

# A test of simultaneous resource and pollen limitation in *Styloidium armeria*

Rowan H. Brookes<sup>1</sup>, Linley K. Jesson<sup>2</sup> and Martin Burd<sup>1</sup>

<sup>1</sup>School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia; <sup>2</sup>Department of Biology, University of New Brunswick, PO Box 4400, Fredericton, Canada E3B 5A3

Author for correspondence

Rowan Brookes

Tel: +61 3 9905 5667

Fax: +61 3 9905 5613

Email: rowan.brookes@sci.monash.edu.au

Received: 14 December 2007

Accepted: 27 February 2008

## Summary

- This study tests the Haig–Westoby model, which predicts that seed output will be limited simultaneously by pollen and resources when plants optimally distribute their reproductive investment.
- The test was conducted over 2 yr using *Styloidium armeria* in a factorial design that fully crossed three pollination levels (small stigmatic loads, open pollination, and supplementation of natural loads) with three levels of resource availability (reduction through partial defoliation, unmanipulated resource conditions, and supplementation through nitrogen, phosphorus and potassium (NPK) addition).
- There was no evidence of pollen limitation from supplemental pollination; however, pollen reductions (to about half the normal mean stigmatic loads) sharply reduced seed output. There was no evidence of resource limitation, in that NPK addition did not, by itself, significantly elevate seed output in either year of the study, while resource reduction by defoliation lowered seed output in the second year. Simultaneous addition of both pollen and resources strongly and significantly increased seed production.
- These results match the direction of effects predicted by the Haig–Westoby model, and suggest that *S. armeria* plants at our site are at or near an equilibrium of joint limitation of seed production by pollen capture and resource availability.

**Key words:** Haig–Westoby model, pollen limitation, pollination, reproductive success, resource limitation, seed set.

*New Phytologist* (2008) doi: 10.1111/j.1469-8137.2008.02453.x

© The Authors (2008). Journal compilation © *New Phytologist* (2008)

## Introduction

The model of Haig & Westoby (1988) has been very influential in plant reproductive ecology (Ashman *et al.*, 2004). It suggests that female reproductive success should be limited simultaneously by pollen and resources over a plant's lifetime. Under the assumptions of their argument, seed output of a plant with optimal resource investment would not increase if supplemental pollen were received because the plant would lack resources to provision additional seeds. The consequences of resource manipulation are less certain because both pollen receipt (from altered investment in pollinator attraction) and seed provisioning ability could be affected. Nonetheless, the model unambiguously implies that an artificial reduction in

resources should reduce total seed output (by diminishing pollinator attractiveness or compromising seed provisioning or both), while an artificial increase may have no effect (if only provisioning ability is elevated while pollinator attraction and ovule fertilization remain unchanged) or may increase seed yield (if both pollen attraction and seed provisioning are enhanced). Only combined addition of both resources and pollen should consistently and unambiguously raise seed output.

Artificial supplemental pollination has often been used to examine pollen limitation and, contrary to Haig–Westoby predictions, has often produced increases in seed production (Burd, 1994; Larson & Barrett, 2000; Ashman *et al.*, 2004; but see Knight *et al.*, 2006). Researchers have also manipulated

both pollen and resource supplies together (Galen, 1985; Delph, 1986; McCall & Primack, 1987; Campbell & Halama, 1993; Corbet, 1998; Asikainen & Mutikainen, 2005) but seldom have imposed resource reductions in conjunction with pollen manipulations (Ne'eman *et al.*, 2006; Brookes & Jesson, 2007). Experimental restrictions have been placed on pollinator visitation (Caruso *et al.*, 2005), but none, to our knowledge, has included a demonstrated pollen reduction among the manipulations. While most studies of this type have found evidence for one limiting factor on seed set (i.e. pollen or resources), evidence for simultaneous limitation by both pollen and resources is rarer. The Haig & Westoby (1988) model implies that a full exploration of the limiting factors on seed output requires both supplementation and reduction of both stigmatic pollen loads and resource availability. We performed such an experiment and examined flower, fruit, ovule and seed output in *Stylidium armeria* (Stylidiaceae) over 2 yr in a natural population.

## Materials and Methods

### Study species and site description

*Stylidium armeria* Labill. (Stylidiaceae) is a perennial, usually rhizomatous herb occurring in habitats ranging from lowland woods to subalpine snowfields throughout south-eastern Australia. The study population was located at Lake Mountain, Victoria (1433 m above sea level 37°31'S, 45°52'E), an undisturbed subalpine ecosystem dominated by snow gum (*Eucalyptus pauciflora*). At this study site, *S. armeria* flowers between December and February. The pinkish, zygomorphic flowers are presented acropetally on a raceme with an average of 56 flowers per plant (SD = 49, range = 6–330,  $n = 150$  plants). The mean ovule number per flower is 117.6 (SD = 48.1,  $n = 130$  flowers). Stigmatic pollen loads after a single day of exposure to pollinators averaged 55.6 grains per stigma (SD = 75.7, range = 0–393,  $n = 40$ ). The staminate and pistillate tissues have fused to form a gynostemium or 'trigger' that deposits and removes pollen from *Stylidium's* insect pollinators (bees, butterflies and flies: Raulings & Ladiges, 2001). Within a flower, sexual development is protandrous: the anthers are presented for an average of 4.3 d, followed by an average stigmatic phase of 4.8 d (SD = 2.0 and 2.8, respectively,  $n = 1478$  flowers). The fruits form dry, dehiscent capsules.

*Stylidium graminifolium*, from which *S. armeria* has been recently separated (Raulings & Ladiges, 2001), has a mixed mating system (Willis & Ash, 1990). *Stylidium armeria* at Lake Mountain is not autonomously selfing, as bagged plants did not produce seed (R. H. Brookes, pers. obs.). However, plants were not artificially self-pollinated to test compatibility. A voucher specimen of *S. armeria* from this study has been placed at the Victorian National Herbarium, Australia (MEL).

**Table 1** Sample sizes (*Stylidium armeria* plant number) for treatments

Pollen treatment:	–			0			+		
Resource treatment:	–	0	+	–	0	+	–	0	+
2005				13	4	4	7	6	8
2006	6	9	9	12	16	16	13	16	16

Reduction (–), unmanipulated control (0), addition (+).

### Experimental methods

During October and November 2005, before plants bolted, 315 plants were tagged in a study area of 20 × 50 m, providing 35 replicates for each of nine treatments: three resource levels (reduction, control and addition) crossed with three pollination levels (reduction, control and addition). To distinguish between individual genets, a small amount of soil was removed around the base of the plant to ascertain whether rhizomes were connected. If an individual comprised multiple ramets, treatments and measurements were performed on all ramets of the genet, and the measurements combined to yield whole-genet values.

Pollen reduction treatments were applied only in 2006, not in 2005. However, plants assigned to pollen reduction treatments received their scheduled resource manipulation in both 2005 and 2006. All other treatment combinations were applied consistently to the same plants for both years. Only 31% of 467 unmanipulated plants in this population flowered during 2005 (R. H. Brookes, unpublished), producing unequal sample sizes among treatments because plants were tagged and assigned to treatments before flowering (Table 1). A power analysis of the 2005 data suggested a sample size of 17 plants per treatment would provide 80% power for an ANOVA (the within-group variation was based upon mean variation in the resource manipulated plants with an effect size of 15%; the between-group variation was based upon the control treatment). However, mortality and browsing in 2006 resulted in sample sizes again being unequal and fewer than 17 plants per treatment (Table 1).

Resource limitation was assessed by three treatment levels. (1) Resource reduction was imposed by removing 50–60% of leaves from a plant before flowering. Plants did not regrow the leaves that were removed during the flowering season. (2) Control plants were unmanipulated and experienced their natural resource environment. (3) Resource addition was accomplished by applying a liquid nitrogen–phosphorus–potassium (NPK, 9 : 2 : 6) fertilizer (Charlie Carp; Charlie Carp Pty Ltd, Deniliquin, NSW, Australia) around the base of plants once a month during the flowering season at the manufacturer's recommended strength (1% v : v dilution, 20 ml per plant). Between flowering seasons a slow release fertilizer (Osmocote native; Scotts, Australia) was applied to the base of the plants (15 ml per plant).

Pollination treatment levels were conducted as follows. (1) Stigmatic pollen deposition was reduced below natural levels by applying dilute suspensions of pollen grains to receptive stigmas on some flowers of plants placed under mesh enclosures before flowering. For this treatment, anthers from donor plants > 5 m away were placed in a 1:5 mixture of glycerine and water. The suspension was then diluted to a pollen grain density of 100–140  $\mu\text{l}^{-1}$ . Flowers were pollinated by using a micropipette to place a single 0.5  $\mu\text{l}$  drop of the suspension (vortexed for homogeneity immediately before) onto the stigmatic surface of a receptive flower. Thus, stigmatic pollen loads in this treatment were about half the average number of ovules per flower. (2) Control plants were unmanipulated and exposed to natural pollinator visitation and pollen deposition. (3) Supplemental pollen was added to the stigmas of plants using a small paintbrush to apply excess pollen from donor plants to receptive stigmas on treatment plants. To minimize potential inbreeding depression, source plants were at least 5 m from treatment plants. During 2005 pollen was applied to every receptive stigma on the whole plant once a week (resulting in approx. 70% of the flowers supplementally pollinated). As supplemental pollinations had no effect on seed set during this year (see the Results section), pollinations were carried out two or three times each week during 2006 to ensure that 100% of flowers were supplementally pollinated. No flowers were hand-pollinated more than once. The fruits were allowed to mature and then the racemes were removed and the seeds and fruits counted in the laboratory. Aborted ovule number was also recorded, but, as the number of aborted ovules corroborated our other results, these data are not included here.

### Data analysis

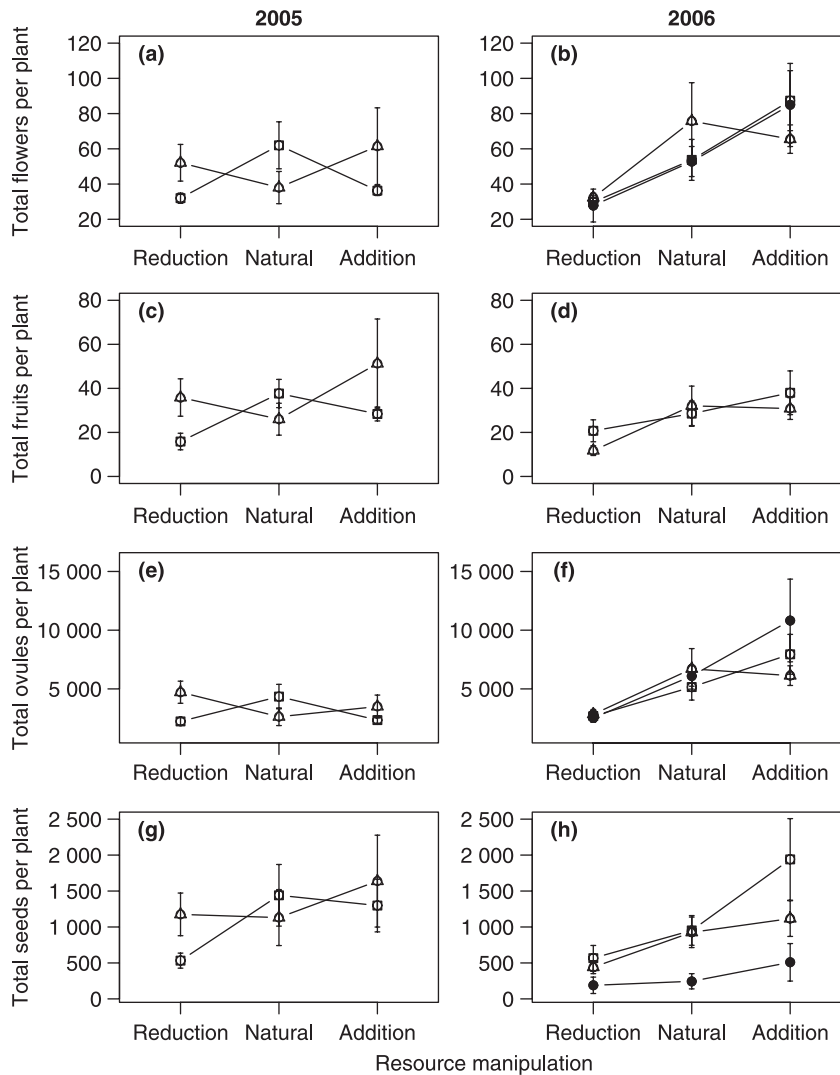
Six variables were used to assess the reproductive response to the pollen and resource treatments: the number of (1) flowers, (2) fruits, (3) ovules and (4) seeds produced per plant, and the proportional (5) fruit set and (6) seed set. Proportional fruit set equalled the number of fruits divided by the total number of flowers (among all racemes on genets with multiple inflorescences) on each plant. However, fruit set in the pollen reduction treatments was not measured, as not all flowers on these plants were artificially pollinated and the mesh enclosures prevented natural pollination. The proportion of ovules setting seed (seed number/ovule number) was determined for all fruits on a plant in 2005, and for every third fruit on one raceme per plant in 2006. Average values of proportion seed set were then calculated for each plant and the plant means used in the statistical analysis described later. To estimate total seed and ovule number for each plant in 2006, the average seed set per fruit and ovule number per fruit were multiplied by the number of fruits or flowers (for total ovule number) per plant. To improve normality, total flower, ovule and seed numbers in the 2005 and 2006 data were square-root transformed, and the proportion seed set data in 2006 were arcsine transformed.

To test the prediction in the Haig & Westoby (1988) model that pollen and resources are simultaneously limiting whole-plant reproductive success, a two-factor ANOVA with R version 3.2.1 (R Development Core Team, 2007) was conducted with pollen and resource treatment as fixed factors, and the six measurements described earlier as dependent variables. Any significant main effects of this model were interpreted in the standard way, using Tukey *post-hoc* contrasts to identify significantly different levels of either the pollen treatment or the resource treatment when pooling all levels of the other treatment.

However, the main treatment effects do not specifically test the Haig & Westoby (1988) hypothesis and are reported largely for their heuristic value. If one assumes that unmanipulated plants in their natural environment are at the equilibrium point of the Haig–Westoby model, four specific predictions can be made about the effects of various treatments relative to unmanipulated controls. (1) Resource reduction should restrict seed provisioning whatever the level of pollen receipt, and therefore decrease mean seed output per plant relative to unmanipulated control plants. (2) Pollen reduction should limit the number of fertilized ovules whatever the resource availability, and therefore reduce mean seed output relative to control plants. (3) Pollen addition in isolation should have no effect, since seed provisioning remains limiting, while resource addition in isolation should have no effect if only provisioning ability is raised, or some effect if both attraction and provisioning ability increase. (4) Only simultaneous increases of both pollen and resources should always enhance mean seed output relative to the controls. Each of these four predictions involves a contrast between a cell or group of cells of the ANOVA and the double-control cell with natural levels of both pollen and resources (see Table 4). We used *a priori* linear contrasts to test the statistical significance of these comparisons. The four contrasts are not orthogonal, but opinion is divided about the need for orthogonality in planned comparisons (Quinn & Keough, 2002). Here, we adopt the advice of Quinn & Keough (2002), who argue that if the number of comparisons is small and there is a firm theoretical basis for them, it is more important to examine all the hypotheses than to insist on orthogonality. Because the Haig–Westoby argument addresses reproductive output rather than reproductive potential (e.g. flower and ovule number), and because the packaging of reproductive output (fruit number or proportional fruit and seed set) is a less direct measure than total seed production itself, we perform the contrasts only on seed number.

### Results

In general, the experiment produced little discernable pattern in the first year, and then a strong effect in the second year. Three explanations can account for this result: some treatment combinations are not expected to affect the response variables; a pollen reduction treatment was not performed in the first



**Fig. 1** (a,b) Total flower number, (c,d) fruit number, (e,f) seed number and (g,h) ovule number per plant (mean  $\pm$  1 SE) of *Styliidium armeria* following the 2005 and 2006 flowering season. Resource manipulations: reduction (60% of foliage stripped), natural (natural conditions) and addition (fertilizer added). Pollen manipulations: pollen reduction (circles), natural pollination (triangles) and pollen addition (squares).

year; and the presence of storage organs may have buffered plants against the resource manipulation in the first year. Because we have evidence for this last possibility, we concentrate on the 2006 results to test the Haig–Westoby model.

Unmanipulated control plants produced an average of 38 flowers (range 18–54) and 1130.75 seeds (range 119–1857) in 2005, and 75.7 flowers (range 12–330) and 925.4 seeds (range 5–2658) in 2006. Given the mean ovule number per flower of 117.6, it is clear that total female reproductive output is normally well below its hypothetical maximum.

The pollen and resource treatments had no significant effect on flower, fruit, ovule or seed production in the first year of the study, and there was no significant interaction of the treatments (Table 2, Fig. 1). The failure of pollen addition to lift seed output in 2005 would be interpreted conventionally as the absence of pollen limitation. The absence of a significant resource treatment effect may be because of storage organs, as noted earlier. This interpretation is supported by the strong

resource effect observed in 2006, following treatments in the previous year (Table 2).

The 2006 results showed a strong and consistent effect of the resource manipulation on all four measures of total reproductive output (Fig. 1). The mean number of flowers per plant differed significantly among the reduction (defoliation) treatment ( $30.4 \pm 2.4$  SE), control ( $62.5 \pm 10.0$  SE), and NPK addition treatment ( $78.1 \pm 8.8$  SE) (Table 2). The pattern of ovule number per plant mirrored that of flower number (Fig. 1), although only the reduction vs addition comparison was significant (Table 2). The main effect of resources on total seed output was less pronounced than it was for flower and ovule number (Fig. 1), with only the contrast between the resource reduction and resource addition treatments being significant (Table 2). However, the resource main effect on seed number is pooled across the pollination treatments, and seed production, unlike flower and ovule number, depends on pollination. Pollination also had a strong main effect on seed set (Table 2).

**Table 2** ANOVAS testing for differences in the total flower, fruit, seed and ovule number per plant over 2 yr of resource and pollen manipulations in *Styloidium armeria*

Effect	Flower number			Fruit number			Ovule number			Seed number		
	SS	df	P	SS	df	P	SS	df	P	SS	df	P
2005												
Pollen	3.3	1	0.323	1 597	1	0.092	605	1	0.167	172	1	0.334
Resource	3.5	2	0.597	937	2	0.424	275	2	0.640	642	2	0.181
Pollen × resource	16.1	2	0.102	1 942	2	0.177	1 599	2	0.086	289	2	0.454
Error	119.2	36		19 208	36		10 951	36		6 447	36	
2006												
Pollen	0.2	2	0.984	441	1	0.443	1 326	2	0.412	4 140	2	0.0002
Resource	154.2	2	< 0.0001	5 726	2	0.025	17 553	2	< 0.0001	2 576	2	0.0039
Pollen × resource	15.6	4	0.647	712	2	0.620	1 750	4	0.670	628	4	0.584
Error	622.6	99		66 727	99		73 233	99		21 742	99	
Reduction vs natural			< 0.01			0.114			< 0.001			< 0.01
Natural vs addition			< 0.001			0.813			0.162			0.149
Reduction vs addition			< 0.001			< 0.01			< 0.05			0.253

Pollen reduction was performed only in the second year. Fruit number does not include plants with pollen reduction in the second year. Values of *F* were estimated using type II sums of squares (SS). Results from Tukey's *post-hoc* contrasts between resource levels for 2006 are presented below the ANOVA.

The relative measures of reproductive output, like the four absolute measures, were insensitive to our initial manipulations. Neither the proportion of fruit set nor the proportion of seed set in 2005 was significantly affected by the pollen treatment, the resource treatment or their interaction (all  $P > 0.05$ ; Fig. 2a,c). In 2006, the proportion of fruit set was lower over all treatments than it was in 2005 (Fig. 2a,b). Mean daily temperatures during the flowering season were similar for both years, but another facet of weather may have been responsible for the low rate of fruit set in 2006. In any case, there were no significant treatment effects on proportion of fruit set in 2006 (all  $P > 0.05$ ). There was a significant pollination effect on proportion of seed set in 2006 (Table 3). Plants in the pollen reduction treatment converted, on average, only 3.4% ( $\pm 1.4$  SE) of their ovules to seeds, which is significantly less than the 25.8% ( $\pm 3.6$  SE) for control plants and 26.4% ( $\pm 3.7$  SE) for hand-pollinated plants. The proportion of seed set was unrelated to total flower number in either 2005 ( $r = 0.022$ ) or 2006 ( $r = 0.062$ ).

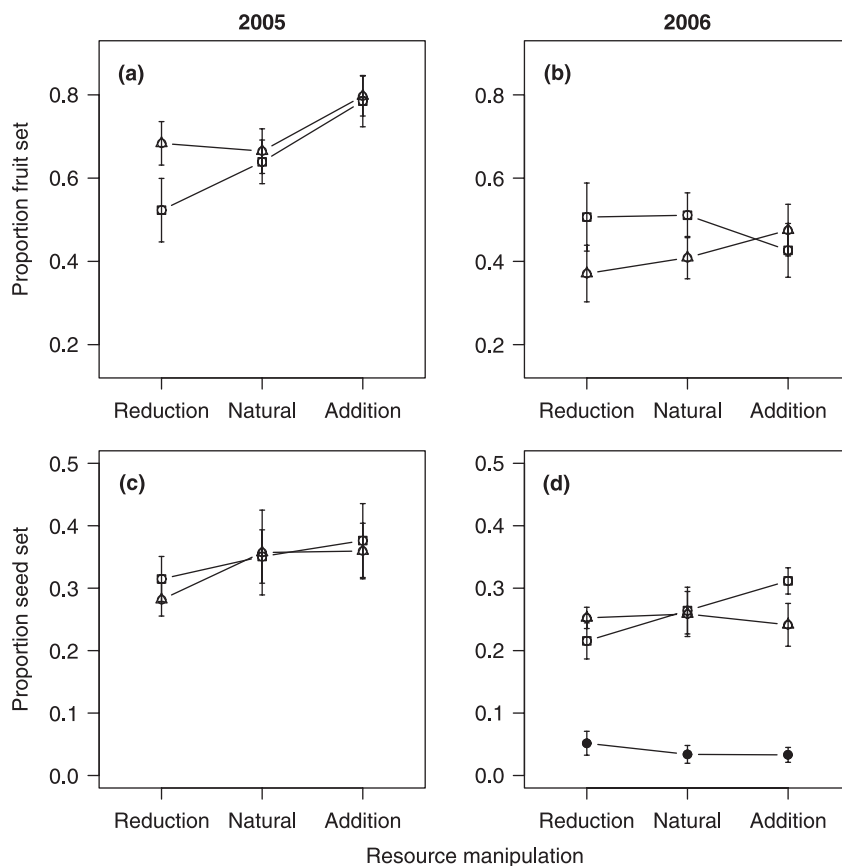
The pattern of mean total seed number per plant in 2006 among each of the nine treatment combinations in the factorial cross (Table 4) allows us to examine our predictions from the Haig–Westoby model. Resource reduction reduced mean seed yield to 20–60% (depending on the associated pollen treatment) of the mean for control plants; pollen reduction produced a similar drop relative to the controls. These differences are qualitatively consistent with the Haig–Westoby model. Combining cells into linear contrasts with the control cell (Table 4) yields a nonsignificant difference between resource reduction plants and controls (contrast 1,  $P = 0.151$ ) but a highly significant difference for the pollen reduction plants versus controls (contrast 2,  $P = 5 \times 10^{-6}$ ). Thus, the statistical evidence

**Table 3** Analysis of variance testing for differences in the proportion of seed set per fruit produced by *Styloidium armeria* during 2006 following pollen and resource manipulations

Source of variation	SS	df	<i>F</i>	<i>P</i>
Pollen	2.366	2	57.81	< 0.0001
Resource	0.0050	2	0.141	0.869
Pollen × resource	0.100	4	1.160	0.333
Error	2.025	99		
Reduction vs natural				< 0.0001
Natural vs addition				0.708
Addition vs natural				< 0.0001

Values of *F* were estimated using type II sums of squares (SS). Results from Tukey's *post-hoc* contrasts between pollination levels are presented below the ANOVA.

partially supports and partially fails to support our expectations. Pollen addition with no resource manipulation produced a mere 3% rise in mean seed number compared with controls, and resource addition with no pollen manipulation produced a 20% rise. The linear contrast combining these outcomes was not significant (contrast 3,  $P = 0.301$ ). However, because our Haig–Westoby expectation is that this difference should be zero, an equivalence test is also needed to explore the contrast. The 95% confidence interval of mean seed number for all pollen addition only and resource addition only plants is (605, 1475) (estimating the error variance from the appropriate  $MS_{\text{Error}}$  calculated from Table 2 and back-transformed from the square-root). While this interval clearly includes the mean seed number for control plants, 925 (Table 4), we would have



**Fig. 2** Whole-plant examination of proportional reproductive outcomes of *Styloidium armeria* in 2005 and 2006. Proportion fruit set per plant for (a) 2005 and (b) 2006; proportion seed set for (c) 2005 and (d) 2006. Symbols indicate the mean ( $\pm 1$  SE). Pollen manipulations: pollen reduction (circles), natural pollination (triangles) and pollen addition (squares). Resource manipulations: reduction (60% of foliage stripped), natural (natural conditions) and addition (fertilizer added). Samples sizes: 2005 ( $n = 42$  plants); 2006 ( $n = 114$  plants).

to accept that seed output up to 1475 (i.e. 60% greater than the observed mean for controls) is biologically irrelevant in order to conclude that there is statistically significant evidence of equivalence. Thus, the result is ambiguous: there is neither significant evidence of difference nor of equivalence for contrast 3 (Table 4). Finally, the combination of both resource and pollen addition more than doubled mean seed output over control levels, which is a significant difference (contrast 4,  $P = 0.030$ ).

## Discussion

This experiment revealed a complex pattern of female reproductive success in *S. armeria*. For the most part, our results – in particular, the results for seed output in the second year – support or are not at odds with the Haig & Westoby (1988) interpretation of simultaneous pollen and resource limitation (Table 4).

*Styloidium armeria* at the Lake Mountain site was not pollen limited in seed set in either year, judging by the conventional standard that supplemental pollination failed to increase mean seed set significantly over natural levels. In this respect, *S. armeria* is in a minority among plant species (Burd, 1994; Larson & Barrett, 2000; Ashman *et al.*, 2004; but see Knight *et al.*, 2006). But at the same time, artificial reduction of

stigmatic pollen loads to about half the number of ovules sharply reduced both proportional seed set (to *c.* 4%, compared with an average of 25% under natural pollination in 2006; Fig. 2) and mean seed number per plant (Table 4). The stigmatic loads in the pollen reduction treatment were similar to 1 d of natural pollen delivery, so, extrapolating the proportional seed set linearly, flowers would need *c.* 6 d of open pollination to achieve natural levels of seed set. In fact, flowers were in their female phase for approx. 5 d. Thus, while *S. armeria* flowers may receive, on average, sufficient pollen for maximum seed set, they may not normally receive large excesses of pollen.

There was no statistically detectable resource effect on any of the six response variables in 2005, but clear effects the following year (Table 2). In particular, resource manipulation affected reproductive potential (number of flowers, and, therefore, ovules) in 2006 (Fig. 1), and probably also attractiveness to pollinators, since inflorescence size is a central feature of attractiveness (Vaughton & Ramsey, 1998; Huang *et al.*, 2006). An increase in flower number and attractiveness in the resource addition/natural pollination treatment may account for the modest 20% elevation (relative to the control) in mean seed number (Table 4). The added pollen/natural resource treatment is never expected to yield an increase in seed number, and the observed effect was quite small (Table 4).

**Table 4** The nine treatment combinations in the factorial experiment

		Resource treatment		
		Reduction	Natural	Addition
Pollen treatment	Reduction	188.7 ± 114.1 (1) (2)	244.9 ± 105.8 (2)	508.7 ± 260.8 (2)
	Natural	441.0 ± 90.4 (1)	925.4 ± 212.0 Control	1116.9 ± 248.2 (3)
	Addition	566.4 ± 176.8 (1)	952.1 ± 206.5 (3)	1940.1 ± 567.1 (4)

The upper entry in each cell gives the mean ± SE of total seed number per *Styloidium armeria* plant in 2006. The lower entry indicates which planned contrast(s) involve that cell. The expected directions of the contrasts and results of the significance tests are: (1) resource reduction lowers seed output relative to the control cell regardless of pollen treatment,  $F_{1,99} = 2.09$ ,  $P = 0.151$ ; (2) pollen reduction lowers seed output regardless of resource treatment,  $F_{1,99} = 23.31$ ,  $P = 0.000005$ ; (3) addition of either resources or pollen alone has no effect,  $F_{1,99} = 1.08$ ,  $P = 0.301$ ; (4) supplemental resources and pollen together increase seed output,  $F_{1,99} = 4.86$ ,  $P = 0.030$ .

Perhaps because we used different methods to alter resources, the effect on mean flower number was somewhat greater in the reduction (defoliation) treatment than in the NPK addition treatment (Fig. 1). Canto *et al.* (2004) observed a similar drop in flower number in *Anthurium schlectendalii* in the year following a reduction in leaf size. Our resource addition treatment implicitly assumed that N, P or K were limiting resources, and the reproductive response in the second year of treatment implies that at least one of these nutrients was limiting at our site. Defoliation, by contrast, should lower the maximum potential carbon gain, and may alter water relations in the plant or induce defense pathways. However, defoliation may also induce elevation of photosynthetic rates or growth rates, compensating for tissue losses in the short term, although we had no evidence of such compensation after 2 yr of treatment in this study. Reduction in resources can also influence the size of flowers, potentially influencing fitness through both male and female functions (Strauss *et al.*, 1996; Strauss, 1997). In any case, our resource treatments produced responses of roughly similar magnitude in the expected directions. Application of the treatments over 2 yr proved essential to the test. Even this 2-yr study highlights the potentially confounding effect of perenniality and storage organs in experimental tests of plant reproductive ecology.

On average, fewer than half of the ovules in *S. armeria* plants became seeds, even with supplemental pollen and resources (Fig. 2). This pattern is typical of pollen supplementation experiments on other species (Burd, 1994) and implies that individual flowers contain excess ovules. A packaging strategy with excess ovules may evolve to buffer seed output against loss of embryos from early acting lethal inbreeding depression (Porcher & Lande, 2005; Harder *et al.*, 2008), to allow scope for selective abortion based on offspring quality (Korbecka *et al.*, 2002), or to maximize seed number as a bet hedging strategy

in the face of inter-floral variation in fertilization success (Burd, 1995). These hypotheses are not mutually exclusive, and the present experiment cannot distinguish among them.

The significant increase in mean seed output in 2006 from the simultaneous addition of pollen and resources (contrast 4 in Table 4) occurred through a combination of higher fruit number and proportion seed set, even though neither of these individually was significantly greater than in control plants (Tables 2 and 3). The sharp increase in total seed number only with simultaneous release from two limiting factors is exactly in accord with the Haig–Westoby model. Previous studies have not found evidence for simultaneous pollen and resource limitation in *Aciphylla squarrosa* (Brookes & Jesson, 2007), *Cynoglossum officinale* (de Jong & Klinkhamer, 1989), *Geranium sylvaticum* (Asikainen & Mutikainen, 2005), *Hyacinthoides nonscripta* (Corbet, 1998), *Lesquerella gordonii* (Delph, 1986), *Maianthemum canadense* (McCall & Primack, 1987) and *Sarracenia purpurea* (Ne'eman *et al.*, 2006). However, pollen and resource limitation has been shown in *Polemonium viscosum* (at differing elevations: Galen, 1985) and in *Ipomopsis aggregata* (Campbell & Halama, 1993). In *Banksia spinulosa*, there was an additive effect of pollen and resource addition on seed set in one of the 2 yr studied, and added resources in that year also increased the following year's fruit production (Vaughton, 1991). Many other studies have found that reducing resources lowers female reproductive success (Gedge & Maun, 1992; Koptur *et al.*, 1996; Susko & Lovett-Doust, 1999; Ne'eman *et al.*, 2006), although lack of an effect may be influenced by perenniality and storage organs (e.g. *Aciphylla squarrosa*; Brookes & Jesson, 2007). In *Asclepias syriaca*, reduced pollinator access did not lower female reproductive success, contrary to expectations, and resource supplementation alone increased the number of fruits initiated (Caruso *et al.*, 2005). However, resource supplementation strongly influenced phenotypic

selection on increases in flower number and other floral traits (Caruso *et al.*, 2005), suggesting there might be a long-term equilibrium consistent with Haig–Westoby predictions.

Haig & Westoby (1988) anticipated that year-to-year stochastic deviations around the optimum in their model could make either pollen or resource availability the limiting factor for female reproduction in any given year. However, Burd (2008) has shown that introducing stochastic variation in pollination and resource availability into the Haig–Westoby model expands the range of conditions under which pollen limitation is expected, rather than inducing alternation of pollen and resource limitation. However, the true situation may be more complex. While both resources and pollen delivery can vary randomly, resource levels in one year may be more indicative of resource availability the next, whereas pollen receipt should be much less predictable. Furthermore, resource abundance in perennial plants can result in storage, growth, or elevated offspring production, whereas favourable pollen receipt can result only in extra offspring, and then only if a plant's resource status allows. Finally, the modular nature of plant reproduction (Wesselingh, 2007) may create a mosaic of pollen and resource effects within a single plant.

Our finding of simultaneous pollen and resource limitation in *S. armeria* after 2 yr of experimental manipulation suggests that both factors have an important effect on lifetime fitness. However, multiple-year studies of pollen and resource limitation are few, even though most experimental evaluations of female reproduction are conducted on perennials. There are practical constraints on the duration of experiments, of course, but we believe that our findings show that fully factorial treatments are best suited to exploring the potentially complex long-term limitations on seed set in flowering plants.

## Acknowledgements

We thank D. Anson, S. Wilson and R. Simpkin for assistance with fieldwork and seed counting. This manuscript was improved by the advice and comments of S. Sultan, R. Wesselingh and an anonymous reviewer. Lake Mountain Alpine Resort and Parks Victoria gave access to the site. We give special thanks to M. Logan for advice on the statistical analysis. This research was supported by a Monash Graduate Scholarship and the Joyce Vickery Scientific Research Fund from the Linnaean Society of New South Wales to R.H.B.

## References

- Ashman T, Knight TM, Steets JA, Amarasekare P, Burd M, Campbell DR, Dudash MR, Johnston MO, Mazer SJ, Mitchell RJ *et al.* 2004. Pollen limitation of plant reproduction: Ecological and evolutionary causes and consequences. *Ecology* 85: 2408–2421.
- Asikainen E, Mutikainen P. 2005. Pollen and resource limitation in a gynodioecious species. *American Journal of Botany* 92: 487–494.
- Brookes RH, Jesson LK. 2007. No evidence for simultaneous pollen and resource limitation in *Aciphylla squarrosa*: A long-lived, masting herb. *Austral Ecology* 32: 370–377.
- Burd M. 1994. Bateman's principle and plant reproduction: The role of pollen limitation in fruit and seed set. *Botanical Review* 60: 83–139.
- Burd M. 1995. Ovule packaging in stochastic pollination and fertilization environments. *Evolution* 49: 100–109.
- Burd M. 2008. The Haig–Westoby model revisited. *American Naturalist* 171: 400–404.
- Campbell DR, Halama KJ. 1993. Resource and pollen limitations to lifetime seed production in a natural plant population. *Ecology* 74: 1043–1051.
- Canto A, Parra-Tabla V, Garcia-Franco JG. 2004. Variations in leaf production and floral display of *Anthurium schlechtendalii* (Araceae) in response to herbivory and environment. *Functional Ecology* 18: 692–699.
- Caruso CM, Remington DLD, Ostergren KE. 2005. Variation in resource limitation of plant reproduction influences natural selection on floral traits of *Asclepias syriaca*. *Oecologia* 146: 68–76.
- Corbet SA. 1998. Fruit and seed production in relation to pollination and resources in bluebell, *Hyacinthoides non-scripta*. *Oecologia* 114: 349–360.
- Delph LF. 1986. Factors regulating fruit and seed production in the desert annual *Lesquerella gordonii*. *Oecologia* 69: 471–476.
- Galen C. 1985. Regulation of seed-set in *Polemonium viscosum*: Floral scents, pollination, and resources. *Ecology* 66: 792–797.
- Gedge KE, Maun MA. 1992. Effects of simulated herbivory on growth and reproduction of two beach annuals, *Cakile edentula* and *Corispermum byssopifolium*. *Canadian Journal of Botany* 70: 2467–2475.
- Haig D, Westoby M. 1988. On limits to seed production. *American Naturalist* 131: 757–759.
- Harder LD, Richards SA, Routley, MB. 2008. Effects of reproductive compensation, gamete discounting and reproductive assurance on mating-system diversity in hermaphrodites. *Evolution* 62: 157–172.
- Huang SQ, Tang LL, Sun JF, Lu Y. 2006. Pollinator response to female and male floral display in a monoecious species and its implications for the evolution of floral dimorphism. *New Phytologist* 171: 417–424.
- de Jong TJ, Klinkhamer PGL. 1989. Limiting factors for seed production in *Cynoglossum officinale*. *Oecologia* 80: 167–172.
- Knight TM, Steets JA, Ashman TL. 2006. A quantitative synthesis of pollen supplementation experiments highlights the contribution of resource reallocation to estimates of pollen limitation. *American Journal of Botany* 93: 271–277.
- Koptur S, Smith CL, Lawton JH. 1996. Effects of artificial defoliation on reproductive allocation in the common vetch, *Vicia sativa* (Fabaceae: Papilionoideae). *American Journal of Botany* 83: 886–889.
- Korbecka G, Klinkhamer PGL, Vrieling K. 2002. Selective embryo abortion hypothesis revisited – a molecular approach. *Plant Biology* 4: 298–310.
- Larson BMH, Barrett SCH. 2000. A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnaean Society* 69: 503–520.
- McCall C, Primack RB. 1987. Resources limit the fecundity of three woodland herbs. *Oecologia* 71: 431–435.
- Ne'eman G, Ne'eman R, Ellison AM. 2006. Limits to reproductive success of *Sarracenia purpurea* (Sarraceniaceae). *American Journal of Botany* 93: 1660–1666.
- Porcher E, Lande R. 2005. Reproductive compensation in the evolution of plant mating systems. *New Phytologist* 166: 673–684.
- Quinn G, Keough M. 2002. *Experimental design and data analysis for biologists*. Cambridge, UK: Cambridge University Press.
- R Development Core Team. 2007. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Raulings EJ, Ladiges PY. 2001. Morphological variation and speciation in *Stylidium graminifolium* (Stylidiaceae), description of *S. montanum* and reinstatement of *S. armeria*. *Australian Systematic Botany* 14: 901–935.

- Strauss SY.** 1997. Floral characters link herbivores, pollinators, and plant fitness. *Ecology* 78: 1640–1645.
- Strauss SY, Conner JK, Rush SL.** 1996. Foliar herbivory affects floral characters and plant attractiveness to pollinators: implications for male and female plant fitness. *American Naturalist* 147: 1098–1107.
- Susko DJ, Lovett-Doust L.** 1999. Effects of resource availability, and fruit and ovule position on components of fecundity in *Alliaria petiolata* (Brassicaceae). *New Phytologist* 144: 295–306.
- Vaughton G.** 1991. Variation between years in pollen and nutrient limitation of fruit-set in *Banksia spinulosa*. *Journal of Ecology* 79: 389–400.
- Vaughton G, Ramsey M.** 1998. Floral display, pollinator visitation and reproductive success in the dioecious perennial herb *Wurmbea dioica* (Liliaceae). *Oecologia* 115: 93–101.
- Wesselingh RA.** 2007. Pollen limitation meets resource allocation: Towards a comprehensive methodology. *New Phytologist* 174: 26–37.
- Willis AJ, Ash J.** 1990. The breeding systems of *Stylidium graminifolium* and *S. productum* (Stylidiaceae). *Australian Journal of Botany* 38: 217–227.