

Resource limitation and pollen source (self and outcross) affecting seed production in two louseworts, *Pedicularis siphonantha* and *P. longiflora* (Orobanchaceae)

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Two animal-pollinated hermaphrodite plants, *Pedicularis siphonantha* and *P. longiflora*, have been used to investigate factors limiting seed production in natural populations. To evaluate the potential seed abortion due to resources limitation, seed development has been observed and seed count conducted twice. Seed production per capsule has been compared when flowers have been removed and in a control group. Open pollination has been investigated and pollen supplementation undertaken to estimate the possibility of pollen limitation. Results show that seed abortion is frequent. Stigmatic pollen load is significantly higher than ovule number per ovary under open pollination for both species. Additional self and outcross pollen did not affect seed production. Flower removal significantly increases seed production per capsule, which indicates that seed production of the studied species is limited by available resources. To detect differences in seed production between flowers pollinated by self and outcross pollen, hand pollination of bagged flowers has also been conducted in natural populations of the two *Pedicularis* species. Compared with open pollination, hand-pollinating self-pollen decreases, while outcross pollen increases seed production per capsule. Such results suggest that inbreeding depression in the two self-compatible species may also result in partial seed abortion under open pollination if mixed pollen is deposited on the stigma. Our results also suggest that pollen interference plays an important role in low female fertility in the two *Pedicularis* species. © 2005 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2005, 147, 83–89.

ADDITIONAL KEYWORDS: hand pollination – inbreeding depression – pollen interference – seed abortion – seed to ovule ratio – stigmatic pollen load.

INTRODUCTION

Seed to ovule ratio in flowering plants is always lower than one (Charlesworth, 1989a; Burd, 1994). The mechanism responsible for uncompleted ovule development can be ascribed to four main categories: (1) pollen limitation (2) resource limitation (3) genetic load, and (4) predation, diseases, or losses by harsh weather conditions (Stephenson, 1981; Charlesworth, 1989b; Ehrlen, 1992). Pollen limitation because of insufficient pollinator service has been most commonly investigated, particularly in animal-pollinated plants (Young & Young, 1992; Burd, 1994; Larson & Barrett, 2000; Suzuki, 2000; Griffin & Barrett, 2002; Ishii & Kadono, 2002). There is also much evidence

that seed production is often limited by resources (Stephenson, 1981; McCall & Primack, 1987; Ehrlen, 1992; Campbell & Halama, 1993; Suzuki, 2000; Griffin & Barrett, 2002). Genetic load is another less studied factor that results in reduced fertility, which is always found in taxa with outcrossing breeding systems (Charlesworth, 1989b; Helenurm & Schaal, 1996). Genetic load (mutational load) is probably the major cause of inbreeding depression (Charlesworth & Charlesworth, 1987).

However, these limiting factors rarely act in isolation. For example, pollen and resource limitation can interact in a complex way (Ehrlen, 1992). A strict dichotomy between pollen and resource limitation is difficult to determine under natural conditions, because a flower producing no seed may not have received appropriate pollen or may have accepted pollen, but could not produce seeds because of resource

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limitation (Campbell & Halama, 1993; Casper & Nielsenbaum, 1993; Burd, 1994; Suzuki, 2000; Goodwillie, 2001; Griffin & Barrett, 2002; Huang & Guo, 2002).

Even though different factors may operate during different phases of ovule development, factors affecting seed production can be detected and classified by experiments (Griffin & Barrett, 2002). *Pedicularis longiflora* Bonati and *P. siphonantha* Don were used in this study. Both of the species are bumblebee-pollinated and occur exclusively in Eastern Himalaya (Wang & Li, 1998). The species are among the most derived taxa in *Pedicularis* (Li, 1951). Plants of the two species have relative large inflorescences, are frequently pollinated geitonogamously by bumblebees, and have been observed to abort ovules during ovule development (C.-F. Yang, pers. observ.). The objective of this study was to determine the factors limiting seed production in the two *Pedicularis* species. Our approach was to manipulate the influence of one limiting factor at a time, to find the difference in seed production between open and artificial pollination, and thereby determine influences on seed production.

MATERIAL AND METHODS

STUDY SPECIES

We studied populations of *Pedicularis siphonantha* and *P. longiflora* in west Sichuan Province in southwest China (30°01'06"N, 101°51'20"E, about 3700 m). Field studies were conducted during the reproductive period of the plants from July to September in 2001–2003.

ESTIMATES OF OPEN POLLINATION

Pollinators were captured and taken back to the laboratory for identification. The insects whose corbicula included pollen of a certain plant and that were detected transferring such pollen to stigmas of that plant were regarded as the effective pollinators. Data on pollination rate, fruit set and stigmatic pollen load of each population under open pollination were collected. To estimate seed production without pollinator service, five randomly chosen individual plants of each population were bagged before flowers opened to harvest fruit for seed count. Mature flowers in the peak of blooming of each population were collected randomly and fixed immediately into formalin-acetic acid-alcohol (FAA) solution [formalin (37–40%), acetic acid and alcohol (50%) at a ratio of 5 : 6 : 89 by volume] for the measurement of stigmatic pollen load and pollination rate in the laboratory. Pistils were dissected carefully from flowers and observed under a fluorescence microscope (Nikon E-600) after being treated by 8 mol l⁻¹ NaOH for 24 h, and then dyed using 0.1% aniline blue for the measurement of pollen germination and pollen

tube growth. In our study, pollination rate means the number of pollinated flowers divided by the total number of measured flowers. At least 25 pollinated flowers in each population were used for the measurement of stigmatic pollen load, and 100 flowers for pollination rate. The ovary was carefully dissected out of each flower and placed into a drop of water on a microscope slide. The entire placenta with attached ovules was removed via a longitudinal slit in the ovary wall. The ovules were carefully loosened from the placenta and spread in the drop of water to be counted at 40× magnification under a dissecting microscope.

More than 25 individual plants of each population were selected randomly to measure flower number per individual plant. These plants were collected when all fruits were mature to calculate fruit set, which equals for each plant the total fruit number divided by total flower number.

ARTIFICIAL TREATMENTS

To detect the potential for resource limitation of seed production, a population of *P. siphonantha* was chosen for a flower removal experiment (see also Ehrlen, 1992). Flower removal was conducted in 30 randomly chosen individual plants during July 13 to August 5, 2003. About one-third of the flowers of each of the plants were cut off just before anthesis. Another 30 individual plants were chosen randomly as controls. Mature fruits of the plants were harvested for seed count.

Four artificial treatments were conducted in this study: (1) open pollination with additional self pollination (pollen from a flower of same inflorescence); (2) open pollination with additional outcross pollination (pollen from the flower of another plant); (3) hand selfing followed by bagging to exclude pollinators; and (4) hand outcrossing followed by bagging to exclude pollinators. At least 30 flowers were used for each treatment. The flowers were marked and collected for seed count when fruits were mature. Additional pollination was conducted during the peak blooming period of the population. Flowers had been previously enclosed in fine bridal veil netting for artificial pollination.

SEED COUNTS

Seed production per capsule for treatments of artificial pollination, flower removal and the control line were quantified when the fruit was completely mature and began to release seeds.

Garwood & Horvitz (1985) showed that ovary enlargement was a valid criterion for assessing fertilization of ovules. Seed counts were conducted for fruits with an expanded ovary. To estimate possible seed abortion during seed development, seed number per

capsule must be counted at different phases during seed development. In the present study, we counted seed number per capsule at the time that the corolla shrivelled and the fruit was beginning expansion (Phase I), and also when the fruit was completely mature and had begun to release seeds (Phase II). At least 40 randomly selected capsules were used for each phase.

DATA ANALYSIS

The SAS program (SAS, 1998) was used in all statistical analyses in this study. ANOVA analysis was conducted to determine the effects of experimental manipulations on stigmatic pollen load, ovules/ovary and seed production.

RESULTS

OPEN POLLINATION AND SEED PRODUCTION

Observations during the whole flowering period in two years indicate that the two *Pedicularis* species are pollinated exclusively by bumblebees (*Bombus* ssp.) (Table 1). Pollinators were observed pollinating many flowers of the same inflorescence or individual plant. Therefore, geitonogamy is probably common in *P. siphonantha* and *P. longiflora*. In addition, flowers bagged early to exclude insects do not set seed, indicating that the reproduction of the two species depends on animal pollination (see also Wang & Li, 1998).

Both *P. siphonantha* and *P. longiflora* have relatively high pollination rates and fruit set (Table 1). Mean stigmatic pollen load of pollinated flowers under open pollination is significantly higher than the mean

number of ovule per ovary for both of the two species ($F = 9.14$, $P = 0.005$ for *P. longiflora*; and $F = 90.90$, $P < 0.0001$ for *P. siphonantha*). Pollen grains deposited on stigmas by open pollination for both of the two *Pedicularis* species germinated successfully and grew pollen tubes (see also Yang *et al.*, 2002). Results show that seed abortion for the two *Pedicularis* species is frequent because seeds per capsule decrease after the initiation Phase I vs. Phase II ($F = 41.96$, $P < 0.0001$ for *P. siphonantha*, and $F = 206.40$, $P < 0.0001$ for *P. longiflora*). We have also noticed that seed production is much lower than ovules per ovary for the two species, even in earliest measurements (Table 1). This means that some ovules are not fertilized and are aborted early in fruit development.

SEED PRODUCTION AFTER ARTIFICIAL TREATMENTS

Results of our flower removal experiment in *P. siphonantha* show that seed production per capsule was significantly higher in plants with flower removal compared with those of controls (9.81 ± 2.43 vs. 5.45 ± 1.84 ; $F = 95.90$, $P < 0.0001$).

Supplementation of self and outcross pollen did not increase seed production per capsule compared with open pollination (Fig. 1A, B). However, exclusive pollination by self-pollen decreased seed production per capsule, while exclusive pollination by outcross pollen increased seed production per capsule (Fig. 1C, D). In addition, final seed production following exclusive pollination by outcross pollen is significantly lower than at seed initiation for both of the species ($F = 206.40$, $P < 0.0001$ for *P. longiflora*, and $F = 27.53$, $P < 0.0001$ for *P. siphonantha*), which indicates that seed abortion has nevertheless occurred.

Table 1. Reproductive trait of open pollination for two *Pedicularis* species in natural populations (mean \pm SD)

Species	<i>P. siphonantha</i>	<i>P. longiflora</i>
Flower number/individual plant ($N > 25$)	78.8 \pm 89.7	31.3 \pm 21.9
Pollinator type	<i>Bombus yunnanicola</i> <i>B. richardsi</i> <i>B. mirus</i>	<i>B. richardsi</i> <i>B. yunnanicola</i> <i>B. atrocinctus</i>
Pollination rate ($N > 100$)	0.909	0.905
Fruit set ($N > 25$)	0.876	0.857
Ovule number/ovary ($N > 30$)	18.7 \pm 2.5	30.6 \pm 2.8
Stigmatic pollen load ($N > 25$) ^a	35.9 \pm 5.8****	36.4 \pm 6.7**
Seed production/capsule ($N > 40$)	Phase I ^b Phase II ^c Under bagged	18.5 \pm 4.1**** 4.6 \pm 2.0**** 0.24 \pm 1.14
	9.7 \pm 1.9**** 5.5 \pm 2.6**** 0.47 \pm 1.49	

^aand ^b: asterisks indicate means that differ significantly from ovule number per ovary; and ^c asterisks indicate means that differ significantly from seed production of phase I. **** $P < 0.0001$; *** $0.0001 < P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$.

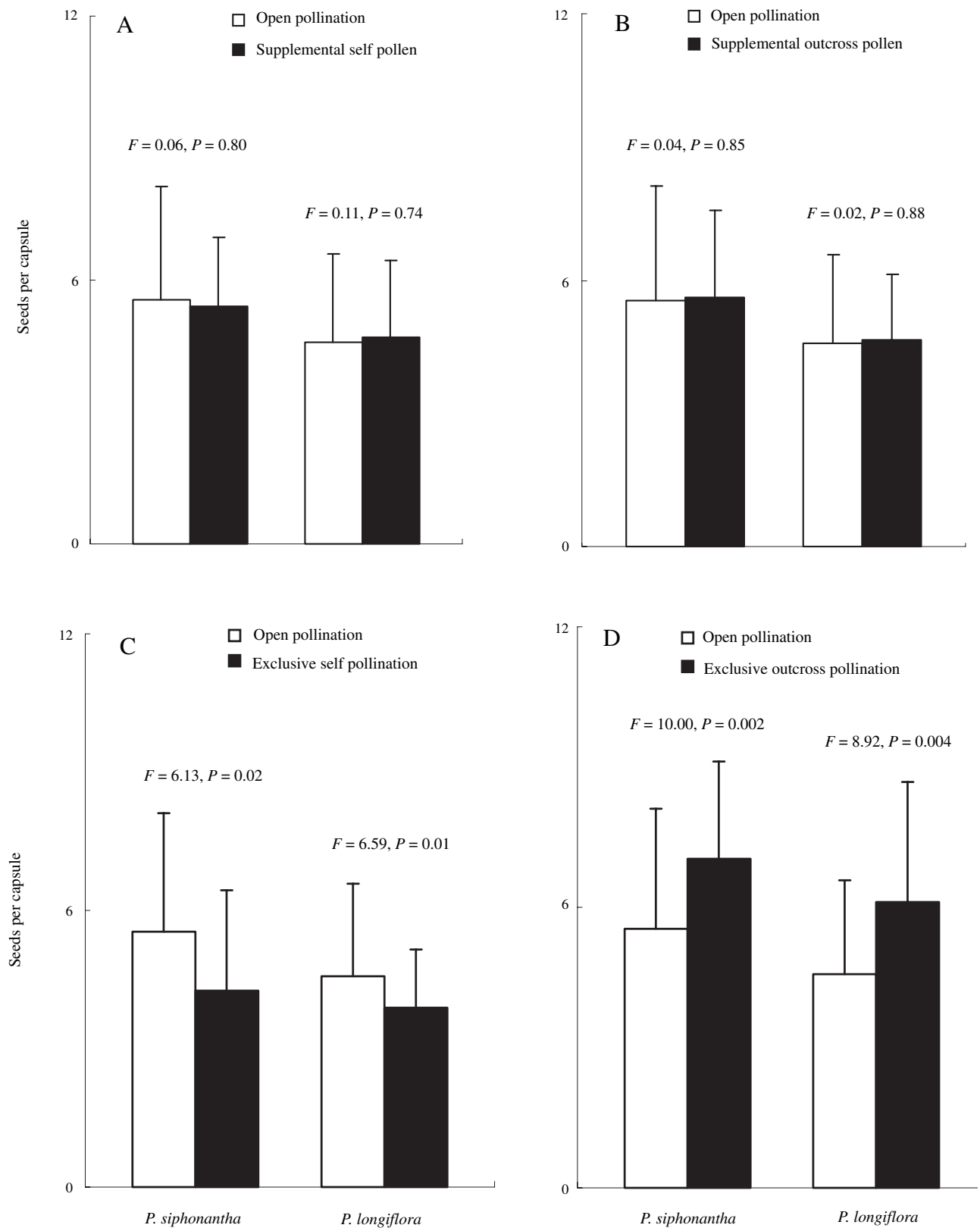


Figure 1. Comparison of seed production per capsule between open pollination and artificial treatments in two species of *Pedicularis* ($N > 30$ for each treatment).

DISCUSSION

Under open pollination, *P. siphonantha* and *P. longiflora* have a relatively high pollination rate and fruit set. Flowers of the two species require insect visits for set seed.

RESOURCE LIMITATION ON SEED PRODUCTION

Yang *et al.* (2002) demonstrated that the stigmas of the two *Pedicularis* species have the highest capability of retaining pollen grains in the genus due to their 'gully like' stigmatic surface. Results of our study also indicate that the number of compatible pollen grains deposited on stigmas by bumblebees exceeds the number of ovules per ovary. Pollen supplementation in our study reveals that seed production is not pollinator limited in either species. Several other studies have also shown that stigmatic pollen load was higher than that required to fertilize all ovules in an ovary (Mulcahy *et al.*, 1983; Spira *et al.*, 1992), and the quantity of compatible pollen deposited on the stigma was found not to affect seed production if compatible pollen on the stigma is enough to fertilize a sufficient number of ovules (Snow, 1986). We therefore suggest that, at the level of a single flower, the number of pollen grains deposited on the stigma under open pollination is not the main factor limiting seed production for the two species.

Even though the number of pollen grains is enough for fertilization of all ovules in the ovary, seed production may nevertheless be limited by resources (Charlesworth, 1989b; Lubbers & Lechowicz, 1989; Campbell & Halama, 1993; Suzuki, 2000; Griffin & Barrett, 2002). In our present study, many immature seeds were found to have been aborted during the process of seed development for the two *Pedicularis* species. Seed production even after pollen supplementation was much lower than early seed set. Gorchov (1988) argued that ovules fertilized but later aborted must be caused by resource limitation. Some other studies also found that many fruits and seeds that were initiated were aborted, probably because of resource limitation (Stephenson, 1981; Sutherland & Delph, 1984; Mazer, 1987). Moreover, we found that removal of some flowers apparently resulted in a translocation of resources that increased seed production among the remaining flowers. Several previous studies have also shown that flower removal may increase seed set because of a smaller need for limited resources (Lee & Bazzaz, 1986; Gorchov, 1988; Ehrlen, 1992). We conclude that resource limitation, rather than pollen limitation, was responsible for the low seed production of the two *Pedicularis* species at the level of a single flower. Working on *P. palustris*, Karrenberg & Jensen (2000) demonstrated resource con-

strained seed production at the level of an individual plant. Unlike our results, they found pollen limitation of seed set existed at the level of a single flower in small population. This difference may be due to the variation of stigma morphology among species of *Pedicularis*. Stigmas of flowers of *P. palustris* have a relatively smooth and small surface, and may therefore have a low capability for retaining pollen grains (Yang *et al.*, 2002).

POLLEN SOURCE AND SEED PRODUCTION

Although resource limitation may be responsible for the low seed production of the species in this study, pollen quality frequently affects seed production in nature (Charlesworth, 1989b; Barrett, 2002). Even when pollen supply is sufficient, seed production may also be reduced by the viability of pollen (Thomson & Thomson, 1992), and the relatedness of the pollen donor (Waser & Price, 1989; Barrett, 2002).

Self-incompatibility and dichogamy are very seldom found in *Pedicularis*, but geitonogamous self pollination is very common (reviewed by Karrenberg & Jensen, 2000). For *P. longiflora* and *P. siphonantha*, self pollen also results in seed set, but lower than that of open pollination and outcross pollination. This suggests the two species are partially self-compatible. Sprague (1962) also indicates that many *Pedicularis* species have reduced self-compatibility and depend on outcrossing for full seed formation. If outcross pollen can increase seed production, why does the supplemental outcross pollen not affect the seed production? The explanation may be that pollinators deposit a mixture of pollen grains on stigmas and clog up the stigmas so that supplemental outcross pollen is ineffective (Shore & Barrett, 1984; Bertin & Sullivan, 1988). Similarly, pollen from the wrong species might occupy stigmas and reduce the chance of successful fertilization (Charlesworth, 1989b). Studies have also shown that interference by self pollen can reduce seed production (Bertin & Sullivan, 1988; Ramsey & Vaughton, 2000). Self-pollen of the two partial self-compatible species may occupy stigmatic area, fertilize the ovule, and thereby disable the outcross pollen grains when they are later brought to stigma. Our fieldwork supports this argument. Flowers of *P. siphonantha* and *P. longiflora* were visited by bumblebees which flew from flowers of other plants, and/or visited many flowers of the same inflorescence (C.-F. Yang, pers. observ.). Therefore, it is unavoidable that some other incompatible or self pollen will have been deposited on stigmas.

Pollination by self-pollen exclusively decreases seed production compared with that of outcross pollination. Similar results have also found in other species of the genus (Karrenberg & Jensen, 2000), and

other taxa (Byers, 1995; Dogterom, Winston & Mukai, 2000; Ramsey & Vaughton, 2000). The transfer of pollen between flowers of the same plant can cause self-fertilization in self-compatible plants and may lead to inbreeding depression (Huenneke, 1991; Young *et al.*, 1996). Genetic load (mutational load) is probably the major cause of inbreeding depression (Charlesworth & Charlesworth, 1987). Our results suggest that, besides resource limitation, inbreeding depression caused by self pollen grains deposited on stigma by bumblebees may induce seed abortion under open pollination (see also Karkkainen *et al.*, 1999). Therefore, the low female fertility of *Pedicularis* species may be explained by genetic load to a certain extent.

Seed production may also be reduced by many other factors, such as seed predation (DiFazio *et al.*, 1998) and ovule infertility (Mogensen, 1975). No invertebrate parasites were seen in ovaries of the two studied species, although they were observed in some other species (C.-F. Yang, pers. observ.). Since stigmatic pollen load is significantly higher than ovules per ovary, while seed initiation is significantly lower than ovules per ovary for the studied species, the low seed production might also be due to the infertility of some ovules. It was observed that ovules of some species mature a few days after anthesis but begin to senesce several days later if not fertilized (Gorchov, 1988 and references therein).

EVOLUTIONARY SIGNIFICANCE

Both *P. siphonantha* and *P. longiflora* produce many flowers per individual plant and the pollination rate and fruit set are relatively high. Plants of the two species must therefore distribute their available resources to many flowers for setting fruit and seed. If the plant distributes limited resources equally to all fertilized ovules, one single ovule might not get enough nutrients for yielding a seed with high quality. It is then possible that all offspring of the plant will have low reproductive fitness (see also Charlesworth, 1989b). Seed abortion may therefore be an adaptive strategy when limited resources are allocated among many flowers because the plant can favour excellent offspring and thereby increase its reproductive success (see also Casper & Wiens, 1981; Charlesworth, 1989b; Karkkainen *et al.*, 1999; Melser & Klinkhamer, 2001).

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