Methods

A piece of the puzzle: a method for comparing pollination quality and quantity across multiple species and reproductive events

Conchita Alonso[1], **Carlos M. Herrera**[1] **and Tia-Lynn Ashman**[2]

[1]Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Américo Vespucio s/n, E-41092, Sevilla, Spain; [2]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA

Author for correspondence:
Conchita Alonso
Tel: +34 954466700
Email: conalo@ebd.csic.es

Received: *2 August 2011*
Accepted: *8 September 2011*

New Phytologist (2012) **193**: 532–542
doi: 10.1111/j.1469-8137.2011.03932.x

Key words: piecewise regression, plant communities, pollen limitation, pollen tubes, pollination, stigmatic pollen load.

Summary

- Understanding how pollination affects plant reproductive success and how changes in pollination service affect plant populations, communities and ecosystems is of increasing concern. Yet supplemental hand-pollination traditionally used to assess pollen limitation is prohibitive for large-scale comparative work. Moreover, it does not differentiate between quality and quantity aspects of pollen limitation, and it may suffer from confounds of post-pollination processes such as resource availability to fill seeds.
- Here, we highlight pollen tubes as the functional link between pollen arrival and seed production and suggest that consideration of pollen tubes leads to a better depiction of limitation at the pre-zygotic (pollination) phase of sexual reproduction.
- We assessed the rigor of piecewise regression to analyze the relationship between the numbers of pollen grains and pollen tubes observed in nonmanipulated wilted flowers. We illustrate how parameters obtained from this analysis provide quantitative insight into the relative relevance of the quantity and quality of pollen receipt in limiting natural pollination success, and can facilitate comparisons among data sets.
- This nonmanipulative method opens up new opportunities for rigorous assessment of the relative importance of the quantity and quality of pollination in limiting plant reproduction, especially from a community-wide perspective.

Introduction

Recently there have been many calls to approach the question of pollen sufficiency of plant reproductive success from a community perspective (e.g. Ashman *et al.*, 2004; Hegland & Totland, 2008; García-Camacho & Totland, 2009; Dauber *et al.*, 2010), and an equal number emphasizing the contributions of both pollen quantity and quality to pollen limitation (PL) of seed production (e.g. Ashman *et al.*, 2004; Aizen & Harder, 2007; Alonso *et al.*, 2010). However, analyzing the severity of PL and the relative roles of components of PL by applying the traditional experimental hand-pollination approach to multiple species may be complicated for several reasons. First, manipulation of pollen loads via hand-pollination on a wide range of species requires knowledge of the floral biology (e.g. timing of pollen dehiscence and stigma receptivity, and pollen viability), as well as the effects of emasculation on each (see the third paragraph of the Introduction section). Secondly, such community studies must include even 'difficult to pollinate flowers' (e.g. very small, short-lived flowers and/or those with complex or 'closed' floral morphology). Therefore, achieving equivalent application of hand-pollination treatments across numerous varied species of a community may be impossible. As a consequence, species-specific results may ultimately be a function of the interaction between ease of manipulation and the species itself.

In addition to the practical issues addressed above, the standard supplemental hand-pollination approach requires many considerations to reliably assess the PL of seed production (see discussions in Young & Young, 1992; Ashman *et al.*, 2004; Wesselingh, 2007), of which we will highlight two. First, it can suffer from the confounding effects of resource limitation and/or reallocation of resources among flowers to fill seeds after heavy hand-pollinations (Knight *et al.*, 2006; Wesselingh, 2007). Secondly, it does not distinguish between the quantity and quality components of PL, which is required to understand the repercussions of PL, especially when comparing plants within communities (Aizen & Harder, 2007; Alonso *et al.*, 2010). The quantity component is directly related to factors such as pollen availability, pollinator visitation rates and the number of ovules per carpel (Burd *et al.*, 2009). The quality component involves differences in pollen viability, germination, and tube development which affect fertilization potential. These components of quality can have a genetic or environmental basis (e.g. Young & Stanton, 1990; Walsh & Charlesworth, 1992; Dafni & Firmage, 2000; Edlund *et al.*, 2004; Mazer *et al.*, 2010; Lankinen & Madjidian, 2011 and references therein). Quality-related differences at the pollen–stigma interface or among tubes within the style are not only expected between compatible and incompatible donors in self-incompatible species (see Busch *et al.*, 2010 and references therein), or between self and cross pollen in species with mixed mating systems (e.g. Lankinen & Armbruster, 2007; Mazer *et al.*, 2010; Lankinen & Madjidian, 2011), but may also arise among pollen grains from different individuals (e.g. Marshall *et al.*, 2007; Lankinen & Madjidian, 2011), or even among pollen grains from a single donor (Lankinen *et al.*, 2009).

One proposal to overcome some of these limitations has been to assess the relative successes of open- and outcross-pollinated flowers (i.e. not supplemental pollination), combining the quantification of the relationship between natural pollen receipt and seed output with data on seed set after hand-pollination with outcross pollen only (Aizen & Harder, 2007). While this approach may better distinguish the quality and quantity components of PL, it does not alleviate the difficulties associated with floral manipulation (emasculation, effective collection and application of pollen to stigmas) of many species in community studies. It also requires enumeration of seeds, and thus still confounds post-zygotic effects of quality with pre-zygotic ones, as well as potential resource reallocation among flowers during seed filling. Another proposal for assessing the quantity and quality of PL is to record seed production of emasculated and control open flowers (Vaughton & Ramsey, 2010). However, this can be misleading if emasculation directly affects pollinator behavior, particularly in plants where pollen is the main reward to pollinators, and/or indirectly impairs pollen tube growth and ovule maturation, and thus fruit set (see Hedhly *et al.*, 2009). Here, we propose an alternate nonmanipulative model-fitting approach for assessing the relative importance of the quantity and quality of pollen receipt in determining the natural pollination success of the pre-zygotic stage.

Our proposal involves analysis of pollen grain–pollen tube dose–response curves of naturally open-pollinated flowers. Thus, it does not require any floral manipulation or subsequent monitoring of seed production. As a result, it overcomes the limitations mentioned above in attempting (but not necessarily achieving) equivalent application of experimental manipulations across diverse sets of flowering species, and the confounding effects of resource reallocation during seed maturation. Further, it also avoids the inherent risk of losing experimentally pollinated flowers after pollinations as a result of the hazards experienced by marked plants in open wild habitats; for example, herbivory (Cahill *et al.*, 2001).

The operational definition of pollen limitation tested through customary experimental pollinations referred to above analyzes plant reproduction as an input (pollen arrival)–output (seed production) process (Ashman *et al.*, 2004). Pollen tubes represent the underappreciated intermediate phase between pollen arrival and seed production that may better characterize the pollination phase of this two-stage process, without the confounding effects of the second phase (filling seeds after ovule fertilization) in which pollination success interacts with resource availability (Ashman *et al.*, 2004; Wesselingh, 2007). The importance of pollen load size for pollen tube numbers has been extensively studied and pollen tubes have been recognized as a better proxy to estimate the reproductive success of flowers (Herrera, 2002 and references therein; Bernasconi *et al.*, 2007). But the shape of the relationship between the number of conspecific pollen grains on the stigma and the number of pollen tubes in the style remains poorly understood in naturally pollinated plants (Aizen & Harder, 2007).

Our model proposes that a change in the relative importance of two factors – the quantity and quality of pollen receipt – underlies the shape of natural pollen grain–pollen tube dose–response curves, and that this can be deduced by use of breakpoint/piecewise regression analysis. We applied our method to two different study cases to demonstrate that piecewise regression is a robust model-fitting approach, which is at least as good as the global nonlinear functions previously applied to similar dose–response curves (e.g. Mitchell, 1997; Alonso, 2005; Aizen & Harder, 2007). The examples also illustrate how the parameters derived from this analysis can facilitate comparisons among morphs, populations, and/or species to discern whether and how they differ in the relative importance of pollen quantity or quality aspects of pollination success. Although not explored in detail here, we note that this approach can also be used to discern the effects of contextual features on pollen quality and quantity. For example, one can combine this analytical approach with experimental manipulations, such as differential pollinator exclosures, to assess the effects of certain floral visitors on pollen quality and quantity. Finally, we discuss some limitations in the application of the model and suggest further ways to relate the quantity and quality components of pollination success to the PL of plant reproduction and recruitment. The distinction between the quality of the pollen vectors and the overall quantity of floral visitors has been acknowledged (e.g. Herrera, 1987, 1988, 1989). However, the links between such quality and quantity components of pollination and plant reproduction are still understudied (but see e.g. Herrera, 2000; Gómez *et al.*, 2007), and we suggest that this method can help to bridge this gap.

Description

Concept

Our conceptual model relies on the simple fact that as pollen receipt increases pollination success is also expected to increase. Initially this increase is closely tied to the alleviation of quantity limitation of pollination, and as pollen receipt increases the quantity effect (Qt, hereafter) is expected to decline. At the same time, however, the importance of pollen quality, that is, the quality effect (Ql), is expected to increase. The importance of quality increases as the number of pollen tubes increases to its maximum – beyond which the number of tubes does not further increase (and may even decrease, because of crowding; Young & Young, 1992)

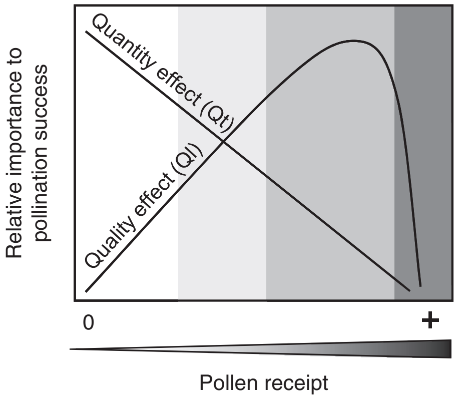

Fig. 1 Conceptual model of the relative effects of pollen quality and quantity on pollination success with increasing pollen receipt. As pollen receipt increases, the effect of quantity limitation on pollination success (Qt) decreases monotonically, whereas the effect of quality limitation (Ql) increases to a maximum and then declines. Quantity effects are expected to predominate at low pollen receipt (white section), whereas quality limitation dominates at higher pollen receipt (dark gray section). The region surrounding the intersection of effects (light gray section) reflects the transition in dominance of the two processes and here both processes are contributing similarly to the limitation of pollination success. At the highest levels of pollen receipt (darkest gray section) neither quantity nor quality is limiting pollination success.

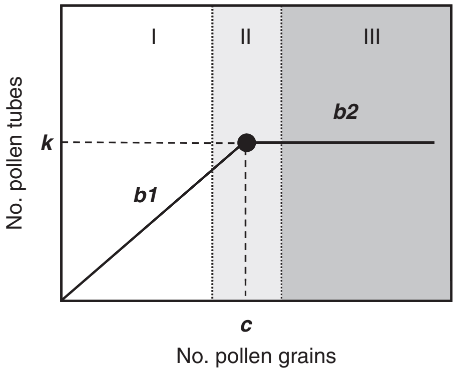

Fig. 2 Depiction of how piecewise regression of the pollen grains to tubes relationship can be used to assess the relative importance of processes underlying pollination success. The relationship between pollen tubes and grains is defined by two linear regressions with different slopes (*b*1 and *b*2) and the 'breakpoint' (*c*,*k*) where the two lines intersect. The confidence limits (dotted vertical lines) around *c* delineate three regions differing in the relative importance of Qt and Ql components (I, Qt > Ql; II, Qt ~ Ql; III, Qt < Ql).

in response to increased stigmatic pollen loads. It is here that the possibilities for selection for the highest quality pollen phenotypes are expected to be strongest as a result of competition among tubes (e.g. Mitchell, 1997; Kalla & Ashman, 2002; Lankinen & Armbruster, 2007). At the highest levels of pollen receipt both quantity and quality effects diminish, as neither limits pollination success.

We expect a shift in the relative importance of the two processes as pollen receipt increases (Fig. 1). First, at low stigmatic pollen loads (Fig. 1; white section) Qt exceeds Ql. At higher pollen loads, quantities have reached the point at which the Qt on pollination success is diminished in relevance relative to that of Ql. Here, a 'transitional phase' of similar relevance of the two effects is expected to occur (Fig. 1; light gray section). At even higher levels of pollen receipt, the difference in the relative relevance of the two factors is further enhanced, and Ql far exceeds Qt in its influence on pollination success (Fig. 1; dark gray region). Barring other detrimental effects of very high pollen loads (Young & Young, 1992), at the highest levels of pollen receipt neither the quality nor the quantity of pollen limits pollination/fertilization success, and both Ql and Qt will thus approach zero (Fig. 1; darkest gray region), although we note that few flowers may achieve this very highest level of pollen receipt. In the next section, we propose a statistical procedure based on the application of piecewise regression to identify objectively these regions and thus characterize the mechanistic features that underlie the pollen grains–pollen tubes relationship.

Piecewise regression approach

Piecewise regression is a technique that allows the inclusion of break points in linear regression when an abrupt change in the slope of the relationship is predicted and allows for the simultaneous evaluation of the location and the standard error of such a break point (Ryan & Porth, 2007; Ficetola & Denoël, 2009). This method has been used to describe abrupt changes in community composition at habitat edges (Toms & Lesperance, 2003; Ficetola & Denoël, 2009) and also when the objective is to determine the onset of different phases underlying a particular process (Ryan & Porth, 2007). The relationship between the dependent and independent variables can thus be interpreted as two linear regressions with different slopes (*b*1 and *b*2) and the point at which these two slopes meet is the 'breakpoint' (*c*), that is, the point at which differences in the relationship occur (Fig. 2). Bootstrapping techniques can be used to obtain approximate confidence intervals (referred as BCa from hereafter) for all three parameter estimates (*b*1, *b*2 and *c*) in the piecewise regression. Thus, the assumptions of normality and homogeneous variance of data required for proper parametric assessment are relaxed and BCa intervals are better bias-corrected, and are accurate in a wide variety of settings (Toms & Lesperance, 2003; Ryan & Porth, 2007).

We propose that a piecewise analysis of the functional relationship of pollen grains to tubes can provide information about the relative importance of pollen quantity and quality in determining pollination success (Fig. 2). Specifically, the initial slope (*b*1) provides information about the quality of the pollen received: a high *b*1 (steep rise) reflects receipt of high-quality pollen, and a low *b*1 (shallow rise) reflects the receipt of low-quality pollen. The value of *c* reflects the point at which the initial slope (*b*1) ceases to fit the data and the second regression (*b*2) becomes more appropriate. The region defined by the confidence limits around *c* denotes the range of pollen receipt levels over which Ql surpasses Qt in influence on pollination success. The width of the confidence interval around *c* reflects both the certainty of its location and the range of pollen load sizes for which Ql and Qt are contributing with similar importance to pollination success. The slope *b*2 reflects the degree to which, even after the quantity effect is diminished (tends toward 0), pollen quality still plays a role

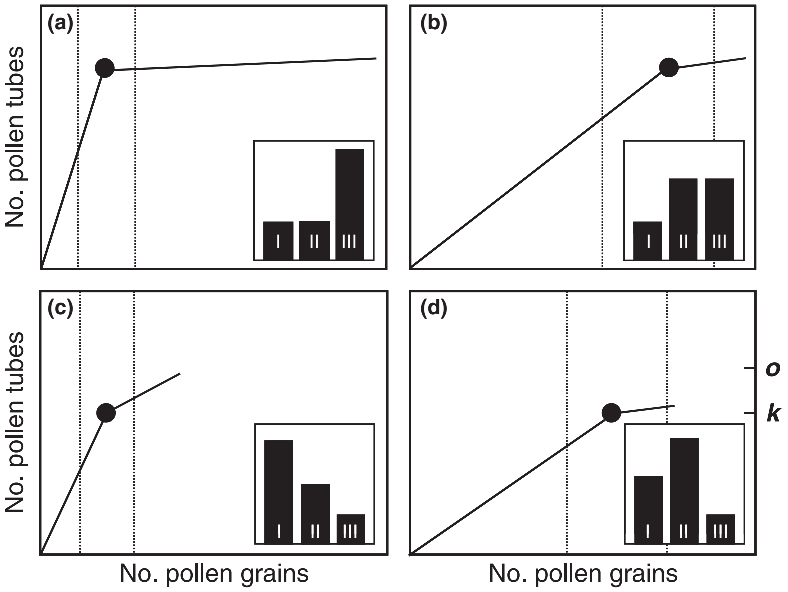

Fig. 3 Illustration of four hypothetical populations of plants that differ in the quantity and quality of pollen received and their predicted effect on pollen tubes following piecewise regression. Bar graphs in the inserts depict the relative frequency of data points falling into each of the regions I–III. Comparisons between left and right panels (a vs b; c vs d) illustrate differences between populations in the magnitude of quality effects (high vs low). Comparisons between top and bottom panels (a vs c; b vs d) illustrate differences in the magnitude of quantity effects (high vs low). Comparisons between panels on the diagonal (a vs d; c vs b) illustrate the expectations when the magnitudes of both components differ between data sets. In panel (d) the number of pollen tubes at the breakpoint (*k*) is expected to be significantly lower than the number of ovules per flower (*o*). See text for more detail.

(positive values), or that additional pollen has a detrimental effect on pollen tube recruitment (negative values). While we acknowledge that the relative strength of Qt and Ql represents a continuum, the frequency of actual data points in the three regions (Fig. 2; I, II and III) demarcated by the lower and upper BCa around *c* reflects the frequency with which the pollination success of a given sample is limited primarily by quantity (I), quality (III) or both (II). A chi-square test can be used to test for differences among samples in the relative frequencies of data points in the three regions, and be interpreted as differences in the relative relevance of the two underlying processes to pollination success of a given population, species or time period. The example in Fig. 2 reflects an idealized case where both Qt and Ql are equally important to pollination success and thus the three regions are expected to contain similar numbers of data points. However, the approach is most informative in a comparative context, such as the four hypothetical populations depicted in Fig. 3. In population (a), flowers receive a high quantity of high-quality pollen and their pollen grains–pollen tubes relationship is characterized by high *b*1 and low *c*, and most of the sampled flowers fall into region III (Fig. 3a). In population (b), plants receive a high quantity of low-quality pollen, so their pollen grains–tubes relationship is characterized by low *b*1 and high *c*, and few data points are found in region I compared with region II or III (Fig. 3b). Pollination success in population (c) is limited by pollen quantity even though pollen quality is high. Here, the pollen grains–tubes relationship is characterized by high *b*1 and low *c* but, in contrast to population (a), most flower samples occupy region I (Fig. 3c). Lastly, in population (d) flowers receive low quantities of low-quality pollen, and their pollen grains–tubes relationship is characterized by low *b*1 and high *c*, and very few data points occur in region III compared with region I or II (Fig. 3d). Moreover, the regression-predicted number of pollen tubes at the breakpoint (*k*) will be further indication of pollination limitation of seed production whenever the value of *k* is significantly lower than the average number of ovules per flower (*o*) (shown in Fig. 3d).

Regions I to III would roughly correspond to the three regions proposed by Mitchell (1997) with respect to offspring quality (e.g. 'filling', 'sorting' and 'surplus'). Thus, the quality and quantity components of pollination success and of PL of seed production are linked. It will be highly desirable to make this connection when monitoring of seed production is feasible. For instance, flowers sampled from region III are expected to have a higher fertilization success (i.e. proportion of seeds per ovule). In addition, the stronger pollen competition in region III will lead to higher quality offspring than those produced by flowers sampled from region I (see Kalla & Ashman, 2002).

Intraspecific comparisons will not require any standardization of data and comparisons among populations or years can be performed using raw data. However, interspecific comparisons should account for differences among species in the number of ovules per flower, as this determines the minimum number of pollen tubes required for full fertilization (and thus the location of *c*). Estimates of pollen tubes per ovule can be easily obtained when species exhibit a fixed number of ovules per flower by dividing the number of pollen tubes by the number of ovules characteristic of each species. However, it is necessary to take additional steps when comparisons involve highly variable species or species that differ by orders of magnitude (Burd *et al.*, 2009). Specifically, when intraspecific variation is rather limited, the average number of ovules per flower can be estimated for each species from an independent sample of flowers. Otherwise, in species with large variation in the number of ovules per flower, both ovule number and pollen tubes should be recorded for the same individual flowers or a tractable covariate of ovule number/flower should be recorded for each flower.

Practical issues

Estimation of pollination success based on the analysis of the relationship between pollen grains and tubes in styles of open-pollinated flowers is technically less challenging than hand-pollinations, although we must be aware of several issues. First, because the speed of germination and pollen tube growth may vary among species (Mazer *et al.*, 2010), styles must be collected from randomly selected wilted flowers to ensure full development of pollen tubes. Secondly, while pollen tubes are always well preserved within the style, pollen grains may be hidden or become dislodged from the stigma and thus the number of pollen grains deposited on the stigma may be underestimated (cf. Kearns & Inouye, 1993). Enumeration of pollen tubes can also become less precise at very high numbers of tubes. The technical problem of pollen underestimation can be partially corrected during the analysis by constraining the number of grains to be equal to or greater than the number of tubes. Deletion of single data points for which pollen grains appeared grossly underestimated (i.e. pollen tubes >> pollen grains) must be considered. Once corrected, a first quantity component of pollen limitation can be estimated as the frequency of flowers without pollen grains. In animal-pollinated species (not capable of autonomous autogamy) this would be equivalent to the proportion of unvisited flowers in the sample (Alonso, 2005). Lastly, because the aim is to describe the relationship between pollen grains and tubes of *visited* flowers, both the unvisited flowers (no pollen grains) and the intercept must be removed from the analyses. This is because the relative frequency of zeros can alter the goodness-of-fit of different models in nonequivalent ways.

Comparing piecewise regression to other statistical methods modeling the pollen grains–tubes relationship

To assess the rigor and comparative value of the piecewise approach, we compared the fit of this model to that of a linear regression model, as well as those of other nonlinear models commonly used to evaluate dose–response relationships. The linearity of the relationship between pollen grains and tubes of visited flowers can be assessed with generalized additive models (GAMs). These models explore data nonparametrically, with the assumption of linearity being relaxed, and the model being fitted locally, thus enabling detection of structure in the relationship between the independent and dependent variables (Hastie & Tibshirani, 1990). A cubic smoothing spline with Poisson error distribution and log link function was selected for this purpose. A significant spline component indicates a better fit when the model includes a nonlinear local component than when it only includes a global linear component.

A nonlinear saturating negative exponential equation ($\text{tubes} = k0(1 - \exp(-s1 \times \text{grains}))$) is often used to model a dose–response relationship, and, when applied to the number of pollen tubes in the style and the number of pollen grains on the stigma, it has been used to compare the quality of pollination (Alonso, 2005; Richards *et al.*, 2009). Similar exponential models have been used to model the relationship between seed set and pollen loads after experimental hand-pollinations (e.g. Mitchell, 1997; Cane & Schiffhauer, 2003) and in species whose styles can be detached without damaging the growing fruit (Aizen & Harder, 2007).

To compare the fits of linear, exponential and piecewise regression models we calculated the Akaike information criterion, $\text{AIC} = 2k + n\ [\log_e(\text{RSS}/n)]$, where k indicates the number of parameters estimated by the model, n indicates sample size and RSS is the residual sum of squares of the model. The AIC quantifies model parsimony, while accounting for the trade-off between the ability of a model to fit data and its degree of complexity. Differences between models that are < 2 units are considered nonsignificant and models differing in < 10 units are considered to be nonsubstantially different (Burnham & Anderson, 2004).

All analyses were conducted with the SAS package (SAS Institute, 2008) and the SAS code for a step-by-step protocol is provided as Supporting Information Notes S1. GAMs were fitted using the GAM procedure and both piecewise and negative exponential models using the NLIN procedure. A broad grid search of initial parameters and the iterative Marquardt method for model optimization were used because poor starting parameters can result in nonconvergence. For the piecewise analysis, initial parameters were obtained by graphically exploring the data, searching for a suitable range for breakpoint location, estimating a simple linear model separately above and below a given value of breakpoint, and using the slopes of the two regressions as the initial values for the grid search (Ryan & Porth, 2007).

Robustness of piecewise approach to outliers and sample size

As in any regression model, to assess the robustness of the method we must evaluate the effects of potential outliers and leverage points on model fit and parameter estimation. Graphic and diagnostic tools can be used to detect putative outliers (Zuur *et al.*, 2010). In our use of piecewise regression we must be cautious with study cases where most of data points fall in the first phase of the process (i.e. region I in Fig. 2), because the outlying data points may reflect the limited sampling of the second phase (i.e. region III) and thus must not be removed (Ryan & Porth, 2007).

To establish guidelines for sample sizes necessary for application of the piecewise approach, we determined how sample size affects model fitting. We analyzed data sets of various sizes (n = 85, 100, 150 and 200). We created these data sets from each of the raw data sets (described in the following section) by random subsampling of data points. In order to ensure that each subsample contained data from the entire range, we grouped the original data into three bins each comprising one-third of the original data based on pollen grains. A data set was generated by randomly sampling without replacement the same number of points from each of the three bins and a piecewise regression model was run, with the grid search of initial parameters conducted between the BCa limits estimated from the complete data set. We repeated the process until 10 subsampled data sets per sample size converged and for each fitted model we recorded whether the piecewise parameter estimates were within the confidence intervals of the original data set. Furthermore, in each case we also fit the negative exponential model and used ΔAIC to compare the two models. The number of data sets that failed to converge was also recorded to detect differences among the study data sets in likelihood of convergence.

Data used for testing the model

Two study cases for which we had large comparable data sets were used to assess the rigor and utility of piecewise regression in modeling the relationship between pollen grains and tubes and to compare the resultant estimates of Qt and Ql between species or morphs.

The first study case consisted of two species of mint (Lamiaceae), *Lavandula latifolia* Medicus and *Rosmarinus officinalis* L., which are both self-compatible and have four ovules per flower. Previous pollination studies indicate that these two species differ in pollination success because *L. latifolia* has, on average, twice as many pollen tubes per style as other species in its family, including *R. officinalis*, and a higher proportion of styles with more pollen tubes than ovules (Herrera, 2004). In 1996 and 2002, respectively, newly withered corollas that had fallen to the ground beneath plants in *R. officinalis*, and dry corollas remaining attached to the calyx after anthesis in *L. latifolia*, were collected on single dates shortly after flowering peak at a single Spanish population from 20 different plants (10–21 corollas per plant) chosen as widely spaced as possible (see Herrera, 2004 for further details). Total sample sizes were 355 and 198 flowers for *L. latifolia* and *R. officinalis*, respectively. There were 111 and 24 cases, respectively, in which the recorded number of pollen grains was less than the number of pollen tubes and these were made equal to the number of tubes before further analyses.

The second study case included data on pollen grain and tube numbers for female and hermaphrodite individuals of the self-compatible, gynodioecious shrub *Daphne laureola* L. (Thymelaeaceae). The data came from ten populations along an altitudinal gradient in the south-east of Spain where pollination success is consistently higher for hermaphrodites than for females. At the end of the 2001 flowering period, 10–12 wilting flowers were collected from five to 12 female and hermaphrodite plants per site (see Alonso, 2005 for further details). Total sample sizes were 640 and 629 flowers for females and hermaphrodites, respectively. There were 82 and 13 cases, respectively, in which the recorded number of pollen grains was less than the number of pollen tubes and these were made equal to the number of tubes before further analyses. These data correspond to one of the study years analyzed in Alonso (2005).

Flowers were stored in vials filled with 2.5 : 2.5 : 95% formaldehyde : acetic acid : ethyl alcohol solution (FAA). We used epifluorescence to visualize pollen tubes (see Kearns & Inouye, 1993 for details). Styles were softened in 1 N KOH at 65°C for 20 min, rinsed with distilled water, and stained for 20 min at 65°C in decolorized aniline blue. Pollen grains and tubes were counted under a microscope without destroying the tissues. Pollen tubes were counted at the base of the style.

Results

Lamiaceae species

Mean pollen loads on the stigma were larger in *L. latifolia* (mean ± SE 30.0 ± 1.6 vs 8.7 ± 0.7 pollen grains), and the frequency of unvisited flowers was higher in *R. officinalis* (10.6 vs 4.2%), which suggests that floral visitation rates were higher in *L. latifolia* than in *R. officinalis* (see also Herrera, 2004).

Absence of continuous linearity in the relationship between pollen grains and tubes of visited flowers was confirmed in both species by the analysis of deviance (spline component: $\chi^2_{13.4} = 433.90$, $P < 0.0001$ and $\chi^2_{5.8} = 163.7$, $P < 0.0001$ for *L. latifolia* and *R. officinalis*, respectively). Both negative exponential and piecewise models provided a much better fit than the linear regression (Table 1). Differences between piecewise and negative exponential models were not as large, but the piecewise model had a lower AIC than the negative exponential model in *L. latifolia*, suggesting a better fit, although the opposite was true for *R. officinalis* (Table 1).

The relative frequency of samples falling in the three regions defined by the BCa intervals around the breakpoint differed widely between the two species ($\chi^2_2 = 112.66$, $P < 0.0001$, Fig. 4). Qualitative limitation was more common in *L. latifolia*, with 49.7% of flowers sampled falling above the upper BCa limit and 37.6% of samples below the lower BCa limit. By contrast, quantitative limitation was more frequent in *R. officinalis*, with 53.1% of samples falling below the lower BCa limit and only 7.3% of samples above the upper BCa limit. Also, the frequency of samples in which both quantitative and qualitative limitations combined was lower in *L. latifolia* (12.6%) than in *R. officinalis* (39.5%).

Table 1 Model-fitting comparison in *Lavandula latifolia* (n = 340 visited flowers) and *Rosmarinus officinalis* (n = 177 visited flowers)

Species	Model	Residual SS	AIC	First parameter[1]	Breakpoint (BCa)[2]	Second parameter[3]
L. latifolia	Linear	25 842.0	1476.48	0.26		
	Negative exponential	7610.6	1060.89	0.082		17.00
	Piecewise	7434.5	1054.87	0.87	17.43 (15.58–20.44)	0.025 ns
R. officinalis	Linear	2097.2	441.58	0.41		
	Negative exponential	1003.4	311.10	0.076		12.27
	Piecewise	1079.2	325.98	0.68	13.09 (7.66–21.62)	0.082 ns

[1] The first parameter estimates the slope of the relationship in the linear model, and the slope of the relationship in the initial portion of data in the two nonlinear models.

[2] The breakpoint is the number of pollen grains at which the slope of the relationship changes. Ninety-five per cent approximate bootstrapping confidence intervals (BCa) were estimated with n = 1000 bootstraps.

[3] The second parameter is the asymptotic value in the negative exponential model and the slope of the second regression in the piecewise model.

ns, not significant.

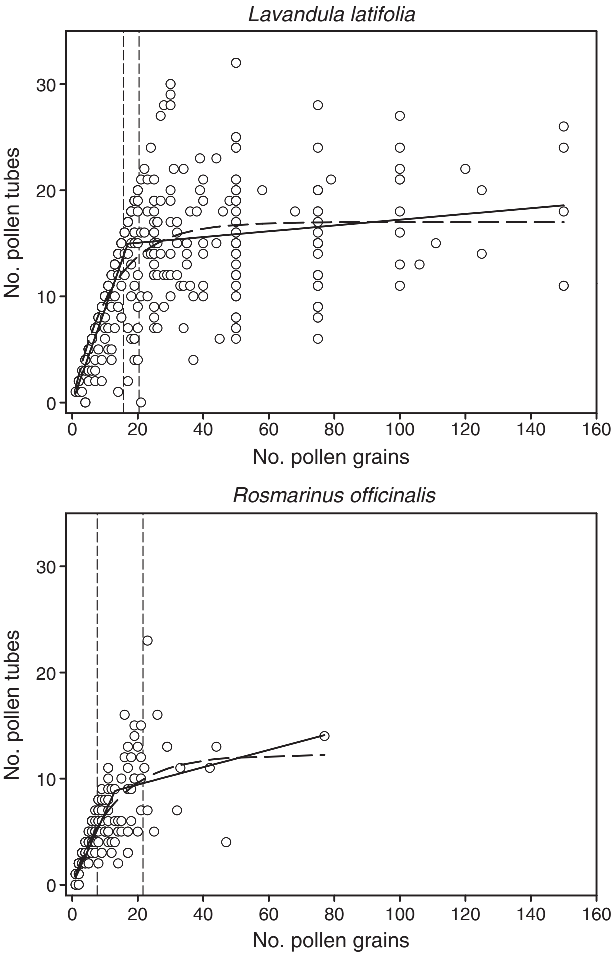

Fig. 4 Piecewise regression (solid line) and nonlinear saturating negative exponential model (dashed line) of the relationship between the numbers of pollen grains and pollen tubes for *Lavandula latifolia* and *Rosmarinus officinalis*. Each symbol corresponds to an individual flower. Vertical dashed lines denote the 95% approximate bootstrapping confidence intervals (BCa) of the breakpoint estimated by piecewise regression with 1000 bootstraps.

The slope of the relationship to the left of the breakpoint (*b*1) was significantly higher in *L. latifolia*, suggesting a better quality of the pollen received since the style saturated with tubes faster than in *R. officinalis* (Table 1). Interestingly, the slope of the relationship to the right of the breakpoint (*b*2) did not statistically differ from 0 in either species, suggesting that the number of pollen tubes was fully saturated in both cases (Table 1). The number of tubes corresponding to the breakpoint (*k*) was 15 in *L. latifolia* and 8.8 in *R. officinalis*, almost four and two times higher than the number of ovules per flower, respectively.

Daphne laureola sex morphs

Mean pollen loads were much larger in hermaphrodites (84.5 ± 4.2 pollen grains) than in females (4.6 ± 0.5 pollen grains) and unvisited flowers were much less frequent in hermaphrodites (6.5%) than in females (60.6%).

The absence of continuous linearity in the relationship between pollen grains and tubes of visited flowers was confirmed for both sex morphs (spline component: $\chi^2_{6.1} = 965.58$, $P < 0.0001$ and $\chi^2_{27.1} = 5853.62$, $P < 0.0001$ for females and hermaphrodites, respectively). Both negative exponential and piecewise models provided a much better fit than the linear regression (Table 2). There were almost no differences between piecewise and negative exponential models in the female data set. The negative exponential model had a lower AIC than the piecewise regression in the hermaphrodite data, and thus a better fit, but nonsubstantially so (Table 2).

The relative frequency of samples falling in the three regions defined by the BCa intervals around the breakpoint differed widely between sexes ($\chi^2_2 = 60.70$, $P < 0.0001$, Fig. 5). Quantitative limitation was frequent in both, with 80.6 and 62.1% of female and hermaphrodite samples, respectively, falling below the lower BCa. However, 29.8% of hermaphrodite samples were above the higher BCa, in contrast to only 5.6% of female samples. Also, the frequency of samples with combined quantitative and qualitative limitations was somewhat lower in hermaphrodites (8.2%) than in females (13.9%).

The slope of the relationship to the left of the breakpoint (*b*1) was twice as high for females as for hermaphrodites, indicating either a higher quality of pollen received by females or a stronger discrimination capacity at female stigmas and styles (Table 2). Further, the slope of the relationship to the right of the breakpoint (*b*2) did not statistically differ from 0 in either sex. This suggests that the styles were fully saturated with pollen tubes,

Table 2 Model-fitting comparison in hermaphrodite (*n* = 588 visited flowers) and female plants (*n* = 252 visited flowers) of *Daphne laureola*

Sex	Model	Residual SS	AIC	First parameter[1]	Breakpoint (BCa)[2]	Second parameter[3]
Hermaphrodite	Linear	17 8251.0	3363.95	0.16		
	Negative exponential	10 2006.0	3035.85	0.01		43.64
	Piecewise	10 3152.0	3044.24	0.33	104.3 (87.76–118.96)	0.017 ns
Female	Linear	7699.72	865.75	0.52		
	Negative exponential	6126.60	808.06	0.01		58.23
	Piecewise	6071.40	807.81	0.67	38.69 (18.35–51.70)	0.24 ns

[1] The first parameter estimates the slope of the relationship in the linear model, and the slope of the relationship in the initial portion of data in the two nonlinear models.

[2] The breakpoint is the number of pollen grains at which the slope of the relationship changes. Ninety-five per cent approximate bootstrapping confidence intervals (BCa) were estimated with *n* = 1000 bootstraps.

[3] The second parameter is the asymptotic value in the negative exponential model and the slope of the second regression in the piecewise model.

ns, not significant.

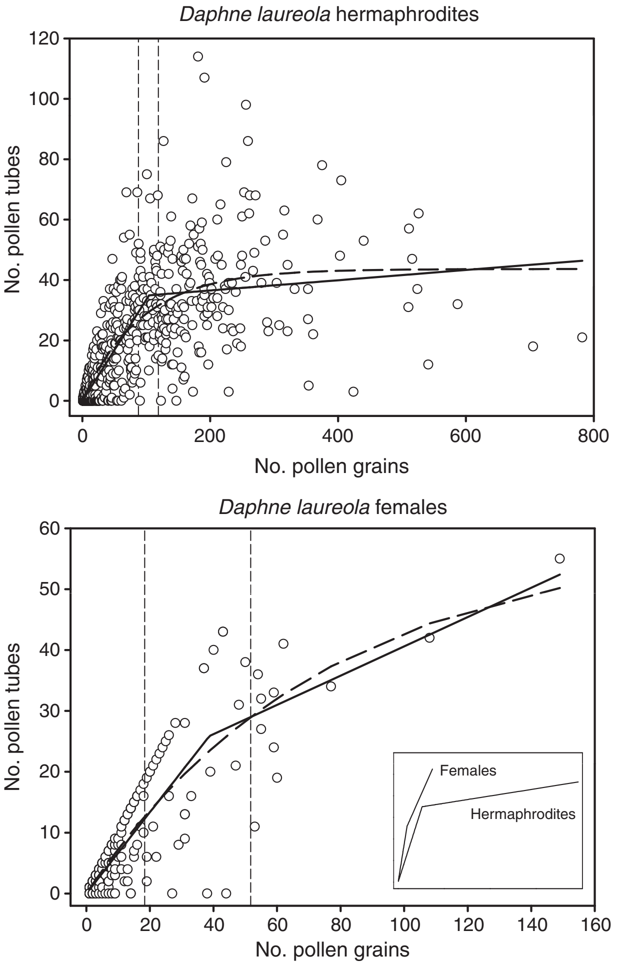

Fig. 5 Piecewise regression (solid line) and nonlinear saturating negative exponential model (dashed line) of the relationship between the numbers of pollen grains and pollen tubes for hermaphrodite and female individuals of *Daphne laureola*. Each symbol corresponds to an individual flower. Vertical dashed lines denote the 95% approximate bootstrapping confidence intervals (BCa) of the breakpoint estimated by piecewise regression with 1000 bootstraps. Differences in the scale of the axes reflect the natural differences in data range. For comparison, the two piecewise models are presented on a common scale in a small insert within the bottom panel.

although the number of tubes corresponding to the breakpoint was 35 in hermaphrodites and 26 in females, much higher than the single ovule per flower.

Outlier and sample size effects

Scatter plots of raw data (Figs 4, 5) and examination of the frequency distribution of residuals did not detect outliers in any of our study cases. Results of the effect of sample size on model fitting are summarized in Table 3. The frequency with which parameter estimates of reduced data sets fell within the confidence intervals estimated from the original data set increased for the largest sizes tested (n = 150 and 200) and was consistently higher in the Lamiaceae subsamples. Piecewise models fit the data similarly or with a significantly lower AIC than exponential models, at all sizes for all study cases, except *R. officinalis*.

Table 3 Convergence percentage of the piecewise regression method applied to subsamples with different sizes (n = 85, 100, 150 and 200 data points) obtained by random subsampling of the original data sets, and the percentage of converged models for which parameter estimates c, $b1$ and $b2$ were within the confidence intervals of the original data set

			Parameter estimates within BCa			
Study case	n	Convergence (%)	c	$b1$	$b2$	ΔAIC
Lavandula latifolia	85	80	80	60	80	−2.26
	100	90	100	80	70	−2.59
	150	70	90	80	70	−2.96
	200	70	100	100	100	−2.31
Rosmarinus officinalis	85	70	60	80	70	0.31
	100	70	80	80	90	5.01
	150	80	90	100	100	8.85
	200*	70	100	100	90	12.92
Daphne laureola Hermaphrodites	85	60	50	40	50	2.00
	100	60	50	90	70	1.97
	150	70	50	60	70	1.21
	200	80	90	70	90	−1.19
D. laureola Females	85	50	80	80	80	−3.67
	100	60	80	100	70	−2.46
	150	60	80	100	90	−3.38
	200	20	100	100	100	−6.88

Estimates were based on 10 repetitions per category (see the model Description section for details).

Negative ΔAIC indicates that on average the piecewise model had a lower AIC; differences < 2 units are not significant.

*The original sample was randomly extended until the desired size was reached.

One effect of reduced sample size was to impair model convergence. This was most apparent in the female *D. laureola* data set, which had the fewest data points located to the right of the breakpoint upper BCa limit (Fig. 4). This suggests that convergence problems in piecewise regression may ultimately reflect the extreme dominance of one of the two components of pollination in a particular study.

Discussion

The piecewise model-fitting approach provides an objective means to estimate the relative relevance of Qt and Ql during a plant reproductive event. In our study cases, piecewise regression provided a goodness of fit similar to the commonly used negative exponential nonlinear regression. However, it also has the distinct advantage of producing parameters that can be explicitly linked to underlying biological processes, and can be compared across multiple data sets in a straightforward fashion. In the following paragraphs we illustrate the utility of the proposed method for understanding how morphs, species, reproductive events, or environments can differ in the relative importance of the quantity and quality of pollen receipt as limiting factors of natural pollination success.

In the Lamiaceae example, the pollination success of *R. officinalis* was more limited than that of *L. latifolia*, and both quantitative and qualitative effects underlie this difference.

Rosmarinus officinalis had a lower percentage of visited flowers, lower average pollen loads, and a lower frequency of visited flowers falling into region III where Qt < Ql (7.3% vs 49.7%; Fig. 4) than *L. latifolia*. Furthermore, the piecewise analysis indicated that the quality of pollen received by *L. latifolia* flowers was on average higher than in *R. officinalis* because their styles saturated more rapidly and with a higher number of tubes. This latter result in particular demonstrates the value of the piecewise approach, as previous analyses of pollen tubes alone (Herrera, 2004) did not reveal the pollination quality difference between the two species that was uncovered here.

The two sexual morphs of the gynodioecious *D. laureola* exhibited widely different patterns. Female flowers were quantitatively less successful than those of hermaphrodites, which conformed to expectations given that they are the less preferred morph by the main pollinator in the study area (Alonso, 2004). Specifically, females had ten times as many unvisited flowers, much lower pollen loads and a higher percentage of flowers falling into region I compared with hermaphrodites (Fig. 5). However, the initial slope of the pollen grains–tubes relationship was twice as steep in females as in hermaphrodites, indicating either a higher quality of pollen received or a stronger discrimination capacity of female styles. Females are expected to receive a higher quality of pollen than hermaphrodites because they receive only outcross pollen. Also, females may have more restrictive stigmas or more discriminating styles because their styles saturated at a lower number of pollen tubes. Experimental studies of pollen tube development are required to ascertain the mechanisms behind these patterns (see e.g. Lankinen & Madjidian, 2011).

Recommendations for use

Given the intrinsic variability among flowers in pollen grains and tubes, achieving convergence and narrow confidence intervals for parameters of the piecewise analysis requires large sample sizes, larger than in most studies that use the standard hand-pollination approach. From our intensively studied cases, we conclude that a sample size of *c.* 150–200 *visited* flowers can be used as a rough guide. Lack of convergence emerged as the most common problem with small or largely unbalanced data sets, as a result of reduced possibilities of collecting samples in all the regions of the pollen grains–tubes relationship. This problem may be overcome with larger sample sizes (e.g. n = 200–600), a judicious analysis of outliers, deletion of single data points for which pollen grains appeared underestimated (i.e. pollen tubes >> pollen grains), and/or intensive grid searches of the initial parameters.

One should acknowledge, however, that absence of convergence of the piecewise model may actually indicate a strong influence of quality or quantity effects on pollination success on a given data set. This can be evaluated by plotting all data on a common scale. Indeed, a balance between quantity and quality effects would be predicted mainly in species with mixed mating systems (Goodwillie *et al.*, 2005). In these species, stigmas are expected to receive mixtures of self and outcross pollen from many genetically distinct conspecific individuals and thus the broadest variation in pollen tube performance (Mazer *et al.*, 2010). Species with a strong propensity to autonomously self-pollinate may be the most problematic, because the amount of pollen received would not directly reflect the activity of pollinators. In extreme cases, for example, species with closed flowers, or prior self-pollination, it may not be possible to fit a curve to the relationship between pollen grains deposited on the stigma and pollen tubes in the style. One option in these species is to emasculate flowers before anthesis (if pollen is not the main reward for pollinators) and/or just before the assurance mechanism is triggered (in species with delayed autonomous selfing) (Fenster & Martén-Rodríguez, 2007) so that only pollinator-mediated pollen deposition is assessed.

Linkages of Qt and Ql with seed production

The relationship between components of pollination success and plant reproduction can be verified by relating pollen receipt to seed production (Mitchell, 1997; Kalla & Ashman, 2002; Aizen & Harder, 2007). The piecewise approach provides an explicit connection between these two stages of the process. As a first step, a low percentage of unvisited flowers suggests little influence of pollen quantity on limitation of seed production in species with a single ovule per flower. In addition, and perhaps more importantly, we can predict that, in species with multiple ovules per flower, flowers to the left of the breakpoint will have fewer seeds per fruit than those to the right of the breakpoint. We explored this prediction with an *L. latifolia* data set (from the same population but a different year) where styles and ripe fruits were collected from the same flowers. Based on pollen tubes (pollen loads were not scored) we divided the sample into two regions defined by the number of tubes at breakpoint (k = 15) as the BCa were very small and few flowers fell in the narrow region II (Fig. 4). As predicted, the percentage of ovules producing seeds was significantly lower in flowers from below than above the breakpoint (25.3% ± 2.3 vs 43.2% ± 4.1; $F_{1,123}$ = 15.47, P < 0.0001). Furthermore, the relationship between the percentage of ovules leading to seeds and the number of tubes was highly significant below ($F_{1,87}$ = 49.57, P < 0.0001, adjusted without intercept), but not above ($F_{1,35}$ = 0.35, P = 0.55) the breakpoint. These results indicate a strong link between the quantity and quality limitation at the pre-zygotic and early post-zygotic phases of pollination success in *L. latifolia*. Such relationships are also likely to hold in other species (see also Kalla & Ashman, 2002).

Concluding remarks

Both pollination quantity and quality have consequences for seed production and potentially for population recruitment. The study of the two-stage relationship between pollen grains and pollen tubes and then between tubes and the quantity and quality of seeds under natural conditions will be logistically limited to species suitable for individual flower monitoring after style collection and environments with a low risk of predation on developing fruits. Piecewise regression provides a tractable quantitative method with which to characterize the extent to which the first step in the pollination process is achieved, and to predict the pollen load threshold at which the pollination process changes from being dominated by quantity effects to being dominated by quality effects. The method provides a direct means to objectively compare multiple species, populations or reproductive events. In particular, we foresee that, by simultaneously scoring pollen grains and tubes of wilted naturally pollinated flowers and analyzing their relationship with piecewise regression, we will be able to more rigorously assess the effects of pollination quantity and quality in plant reproduction from a community perspective. Such work is imperative if we are to better understand this important ecosystem service and the potential consequences that pollinator disruptions may impose (Kremen & Ricketts, 2000 and references therein).

Acknowledgements

We are grateful to Víctor Parra-Tabla and Luis Abdala-Roberts for very fruitful discussion on this topic, to Gerardo Arceo-Gómez, Renate A. Wesselingh and two anonymous referees for comments that improved the manuscript, and to the Consejería de Medio Ambiente and Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía, for authorizing our work in Cazorla and providing invaluable facilities. The study was supported by FBBVA through the research project ENDLIMIT (BIOCON08/125). T.-L.A. was also supported by NSF OIS 0852846.

References

Aizen MA, Harder LD. 2007. Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. *Ecology* **88**: 271–281.

Alonso C. 2004. Early blooming challenges: extended flowering season, diverse pollinator assemblage, and the reproductive success of gynodioecious *Daphne laureola*. *Annals of Botany* **93**: 61–66.

Alonso C. 2005. Pollination success across an elevation and sex ratio gradient in gynodioecious *Daphne laureola*. *American Journal of Botany* **92**: 1264–1269.

Alonso C, Vamosi JC, Knight TM, Steets JA, Ashman T-L. 2010. Is reproduction of endemic plant species particularly pollen limited in biodiversity hotspots? *Oikos* **119**: 1192–1200.

Ashman T-L, Knight TM, Steets JA, Amarasekare P, Burd M, Campbell DR, Dudash MR, Johnston MO, Mazer SJ, Mitchell RA *et al.* 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* **85**: 2408–2421.

Bernasconi G, Lang DJ, Schmid B. 2007. Microgametophyte population sizes and plant reproductive output in the insect-pollinated *Prunella grandiflora* (Lamiaceae). *New Phytologist* **173**: 393–400.

Burd M, Ashman T-L, Campbell DR, Dudash MR, Johnston MO, Knight TM, Mazer SJ, Mitchell RJ, Steets JA, Vamosi JC. 2009. Ovule number per flower in a world of unpredictable pollination. *American Journal of Botany* **96**: 1159–1167.

Burnham KP, Anderson DR. 2004. Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods Research* **33**: 261–304.

Busch JW, Joly S, Schoen DJ. 2010. Does mate limitation in self-incompatible species promote the evolution of selfing? The case of *Leavenworthia alabamica Evolution* **64**: 1657–1670.

Cahill JF, Castelli JP, Casper BB. 2001. The herbivory uncertainty principle: visiting plants can alter herbivory. *Ecology* **82**: 307–312.

Cane JH, Schiffhauer D. 2003. Dose-response relationships between pollination and fruiting refine pollinator comparisons for cranberry (*Vaccinium macrocarpon* [Ericaceae]). *American Journal of Botany* **90**: 1425–1432.

Dafni A, Firmage D. 2000. Pollen viability and longevity: practical, ecological and evolutionary implications. *Plant Systematics and Evolution* **222**: 113–132.

Dauber J, Biesmeijer JC, Gabriel D, Kunin WE, Lamborn E, Meyer B, Nielsen A, Potts SG, Roberts SPM, Sober V *et al.* 2010. Effects of patch size and density on flower visitation and seed set of wild plants: a pan-European approach. *Journal of Ecology* **98**: 188–196.

Edlund AF, Swanson R, Preuss D. 2004. Pollen and stigma structure and function: the role of diversity in pollination. *The Plant Cell* **16**(Suppl.): S84–S97.

Fenster CB, Martén-Rodríguez S. 2007. Reproductive assurance and the evolution of pollination specialization. *International Journal of Plant Science* **168**: 215–228.

Ficetola GF, Denoël M. 2009. Ecological thresholds: an assessment of methods to identify abrupt changes in species-habitat relationships. *Ecography* **32**: 1075–1084.

García-Camacho R, Totland O. 2009. Pollen limitation in the Alpine: a meta-analysis. *Arctic, Antarctic, and Alpine Research* **41**: 103–111.

Gómez JM, Bosch J, Perfectti F, Fernández J, Abdelaziz M. 2007. Pollinator diversity affects plant reproduction and recruitment: the tradeoffs of generalization. *Oecologia* **153**: 597–605.

Goodwillie C, Kalisz S, Eckert CG. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution and Systematics* **36**: 47–79.

Hastie TJ, Tibshirani RJ. 1990. *Generalized additive models*. London, UK: Chapman and Hall.

Hedhly A, Hormaza JI, Herrero M. 2009. Flower emasculation accelerates ovule degeneration and reduces fruit set in sweet cherry. *Scientia Horticulturae* **119**: 455–457.

Hegland SJ, Totland O. 2008. Is the magnitude of pollen limitation in a plant community affected by pollinator visitation and plant species specialisation levels? *Oikos* **117**: 883–891.

Herrera CM. 1987. Components of pollinator 'quality': comparative analysis of a diverse insect assemblage. *Oikos* **50**: 79–90.

Herrera CM. 1988. Variation in mutualisms: the spatio-temporal mosaic of a pollinator assemblage. *Biological Journal of theLinnean Society* **35**: 95–125.

Herrera CM. 1989. Pollinator abundance, morphology, and flower visitation rate: analysis of the 'quantity' component in a plant-pollinator system. *Oecologia* **80**: 241–248.

Herrera CM. 2000. Flower-to-seedling consequences of different pollination regimes in an insect-pollinated shrub. *Ecology* **81**: 15–29.

Herrera CM. 2002. Censusing natural gametophyte populations: variable spatial mosaics and extreme fine-graininess in winter-flowering *Helleborus foetidus*. *American Journal of Botany* **89**: 1570–1578.

Herrera CM. 2004. Distribution ecology of pollen tubes: fine-grained, labile spatial mosaics in southern Spanish Lamiaceae. *New Phytologist* **161**: 473–484.

Kalla SE, Ashman T-L. 2002. The effects of pollen competition on progeny vigor in *Fragaria virginiana* (Rosaceae) depend on progeny growth environment. *International Journal of Plant Sciences* **163**: 335–340.

Kearns CA, Inouye DW. 1993. *Techniques for pollination biologists*. Niwot, CO, USA: University Press of Colorado.

Knight TM, Steets JA, Ashman T-L. 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.

Kremen C, Ricketts T. 2000. Global perspectives on pollination disruptions. *Conservation Biology* **14**: 1226–1228.

Lankinen A, Armbruster WS. 2007. Pollen competition reduces inbreeding depression in *Collinsia heterophylla* (Plantaginaceae). *Journal of Evolutionary Biology* **20**: 737–749.

Lankinen A, Maad J, Armbruster WS. 2009. Pollen-tube growth rates in *Collinsia heterophylla* (Plantaginaceae): one-donor crosses reveal heritability but no effect on sporophytic-offspring fitness. *Annals of Botany* **103**: 941–950.

Lankinen A, Madjidian JA. 2011. Enhancing pollen competition by delaying stigma receptivity: pollen deposition schedules affect siring ability, paternal diversity, and seed production in *Collinsia heterophylla* (Plantaginaceae). *American Journal of Botany* **98**: 1191–1200.

Marshall DL, Shaner MGM, Oliva J-P. 2007. Effects of pollen load size on seed paternity in wild radish: the roles of pollen competition and mate choice. *Evolution* **61**: 1925–1937.

Mazer SJ, Hove AA, Miller BS, Barbet-Massin M. 2010. The joint evolution of mating system and pollen performance: predictions regarding male gametophytic evolution in selfers vs outcrossers. *Perspectives in Plant Ecology, Evolution and Systematics* **12**: 31–41.

Mitchell RJ. 1997. Effects of pollination intensity in *Lesquerella fendleri* seed set: variation among plants. *Oecologia* **109**: 382–388.

Richards SA, Williams NM, Harder LD. 2009. Variation in pollination: causes and consequences for plant reproduction. *The American Naturalist* **174**: 382–398.

Ryan SE, Porth LS. 2007. *A tutorial on the piecewise regression approach applied to bedload transport data*. Gen. Tech. Rep. RMRS-GTR-189. Fort Collins, CO, USA: United States Department of Agriculture, Forest Service, Rocky Mountain Research Station. http://www.fs.fed.us/rm/pubs/rmrs_gtr189.html.

SAS. 2008. *SAS for Windows (version 9.2)*. Cary, NC, USA: SAS Institute.

Toms JD, Lesperance ML. 2003. Piecewise regression: a tool for identifying ecological thresholds. *Ecology* **84**: 2034–2041.

Vaughton G, Ramsey M. 2010. Floral emasculation reveals pollen quality limitation of seed output in *Bulbine bulbosa* (Asphodelaceae). *American Journal of Botany* **97**: 174–178.

Walsh NE, Charlesworth D. 1992. Evolutionary interpretations of differences in pollen tube growth rates. *Quarterly Review of Biology* **67**: 19–37.

Wesselingh RA. 2007. Pollen limitation meets resource allocation: towards a comprehensive methodology. *New Phytologist* **174**: 26–37.

Young HJ, Stanton ML. 1990. Influence of environmental quality on pollen competitive ability in wild radish. *Science* **248**: 1631–1633.

Young HJ, Young TP. 1992. Alternative outcomes of natural and experimental high pollen loads. *Ecology* **73**: 639–647.

Zuur AF, Ieno EN, Elphick CS. 2010. A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution* **1**: 3–14.

Supporting Information

Additional supporting information may be found in the online version of this article.

Notes S1 Annotated step-by-step procedure to apply the piecewise method to the analysis of pollen grains and tubes using the SAS program.