calanthe* spp. contains eight hard, relatively large pollinia (Dressler, 1993). It should be easy to count the number of pollinia deposited by pollinators on receptive stigmas. The recently described *Calanthe yaoshanensis* Z.X.Ren & H.Wang (Ren *et al*., 2011b) should be a particularly useful model to test hypotheses regarding reproductive success, as it is known from only one population and is regarded as rare and endangered. In this article, we present a detailed study of the pollination biology, breeding system and reproductive success of *C. yaoshanensis*. We address the following questions. (1) Is *C. yaoshanensis* self-compatible? If it is, do self-pollinated seeds suffer inbreeding depression? (2) Does *C. yaoshanensis* self-pollinate mechanically in the absence of pollinia vectors? If not, which insects pollinate it in the wild? Is pollinia viability a limiting factor? (3) Do environmental factors (anthropogenic activity, frost and herbivores) rather than pollinator activity lower or increase fruit set?

MATERIAL AND METHODS

Study species and study site

Calanthe yaoshanensis is currently restricted to the Yaoshan Mountain, Yaoshan National Natural Reserve, Yunnan, China. Its conservation status has been assessed as critically endangered (Ren *et al*., 2011b). This understorey herb grows on high-mineral, humus-rich soils on limestone mountain cliffs at elevations of 2800–3000 m. Ren *et al*. (2011b) found it growing in small patches of 1–24 individuals. It has a multi-flowering raceme producing greenish yellow flowers and a pleasant odour (Fig. 1).

Observations and experiments in the field were conducted on the north-eastern slope from 2850 to 2900 m. About 200 individuals of *C. yaoshanensis*

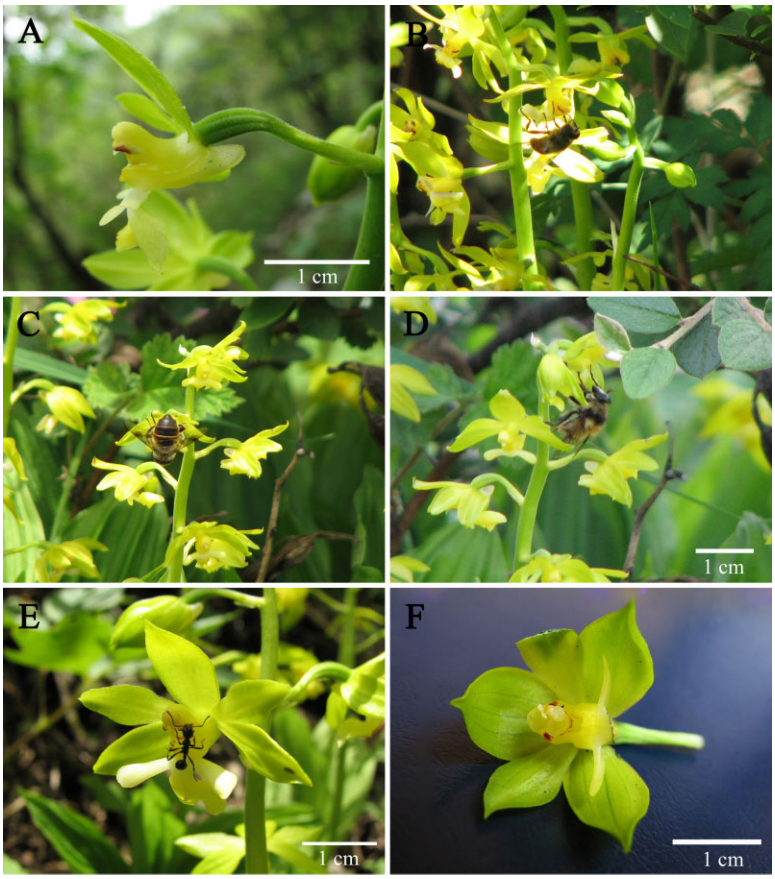

Figure 1. The flower and insect visitors of *Calanthe yaoshanensis*. A, Lateral view of a flower with a lateral sepal and one lateral petal removed to expose the short spur. B, C, *Eristalis tenax* visiting orchid flowers (note the different visiting behaviours): B, a fly lands on the labellum and inserts its proboscis down into the spur; C, a fly crawls on the flower upside down (this mode of visitation may effect pollinator-mediated self-pollination). D, A specimen of *Criorhina* sp. visits the flower. E, An unidentified ant on a flower. F, A self-pollinated flower in which the viscidium is retained on the rostellum, but two pollinia germinate on the receptive surface of the stigma (one lateral petal and the mid-lobe of the labellum were probably destroyed by herbivorous animals).

were found in 1 km^2 at the study site. Yaoshan Mountain has a montane, monsoon climate. The annual average temperature is 7–11 °C. The coldest monthly temperature does not exceed 5.2 °C. The annual rainfall is *c*. 1100–1200 mm, and the rainy season begins in mid-June. The site is covered by deciduous broadleaf forest dominated by *Quercus spinosa* David and *Corylus yunnanensis* (Franch.) A.Camus. *Magnolia wilsonii* Rehder, *Rhododendron argyrophyllum* Franch. and *R. siderophyllum* Franch. dominate the shrub layer. In early spring, several orchid species bloom on the forest floor, including three *Calanthe* species: *C. alpine* Hook.f. ex Lindl., *C. brevicornu* Lindl. and *C. tricarinata* Lindl. Other terrestrial orchids include *Cypripedium fargesii* Franch., *Cypripedium fasciolatum* Franch., *Cypripedium tibeticum* King ex Rolfe and *Cremastra appendiculata* (D.Don) Makino. *Calanthe tricarinata* and *C. yaoshanensis* co-flower on Yaoshan Mountain in May and early June, but the pollinia of *C. tricarinata* are about double the size of those of *C. yaoshanensis*, and it is easy to distinguish them (Z. X. Ren *et al*., unpubl. data).

Floral traits and phenology

The floral spur was checked for the presence of nectar using the urine glucose-testing strip (Urit Medical Electronic Group Co., Ltd, Guilin, China) spot test for urine sugar (Thakar *et al*., 2003). These strips indicate the presence of reducing sugars, even when the amount of nectar in a sample is small. The pollinarium was removed with a clean toothpick to determine whether the dehiscent anther cap clings to the released pollinarium and to record the change in the position of the eight caudicles in each pollinarium as they dried in the open air.

Phenological data on *C. yaoshanensis* were gathered by visiting the study area every 4 days from May to June in 2008–2010. We recorded the dates on which buds opened and the perianth wilted on 20 tagged flowers on 20 racemes in 2008. We monitored the flowering period of the population from the opening of the first bud to the wilting of the last open flower in 2010. We also recorded the lifespan of single flowers after their pollinaria were removed ($n = 10$). After hand pollination of a flower, we recorded how long it took for the perianth segments to wilt.

Breeding systems

To determine whether flowers of *C. yaoshanensis* require vector-mediated pollination to produce fruit and seeds, we randomly bagged 36 flowering stems under muslin (wedding veil) bags before the buds opened, and subdivided the flower buds into four categories: (1) bagged controls remained under bags and were never manipulated or exposed to insects ($n = 10$ in 2008); (2) self-pollinated flowers were hand pollinated with their own pollinia (same flower; $n = 10$ in 2008); (3) geitonogamous self-pollination included hand pollination of the stigma with pollinia from a second open flower on the same raceme ($n = 6$ in 2008); and (4) cross-pollinated flowers received pollinia from one flower on a second plant taken from a patch located more than 10 m away ($n = 10$ in 2008). We tagged and hand pollinated one to three flowers, and only one treatment was applied for each inflorescence. Natural (insect)-pollinated flowers were not bagged and were readily available to insects throughout their floral lifespans ($n = 91$ in 2008, $n = 209$ in 2009 and $n = 100$ in 2010).

To determine the longevity of individual pollinia, we followed the treatments of Gumbert & Kunze (2001). In 2010, we removed 20 pollinaria from 20 fresh flowers from ten inflorescences (two flower per inflorescence) using separate, clean toothpicks and placed the newly extracted pollinaria in a wood and plastic box at room temperature. These pollinaria were used to cross-pollinate flowers on racemes 24 h later ($n = 5$), and then a second series was used to hand, cross-pollinate flowers 48 h later ($n = 5$). Mature fruits were counted and collected in mid-October, about 4 months after the end of the flowering season.

Seed development and viability

Twenty capsules were selected at random from ovaries fertilized by hand self- and cross-pollination based on pollinia removed and deposited on stigmas on the same day (ten capsules for both self-pollination and cross-pollination). After 4 months, we collected the mature capsules. All the seeds in a capsule were extracted into a separate Petri dish ($n = 10$ for both cross- and self-pollination). We checked the embryonic development of seeds from self- and cross-pollination treatments with a light microscope (Olympus BX51 microscope, Tokyo, Japan) using the methods of Jersáková & Johnson (2006). We assigned seeds to four categories: large embryo, small embryo, aborted embryo (collapsed, reduced and incomplete development) and no embryo. We scored *c*. 100 seeds per capsule.

Seed viability was tested using a modified tetrazolium method (van Waes & Debergh, 1986). We pretreated seeds in 5% $Ca(OCl)_2$ (w/v) + 1% Tween-80 (v/v) for 12 h, and then transferred them to 1% tetrazolium solution for 6 h. The stained embryos were also assigned to three categories: red, pink (partly coloured) and colourless ($n = 8$ self-pollinated capsules and $n = 8$ cross-pollinated capsules). We counted *c*. 100 seeds per capsule.

Pollinator observation

Insect visitation was observed on sunny days from 09:00 to 17:00 h over the flowering periods from 2008 to 2010, representing *c*. 98 h of observation. We discontinued nocturnal observations after three nights (19:00 to 03:00 h) in 2010. Observations were conducted from stationary positions from 2 m away from patches in bloom and recorded with a video camera (Sony DCR-SX43, Shanghai, China). The individual behaviour of floral foragers and the number of flowers and inflorescences visited by an insect were recorded for each foraging bout. When the observed insect left an individual flower and/or a patch, we checked the visited flowers to record the removal of pollinaria from the anther and the deposition of pollinia on stigmas. Insect specimens were collected using butterfly nets and were killed in jars poisoned with fumes of diethyl ether. Pinned and labelled specimens were sent to entomologists at the Zoological Museum of the Institute of Zoology, Chinese Academy of Sciences (CAS) and China Agricultural University for identification. Vouchers were deposited at the Kunming Institute of Botany, CAS, Kunming.

Reproductive success

Reproductive success was subdivided into male success (insect removal of a whole pollinarium from a flower) and female success (deposition of one or more pollinia onto a receptive stigma). From May to June 2008–2010 (three flowering seasons), we mapped and tagged all inflorescences in a 1-km^2 plot. We surveyed the inflorescences every week ($n = 20$ visits over 3 years). The following data were collected: the number

of inflorescences; the number of plants collected or destroyed by human activities; the number of flowers per inflorescence; the number of flowers damaged by frost and herbivores; the number of flowers with pollinaria removed per inflorescence; and the number of flowers with pollinia deposited per inflorescence.

All pollinaria on flowers were subdivided into four categories: not removed (remained under the anther cap); removed (anther empty, anther cap missing); deposition of one or more pollinia on the stigma (pollinia hydrates and dissolves); and deposition on the stigma but flower withered. We also observed and recorded whether the anther cap and pollinarium of a flower were damaged by florivorous insects.

Statistical analyses

Statistical analyses were performed in R (version 3.0.2, R Development Core Team, 2013). We compared results of breeding system experiments (both hand-pollinations, insect/open pollinations) using the odds ratio χ^2 test (Sokal & Rohlf, 2012). We left the results of the bagged controls out of this test so as not to bias our results. We compared the embryo development and viability of cross- and self- pollinated seeds using the Kruskal–Wallis test, a non-parametric analysis of variance. The numbers of flowers on an inflorescence, the percentage of flowers with pollinaria removed for each inflorescence and the percentage of flowers maturing into capsules for each inflorescence over 3 years were compared by one-way analysis of variance (ANOVA). If significant differences were detected, a Tukey honestly significant difference (HSD) *post-hoc* test was used to determine the sources of the differences. As data were recorded as counts, the numbers of embryo development and viability per total number of seeds of each replica were arcsine transformed when we made the box plots. The numbers of flowers were log transformed to meet the assumptions of the test.

RESULTS

Floral presentation and pollinarium

Flowers of *C. yaoshanensis* are predominantly greenish yellow (Fig. 1A), but the condensed column wings and the united area of the column and labellum (around the opening of the spur) are marked by a brick red colour (Fig. 1F). The diameter of an open flower is 3–4 cm and the spur is *c.* 1–3 mm long (Fig. 1A). The distance between the opening of the floral tube and the tip of the spur is > 8 mm. The urine glucose-testing strip spot test showed no sugar-based secretions in any part of the flower, including the spur.

The flowers emitted a pleasant perfume reminiscent of diluted, commercial rosewater, but with an undertone of ethanol, from 9:00 to 15:00 h. The smell was most noticeable from 10:00 to 14:00 h, especially for plants in sunny gaps.

The anther cap covered the pollinarium inside the anther and then continued to cover the eight individual pollinia after the viscidium was withdrawn from the flower using a toothpick. We observed that the anther cap clung loosely to the pollinia for approximately 5 min until it fell off naturally or was gently prised off the pollinia with a second toothpick. We observed eight anther caps under plants in 2010 that had probably dropped off after insects had removed the pollinaria. The length of the caudicle was < 1 mm; once free of the anther cap, the caudicles did not change their positions, and the eight pollinia loosened naturally. The pollinarium consisted exclusively of a single, solid and yellowish white viscidium attached to eight short caudicles terminating in eight separate pollinia.

Flower phenology and stigmatic receptivity

The flowering period of patches of *C. yaoshanensis* was from the end of April to the beginning of June (30–40 days). The peak flowering time was the middle of May. The earliest flowers were usually damaged by frosts. The end of the flowering season was the beginning of the rainy season. When stigmas of the remaining open flowers were washed by rain, their surfaces swelled. Pollinia deposited on a swollen stigma failed to produce fruit. The lifespan of a single flower was 13.80 ± 2.26 days (mean ± SD; n = 20). The flowering period of an inflorescence was 22.45 ± 2.89 days (n = 20), but most flowers in an inflorescence opened within 4–6 days of each other. Hand-manipulated pollinarium removal did not shorten the individual floral lifespan. In contrast, when a flower was pollinated by hand or by native pollinators (see below), the petals and sepals closed within 3–4 days.

Breeding systems

All bagged but unmanipulated flowers (controls) failed to set fruit, indicating a lack of spontaneous and mechanical self-pollination. In contrast, all stigmas hand pollinated with pollinia within 30 min (= fresh pollinia) after the pollinia had been withdrawn from the anthers showed 100% fruit set, regardless of whether self- or cross-pollination was used (Table 1). Stigmas cross-pollinated with pollinia that were withdrawn from anthers 24 h earlier also showed 100% fruit set, whereas stigmas pollinated with pollinia withdrawn 48 h earlier showed 60% fruit set. There was no significant difference (P = 0.22,

Table 1. Results of hand pollinations and open (insect-mediated) pollinations in *Calanthe yaoshanensis* (Yaoshan population, north-eastern Yunnan, China)

Treatments	No. inflorescences	No. flowers	No. capsules	Fruit set (%)
Bagged control	10	20	0	0
Autogamous self-pollination	10	21	21	100
Geitonogamous self-pollination	6	10	10	100
Cross-pollination				
30-min pollinia	10	20	20	100
24-h pollinia	5	10	10	100
48-h pollinia	5	10	6	60.00
Naturally pollinated (2008)	91	630	96	15.23
Naturally pollinated (2009)	209	1170	200	17.09
Naturally pollinated (2010)	100	571	121	21.19

Fisher's exact test; Table 1) probably because of the small sampling numbers. The fruit sets of open flowers from 2008 to 2010 were 15.23%, 17.09% and 21.19%, respectively. The difference between these three flowering seasons was also not significant (odds ratio χ^2 test, $\chi^2 = 0.0353$, d.f. = 2, $P = 0.8126$). However, hand-mediated cross- and self-pollination of flowers always led to significantly higher values than for insect-mediated pollinations ($\chi^2 = 28.90$, d.f. = 2, $P < 0.0001$).

Embryo development and viability

About one-half of the cross-pollinated seeds had large embryos (41.47 ± 5.30%; mean ± SD; $n = 10$), which was significantly higher than the proportion of large embryos in self-pollinated seeds (9.67 ± 2.55%, $n = 10$; Kruskal–Wallis test: $\chi^2 = 14.286$, d.f. = 1, $P < 0.001$; Fig. 2A). Self-pollinated capsules had a higher proportion of small embryos (28.42 ± 5.81%) and aborted (i.e. collapsed) embryos (8.99 ± 3.65%) than did cross-pollinated capsules (small embryos: $\chi^2 = 13.720$, d.f. = 1, $P < 0.001$; aborted embryos: $\chi^2 = 6.228$, d.f. = 1, $P < 0.05$; Fig. 2B, C). More than one-half of the self-pollinated seeds contained no embryos at all (52.92 ± 8.27%). This ratio of empty seeds was far higher than those counted in cross-pollinated capsules ($\chi^2 = 9.605$, d.f. = 1, $P = 0.0019$; Fig. 2D). The results of seed viability tests using the tetrazolium method showed similar results. Cross-pollinated flowers produced a significantly higher proportion of red embryos than seeds in self-pollinated flowers ($\chi^2 = 5.333$, d.f. = 1, $P = 0.021$; Fig. 3A). Conversely, self-pollinated seeds had a higher proportion of colourless embryos ($\chi^2 = 5.333$, d.f. = 1, $P = 0.021$; Fig. 3C). The proportion of partly stained embryos in both pollination treatments did not differ significantly ($\chi^2 = 0.083$, d.f. = 1, $P = 0.773$; Fig. 3B).

Pollinators and behaviours of floral visitors

All nocturnal observations failed to observe any insects visiting flowers of *C. yaoshanensis*. We did not find moth scales or legs on flowers or receptive stigmas. In contrast, 12 insect species were observed during daylight hours (Table 2). The dominant foragers were members of the flower fly (hoverfly) family, Syrphidae. We observed the true drone fly (*Eristalis tenax*), a second congener (*Eristalis cerealis*) and a possibly new species in the genus *Criorhina* (F. C. Thompson, pers. comm.) carrying the pollinaria of the host flower. We also observed and collected gynes of *Bombus patagiatus* (Apidae) visiting the flowers and carrying pollinaria of *C. yaoshanensis*.

Eristalis spp. were the dominant floral visitors to *C. yaoshanensis*. We recorded 40 individuals of *E. tenax* visiting its flowers (Fig. 1B, C). Removal of the pollinarium (with the anther cap attached) by *E. tanax* was recorded only 12 times. When a fly landed on the labellum, it inserted its proboscis into the spur. The viscidium of the stigma became attached to its head and the pollinarium/anther cap unit was deposited on the insect as it backed out of the flower. When the insect visited a second flower, the pollinaria were deposited on its stigma surface. *Eristalis cerealis* had a smaller body than *E. tanax*, but was also observed carrying the pollinaria and anther caps of *C. yaoshanensis* three times. Visits by *Bombus patagiatus* were observed less frequently. We witnessed these bees visiting the flowers 12 times, but only two bees flew off with the pollinarium/anther cap units attached to their heads. We failed to catch the single specimen of *Criorhina* sp., and the specimen was identified by F. C. Thompson based exclusively on a photograph of the fly on a flower of *C. yaoshanensis* (Fig. 1D). We observed this fly species visiting the flowers five times, and two flies removed pollinaria on two separate days.

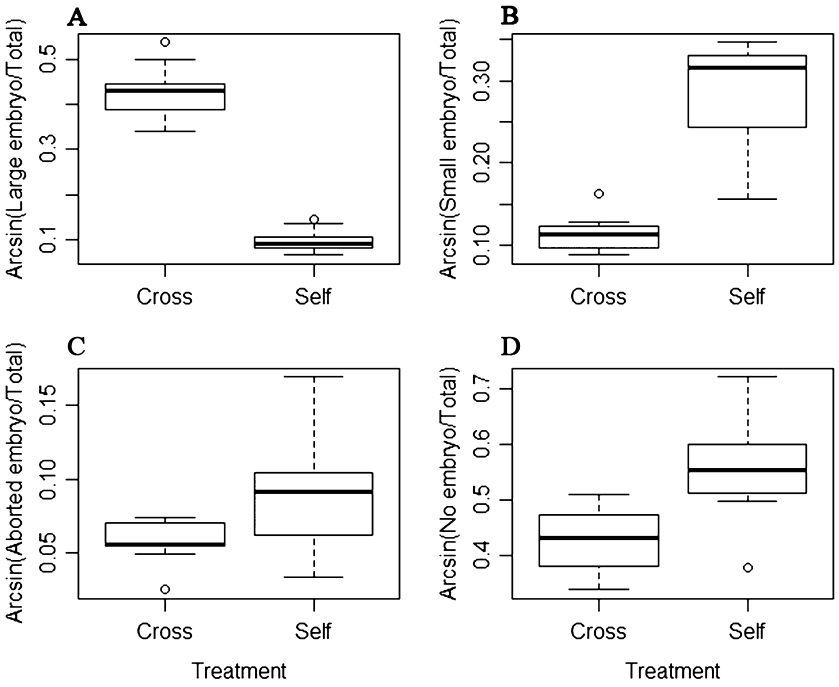

Figure 2. Embryonic development in cross- and self-pollinated seeds. The embryos were assigned to four categories: large (A), small (B), aborted (collapsed; C) and empty (no embryos; D). The proportion of large embryos in cross-pollinated capsules was significantly higher than in self-pollinated ones (Kruskal–Wallis test: $P < 0.001$). Self -pollinated capsules contained more small embryos ($P < 0.001$), aborted embryos ($P < 0.01$) and empty seeds (no embryos; $P < 0.05$) than did cross-pollinated capsules.

All remaining visitors failed to remove the pollinarium/anther cap unit. This included frequent visits ($n = 25$) by tiny hoverflies in the genus *Platycheirus*, which did not contact the viscidium. We also observed the large gyne of *Bombus graham*, but it failed to remove pollinaria. Small bees in the genus *Halictus* were observed to visit flowers, but never contacted the column. Only one pierid butterfly was observed to approach a flower, but never landed on it. Ants (unidentified) also visited the flowers (Fig. 1E), sometimes overturning the anther caps and exposing the pollinaria. The freed pollinia then touched the receptive lobes of the stigma causing self-pollination. We observed that flowers were damaged by unidentified caterpillars that also overturned anther caps, effecting self-pollination (Fig. 1F).

Flower mortality vs. reproductive success

A plant of *C. yaoshanensis* usually produced only one inflorescence, with only a few producing two inflorescences. We counted 1–15 flowers per inflorescence. We recorded 91, 209 and 100 inflorescences, *in situ*, from 2008 to 2010, respectively. The flower numbers per inflorescence were significantly different over 3 years (one-way ANOVA, $F = 8.073$, d.f. = 2, 397, $P < 0.001$) because the number of flowers per inflorescence in 2008 was significantly higher than in the 2 years that followed (2008 vs. 2009, $P < 0.001$; 2008 vs. 2010, $P < 0.01$, Tukey HSD test; Table 3, Fig. 4A). In 2009, we found five inflorescences on five plants in which all the flowers were frozen. In 2009 and 2010, a total of ten and five inflorescences, respectively, appeared to have been damaged by insect-mediated herbivory (see above). In 2010, seven inflorescences were collected illegally (probably by local villagers for medicine).

Over 3 years, the majority of surviving inflorescences (72.23–83.00%) had a minimum of one flower per raceme in which the anther cap was missing and the pollinarium was removed. In each year, slightly more than one-half of all inflorescences (52.15–60.00%) had at least one flower bearing at least one pollinium deposited on its stigma (Table 4).

Within a single inflorescence, the pollinaria of 1.70–2.59 flowers were removed over a flowering season (Table 3). There were significant differences between the 3 years ($F = 12.26$, d.f. = 2, 397, $P < 0.001$; Fig. 4B). Pollinarium removal was significantly lower in 2009 than 2010 ($P < 0.001$), but not different from 2008 ($P = 0.897$, Table 3). The rate of pollinia deposition on stigmas was always lower than the rate of pollinarium removal over 3 years (Table 3). However, there was no significant difference in pollinia deposition on stigmas over 3 years ($F = 2.683$, d.f. = 2, 397,

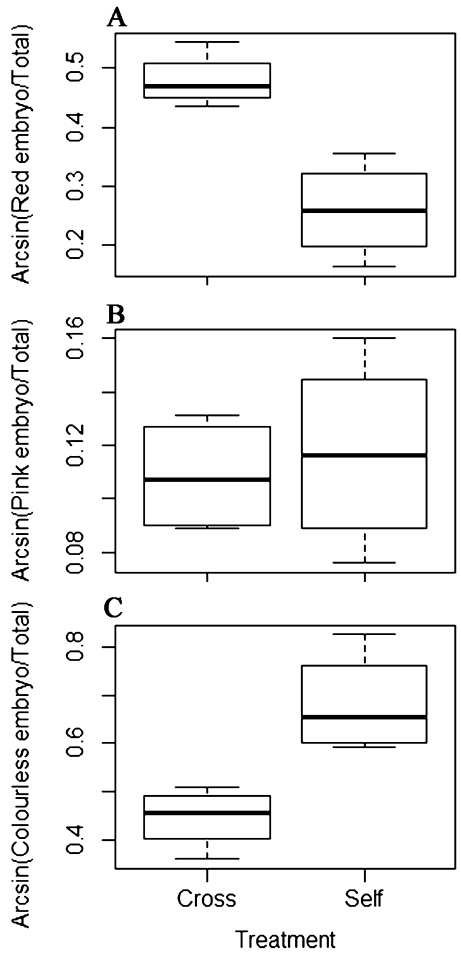

Figure 3. Comparison of embryonic viability in cross- and self-pollinated capsules using the tetrazolium test. A, Cross-pollinated capsules had significantly more red embryos than did self-pollinated capsules (Kruskal–Wallis test: $P < 0.05$). B, The proportion of pink embryos (or partly stained embryos) showed no significant difference ($P > 0.05$). C, Self-pollinated capsules contained more colourless embryos ($P < 0.05$).

$P = 0.0696$) using either one-way ANOVA or the Tukey HSD test (Fig. 4C).

Approximately one-half of all pollinaria removed by insects were lost during the process of transfer to the vector: 45.66% (2008), 43.38% (2009) and 50.96% (2010) (Table 4). Discounting such losses, the grand totals of pollinarium dispersal (male success) rates on insects were 27.46% (2008), 30.34% (2009) and 45.36% (2010). The rates of pollinia deposited on stigmas (female success) were lower at 14.92%, 17.18% and 22.24% from 2008–2010, respectively (Table 4).

We counted one to six pollinia deposited on individual stigmas of *C. yaoshanensis* but, if more than four pollinia were deposited on the receptive lobes, only four pollinia had sufficient stigmatic contact to germinate. The additional pollinia darkened and dried up. We recorded 12 (2009) and seven (2010) flowers with stigmas bearing pollinia from the same flower as a result of insect visitation or herbivore damage (see above; Fig. 2F). We were able to determine that these pollinia came from the same flower, as the viscidium in each flower remained intact and attached to its rostellum. We found that some anther caps (36 in 2009 and 43 in 2010) were overturned or removed, but pollinia remaining in the anthers were now exposed to the air. We also recorded that 5.13%

Table 2. Insect visitors observed and recorded visiting *Calanthe yaoshanensis*

Insect taxa	No. observed	No. carrying pollinaria
Diptera		
Syrphidae		
Criorhina sp. nov.	5	2
Eristalis tenax	40	12
Eristalis cerealis	16	3
Syrphus vitripennis	11	0
Leucozona lucorum	5	0
Rhingia sp.	4	0
Platycheirus sp.	25	0
Small unknown fly	4	0
Hymenoptera		
Apidae		
Bombus grahami	1	0
Bombus patagiatus	12	2
Halictidae		
Halictus sp.	21	0
Formicidae		
Ant (unidentified)	5	0
Unknown bee (small, unidentified)	11	0
Lepidoptera		
Butterfly (unidentified)	1	0

Table 3. Mean and standard deviation of number of flowers, number of flowers with pollinaria removal and pollinia deposition on stigmas of *Calanthe yaoshanensis* (Orchidaceae) in 2008 ($n = 91$), 2009 ($n = 209$) and 2010 ($n = 100$)

Variables (number of)	2008	2009	2010
Flowers	6.92 (2.50)	5.60 (2.54)	5.71 (3.09)
Flowers with pollinaria removal	1.90 (1.67)	1.70 (1.64)	2.59 (2.10)
Pollinated flowers	1.03 (1.35)	0.96 (1.23)	1.27 (1.51)

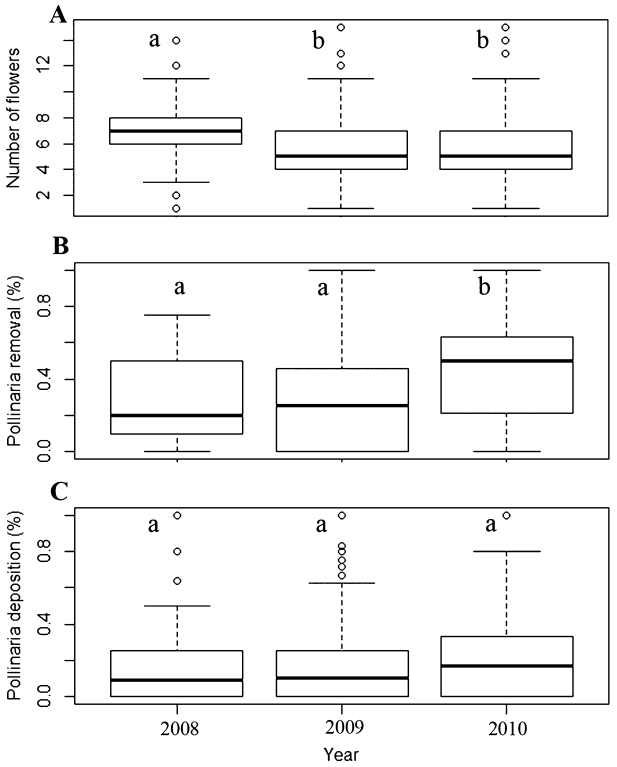

Figure 4. Comparison of the number of flowers per inflorescence and reproductive success of *Calanthe yaoshanensis* over 3 years (2008–2010). Comparison of the number of flowers per inflorescence (A) and percentage of flowers per inflorescence with pollinaria removal (B) and pollinia deposited on stigmas over the same time period (C). Different lower case letters indicate significant differences ($P < 0.05$).

(2009) and 7.18% (2010) of flowers had stigmas bearing pollinia from other flowers, whilst their own anther caps remained intact covering their pollinia. In 2010, we found five stigmas bearing anther caps, but no pollinaria were found under each anther cap (Table 4).

DISCUSSION

Floral presentation, breeding systems/seed viability and fruiting success

The floral spur of *C. yaoshanensis* contains no nectar, as in the five insect-pollinated *Calanthe* spp. studied previously (Zhang *et al*., 2010; Sugiura, 2013; Sakata *et al*., 2014; Suetsugu & Fukushima, 2014). The flower of *C. yaoshanensis* shows much the same suite of floral characters as described in many other epidendroid species that are food mimics and are pollinated by large insects. Their flowers are relatively large, long-lived and showy (Primack, 1985). Although the dominant colour, yellow, should be visible to a wide range of anthophilous insects, past studies have shown that this colour often attracts *Eristalis tenax* (Ilse, 1949; Lunau & Wacht, 1994; Wacht, Lunau & Hansen, 1996; Dinkel & Lunau, 2001).

Most orchid species studied to date appear to lack a pre-zygotic form of self-incompatibility (Edens-Meier *et al*., 2010). In contrast, *C. yaoshanensis* shows a partial trend towards post-zygotic self-incompatibility based on experimental results comparing embryonic development and viability. The population studied here appeared to be incapable of mechanical autogamy, but it is obvious that there is a low but persistent frequency of insect-mediated (forager, herbivore) self-pollination. Although this mode of self-pollination results in a greater number of non-viable embryos and/or 'empty seeds', this may not always be a maladaptive process. As in many food-mimic orchids, pollinator visitations are comparatively low (see below) relative to those in orchids offering rewards and in other angiosperms that secrete nectar (Neiland & Wilcock, 1998; Tremblay *et al*., 2005). Comparatively few flowers on the racemes of this species set fruit each year. Self-pollinated flowers contribute to a decrease in the number of viable seeds per capsule in this species, but they also contribute proportions of perfectly viable seeds, at least based on our tests. We do not know, at present, whether large embryos, produced by self-pollination, germinate and grow at rates comparable with those of embryos produced by cross-pollination.

Fruit set rates in *C. yaoshanensis* remained self-consistent over 3 years, but the population showed a low rate of fruit set, as in the majority of other food-mimic orchid species (Tremblay *et al*., 2005; Bernhardt & Edens-Meier, 2010; Edens-Meier *et al*., 2011). The average fruit set in mimetic orchid species distributed through temperate zones is *c*. 19.5% in North America and 27.7% in Europe (Neiland & Wilcock, 1998). Our fruit sets for insect-pollinated flowers of *C. yaoshanensis* showed a conversion rate in the range 15.23–21.19%. By comparison, as 100% of the hand self- and cross-pollinated flowers set fruit, this indicates that the endangered population is pollinator limited, similar to other food-mimic species. Fruit set in four other *Calanthe* spp. distributed in Japan was also extremely low, ranging from 0–9.0% in *C. reflexa* Maxim. (Sakata *et al*., 2014) to 8.3–17.3% in *C. striata* Lindl. (Sugiura, 2013), 1.20–6.49% in *C. discolor* (Suetsugu & Fukushima, 2014) and *c*. 10.0% in *C. tricarinata* (Kudo, Ida & Tani, 2008). The fruit set of *C. sylvatica* Lindl. on Reunion Island was only 3.08–9.19% (Juillet *et al*., 2010). Some variation may occur according to season and distribution in this genus. For example, on Yaoshan mountain (China), the conversion rate of flowers into fruits for *C. tricarinata* was 1.20–2.96% over three seasons (Z. X. Ren *et al*., unpubl. data).

Table 4. Comparative reproductive statistics of *Calanthe yaoshanensis* in Yaoshan population over three seasons. Percentages are in parentheses, except for the final entry entitled 'Pollinaria removed but lost'. Otherwise, all percentages were calculated by the number of different variables divided by the total number of inflorescences or flowers

	2008	2009	2010
Total number of inflorescences	91	209	100
At least one flower with pollinarium removal	69 (75.82)	152 (72.73)	83 (83.00)
At least one flower with pollinia deposition	48 (52.74)	109 (52.15)	60 (60.00)
Inflorescences damaged (frozen)	NA	5 (2.39)	0
Inflorescences damaged (herbivory)	NA	10 (4.78)	5 (5.00)
Whole plant collected for 'medicine'	NA	0	7 (7.00)
Total number of flowers	630	1170	571
Insect-mediated autogamy	NA	12 (1.03)	7 (1.23)
Pollinaria remained in anther but pollinia deposited on stigma	NA	60 (5.13)	41 (7.18)
Anther cap absent	NA	36 (3.08)	43 (7.53)
Anther cap found on stigma (no pollinia)	NA	0	5 (0.88)
Pollen did not germinate on stigma	NA	1 (0.09)	6 (1.05)
Flowers damaged by herbivorous animals	NA	23 (1.97)	9 (1.58)
Pollinaria removal (grand total)	173 (27.46)	355 (30.34)	259 (45.36)
Pollinia deposition (grand total)	94 (14.92)	201 (17.18)	127 (22.24)
Pollinaria removed but lost (missing)*	79 (45.66)	154 (43.38)	132 (50.96)

*The percentage of pollinaria removed/missing was calculated by (total number of flowers with pollinaria removal – total number of flowers with pollinia deposition)/total number of flowers with pollinaria removal (for each season).

Comparative pollination ecology

Our results are the first to confirm that large-bodied members of the family Syrphidae (hoverflies) comprise a major part of the pollinator vector spectrum (*sensu* van der Pijl & Dobson, 1966) of *Calanthe* species. As *Calanthe* spp. are often sympatric, with overlapping flowering periods in China (Chen *et al*., 2009), a radiation of pollination systems should be selectively advantageous in discouraging interspecific hybridization, as these species are easy to hybridize under cultivation (Cribb & Bailes, 2001).

Pollination by hoverflies is common in angiosperms (Larson, Kevan & Inouye, 2001) and has evolved independently in unrelated lineages in Orchidaceae. Some of these species secrete floral nectar and may have resupinate (Lehnebach & Robertson, 2004; Wilson, 2009) or non-resupinate (Bernhardt & Burns-Balogh, 1986) flowers. *Calanthe yaoshanensis* is also not the first orchid species to 'deceive' hoverflies. For example, *Govenia utriculata* Lindl. (Epidendroideae) produces false 'pollen clusters' on its labellum (Pansarin, 2008) and *Galearis rotundifolia* (Banks ex Pursh) R.M.Bateman (Orchidoideae) has no nectar in its long spur (Catling & Kostiuk, 2011). Brood-site mimesis of gravid hoverflies may occur extensively in the genus *Paphiopedilum* Pfitzer (Cypripedioideae; Bänziger, 1996; Shi *et al*., 2007, 2009) and may vary throughout the distribution of *Epipactis veratrifolia* Boiss. & Hohen. (Epidendroideae; Ivri & Dafni, 1977; Stökl *et al*., 2011; Jin *et al*., 2014). Pollination of orchid flowers by *Eristalis* spp. occurs in the nectar-secreting *Earina autumnalis* Hook.f. (Lehnebach & Robertson, 2004) and in the food mimic *G. rotundifolia*, although they are not the major pollinators in the second species (Catling & Kostiuk, 2011). As in *C. yaoshanensis*, *Prasophyllum odoratum* R.S.Rogers (Orchidoideae) and *G. rotundifolia* are pollinated by a combination of bees and hoverflies (Bernhardt & Burns-Balogh, 1986; Catling & Kostiuk, 2011).

Ineffective transfer of pollinia by pollinators vs. adaptive floral morphology

What we did not anticipate was the frequently unsuccessful movement of pollinaria by hoverflies and bumblebees on flowers of *C. yaoshanensis*. Most observations showed that the visits of these insects failed to accumulate or deposit pollinia. Pollinator–pollinarium transfer/deposition in mimetic orchid flowers usually occurs so rapidly and infrequently that we still lack a common body of literature on the frequency of transfer (Scopece *et al*., 2010). This may be an evolutionary 'trade-off' (Gurevitch, Scheiner & Fox, 2006) and the consequence of a skewed mode favouring male success in orchids. In a cross-pollinated, epidendroid flower with hard, waxy pollinia, all the seeds may be fathered by a single parent. However, the successful reception and transfer of a pollinium to the body of a pollinator requires a self-consistent modification of column (gynostegium) architecture. This may be a far

more fallible adaptation in an orchid with a comparatively narrow diversity of prospective pollinators compared with the transfer of granular pollen grains in the majority of angiosperm species with broader, generalist modes of animal pollination.

At first, the retention of the anther cap on the pollinarium of *C. yaoshanensis* appears to be maladaptive. In the Japanese species, *C. striata*, Sugiura (2013) found that the anther cap clings to the pollinarium, and sometimes prevents pollinium germination on a receptive stigma. Although we found a few anther caps on the stigmas of our species, they did not block receptive sites entirely. Indeed, the anther cap is not retained on a pollinarium for more than 5 min in this species, and so it should have lowered the rates of geitonogamous pollination if the insect visited additional flowers on the same inflorescence and/or members of the same family unit in the same patch. Anther cap retention has been noted in other epidendroid orchid species, and has been interpreted previously as an adaptive feature reducing inbreeding depression (Peter & Johnson, 2006). Roubik (2014) came to the same conclusion regarding anther cap retention in some lineages of Neotropical, epiphytic epidendroids.

Evolutionary ecology of abiotic/biotic reproductive stress

We can now state that herbivores, local herbalists, freezing temperatures and rain remove some flowers from the potential pool of fruit-producing inflorescences. Furthermore, we also suggest that ants and unidentified herbivores may affect the frequency of inbreeding depression by producing less fit seed by some mode of insect-mediated self-pollination. The extent to which these modes of environmental stress affect annual fruit set in this discrete population remains unclear, as rates of reproductive success (conversion of flowers into fruits on an inflorescence) did not vary statistically from year to year. If a second, disjunct population were to be found, it would be possible to compare fruit set frequencies with the first population under dissimilar environmental conditions.

In one respect, *C. yaoshanensis* is no different from the majority of food-mimic orchids. Visitation rates by preferred pollinators were low over three seasons, but even the most common pollinators failed to remove and/or transfer viable pollinaria on the majority of visits to these flowers. Why are hoverflies and bumblebees so clumsy? Are we looking at an orchid species that has lost its primary pollinator and only secondary and/or tertiary pollinators are left in the original vector spectrum? We doubt this interpretation, because we note that the pollinia have such a long period of viability. Even if a 'clumsy and incompetent' fly or bee delays depositing a pollinium on a receptive stigma until 48 h or more later, some seed will be set (Bellusci *et al*., 2010). This could be the evolutionary trade-off (see Gurevitch *et al*., 2006).

The problem is that the rate of pollinarium transfer, followed by the deposition of pollinia on receptive stigmas, remains underexplored (Tremblay *et al*., 2005; Scopece *et al*., 2010). We understand that in, a number of species, rates of removal of pollinia by pollinators of mimetic orchids are far higher than rates of pollinia deposition (Scopece *et al*., 2010; Edens-Meier, Westhus & Bernhardt, 2013). Therefore, this study is one of the few to show how often a potential pollinator visits flowers of one mimetic species, but fails to either disperse its pollinaria or deposit viable pollinia on stigmas.

We suspect that this is the real evolutionary consequence of orchid species offering only mimetic flowers. They are not examples of a co-evolutionary pathway. It is far more likely that they are the result of asymmetric pathways (Tremblay *et al*., 2005; see Roubik, 2014). In this pathway, there is no directional selection of pollinator body size and foraging behaviour in insect populations, as there is no mutualism between the exploited pollinator(s) and the orchid flower (see also Edens-Meier & Bernhardt, 2014). This may be yet another problem in the conservation of orchid species, especially those confined to small, discrete populations not considered by Dixon (2009). As endangered orchid species are returned to restored landscapes, their modes of floral presentation and architecture could be less reproductively fit because their primary pollinators have changed in population density, foraging behaviour and physical parameters.

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