

Effects of herbivores and pollinators on fruit yield and survival in a cleistogamous herb

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Abstract Although the way in which multiple biotic interactions affect plant reproductive success has been assessed in sexually monomorphic plants, little is known about the joint influence of these interactions on the reproductive success and the consequences to the mating system of plants with sexual heteromorphisms. Dimorphic cleistogamy is a sexual heteromorphism where a single plant produces open, potentially out-crossed chasmogamous (CH) flowers and closed, obligately self-pollinated, cleistogamous (CL) flowers. Fruits produced are also dimorphic with CH fruit being larger and having more seeds than CL fruit. The effects of defoliation and enhancement of the pollination environment on CH and CL fruit yield and plant survival were experimentally assessed in a dimorphic cleistogamous herb (*Ruellia nudiflora* Engelm. & Gray). We predicted that defoliation would have a stronger effect on CH fruit than on CL fruit owing to the high cost of maintaining the former.

However, the negative effects of defoliation on CH fruit may be overcome by compensatory mechanisms in an enhanced pollination environment. Lower survival is expected in defoliated plants, particularly in an enhanced pollination environment owing to greater investment in reproduction. As expected, we found that defoliation had a greater negative effect on CH fruit production; however, this effect was absent in the enhanced pollination environment. Enhancement of the pollination environment also increased survival, but only when plants were not defoliated. Although herbivores may increase inbreeding (via reduction of CH fruit production) in plants with dimorphic cleistogamy, this effect may be negligible in environments where pollination service is optimal.

Keywords Cleistogamy · Herbivory · Multiple interactions · Pollination · *Ruellia* · Yucatan

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Introduction

Pollination typically enhances plant reproductive success, while herbivory is usually detrimental to it (Marquis 1984; Hendrix 1988; Crawley 1989; Buchman and Nabhan 1996; Pellmyr 2002; Maron and Crone 2006). Perhaps because different tissues and species are involved, most of the early work looking at the impact of herbivores and pollinators on plant reproductive success has examined these variables

separately (Mothershead and Marquis 2000). More recently, it has been recognized that plants exist in complex environments where they interact simultaneously with antagonisms and mutualisms (Strauss and Irwin 2004; Barber et al. 2012). Final effect of herbivory and pollination on plant reproduction is not easily predicted because the effects of these biotic interactions may not be additive. For instance, Gómez (2005) showed for *Erysimum mediohispanicum* Polakchek that herbivores only had a detrimental effect on plant reproductive success when pollinators were present, and pollinators enhanced reproductive success only when herbivores were excluded. Likewise, the non-additive effects of pollinators and herbivores on the reproductive success have been reported in other studies suggesting that this may be the pattern expected for plants with monomorphic flowers (e.g. Strauss 1997; Herrera 2000; but see Abdala-Roberts et al. 2009). However, there may be another layer of complexity when the plants under study exhibit floral heteromorphism because different floral morphs on a single plant may respond differentially to leaf herbivory (Quesada et al. 1995; Steets and Ashman 2004; Steets et al. 2006; Hlandun and Adler 2009; Parra-Tabla and Herrera 2010) and this may have important consequences for pollination and plant mating systems (Steets and Ashman 2004; Schutzenhofer 2007; revised by Carr and Eubank 2014). For example, when floral morphs differ in their energetic cost, leaf herbivory may have a stronger negative effect on the production of the most expensive floral morph (Steets and Ashman 2004), and this may in turn affect negatively plant reproductive success not only because of the reduction in photosynthetic area but also because pollinators may find the cheaper floral morph less attractive or inaccessible. A plant's mating system may also be affected by leaf herbivory in species where different floral morphs have different breeding systems, i.e., selfing versus outcrossing (Steets and Ashman 2004).

Dimorphic cleistogamy (sensu Culley and Klooster 2007) is a floral dimorphism in which a single plant produces closed, obligately self-pollinated cleistogamous (CL) flowers along with open, potentially out-crossed chasmogamous (CH) flowers (Campbell et al. 1983; Culley and Klooster 2007), and is considered an example of a mixed mating system (Goodwillie et al. 2005; Oakley et al. 2007). Typically, CL flowers and fruits are smaller and energetically less

expensive than CH flowers and fruits (Oakley et al. 2007). Theory predicts that dimorphic cleistogamy is evolutionarily stable when cheaper CL flowers are produced and that CL-derived progeny has a greater fitness advantage under suboptimal environmental conditions, while more costly CH flowers are produced and CH-derived progeny has a greater fitness advantage under optimal conditions (Schoen and Lloyd 1984). Several empirical studies have partially supported what the theory predicts, especially regarding the production of reproductive structures (flowers and fruit): CH and CL structures are produced under optimal and suboptimal environmental conditions, respectively (Campbell et al. 1983; Culley and Klooster 2007; Oakley et al. 2007). Among the environmental variables tested, abiotic variables such as low soil moisture (Schemske 1978; Waller 1980; Bell and Quinn 1987), low light intensity (Schemske 1978; Waller 1980; Bell and Quinn 1987), low nutrient availability (Le Corff 1993), and high plant density (Lu 2000) all decrease the production of CH reproductive structures but not that of CL reproductive structures. However, far less attention has been paid to the influence of the biotic environment. To our knowledge, only two studies have looked at the effect of leaf herbivory on the production of different floral morphs in plants with dimorphic cleistogamy, and both studies show that, as expected, herbivory negatively affects only the production of CH flowers, which also led to greater inbreeding (Steets and Ashman 2004; Schutzenhofer 2007). However, we do not know of any study that has looked at the effect of the pollination environment or the interactive effect of pollinators and herbivores on the production of reproductive structures in any plant with dimorphic cleistogamy.

In this study, our goals were to assess experimentally the effects of leaf herbivory, pollination environment, and their interaction on flower and fruit production, and on survival in a cohort of *Ruellia nudiflora* Engel. & Gray (Acanthaceae), a short-lived perennial herb. We predicted that leaf herbivory would reduce more dramatically the production of the more costly CH fruit than that of the cheaper CL fruits (because of a reduction in plant carbohydrate output resulting from the loss of photosynthetic area). Leaf herbivory is expected to have stronger detrimental effect on fruit than on flowers, because the former demand more resources over longer periods of time.

However, it is also possible that in plants receiving extraordinary pollination service, the detrimental effects of leaf herbivory may be compensated for or cancelled out by compensatory mechanisms (i.e., an increase in photosynthetic activity) triggered by an increased demand from sink organs (i.e., developing fruit and seeds) (Fujii and Kennedy 1985; Lehtilä and Syrjänen 1995; Munguía-Rosas et al. 2012). However, this is only expected for CH fruit production because, in CL plants, pollinators only have access to CH flowers. Also, an enhanced pollination environment may increase the reproductive effort of plants because it is expected that plants in such an environment would produce more CH fruit relative to plants in a pollination environment with no enhancement (Dudash and Fenster 1997). An increase in reproductive effort may reduce the resources available for other functions (e.g. defence against natural enemies), and thus decrease the chance of survival. This may be particularly true for plants undergoing leaf herbivory when resources are even more compromised (García and Ehrlén 2002). Therefore, we also predicted that mortality would be greater for plants in an enhanced pollination environment and with leaf herbivory owing to an increased cost in reproduction which is defined as a reduction in future reproductive opportunities and survival caused by current investment in reproduction (Obeso 2002).

Materials and methods

The study species

Ruellia nudiflora Engel & Gray (Acanthaceae) is a New World herb which naturally occurs in open spaces and disturbed forest (Cervera and Parra-Tabla 2009; Munguía-Rosas et al. 2012) from southern Texas to the Caribbean (Long 1977). Although perennial, this species has a very high mortality rate during its first year (ca. 75 % of plants) under greenhouse conditions (this study). *Ruellia nudiflora* produces fruit and seeds from its first year (Munguía-Rosas et al. 2013a) and has a dimorphic CL reproductive system (sensu Culley and Klooster 2007), meaning that a single plant produces both CL (CL) and CH (CH) flowers. Typically, more CL (ca. 85 %) flowers are produced almost year around, while far fewer CH flowers (ca. 15 %) are produced

opportunistically during one or two short blooming pulses per year (Munguía-Rosas et al. 2013b). Individual CH and CL flowers last less than 1 day (Abdala-Roberts et al. 2014). CH flowers are larger (2.4 ± 0.3 cm; hereafter mean ± 1 SE of the mean; $n = 35$) than CL flowers (0.4 ± 0.1 cm; $n = 38$) (Munguía-Rosas Unpublished Data). Fruits are energetically more expensive in terms of resources than flowers because both CH and CL fruits remain on the plant for about 1 week before seed dispersal. However, the two types of fruits differ substantially in terms of size (CH fruit = 28.7 ± 0.46 mg vs. CL fruit = 16.61 ± 0.22 mg; Munguía-Rosas et al. 2013a) and seed number (CH fruit = 11.51 ± 0.17 seeds vs. CL fruit = 8.14 ± 0.07 seeds; Munguía-Rosas et al. 2013a). Both fruit types (CH and CL) are dry capsules, with ballistic dispersal. CL flowers never open and obligately self-pollinate, while CH flowers do open and can be out-crossed. CH flowers are completely self-compatible and are able to self-pollinate autonomously; however, out-crossed CH flowers have a substantially larger fruit set (78 %) than selfed CH flowers (38 %) (Abdala-Roberts et al. 2014). On the Yucatan Peninsula, pollinators include native and exotic bees (*Apis mellifera* L., *Trigona fulviventris* Guérin), as well as butterflies (*Microtia elva* Bates) (Abdala-Roberts et al. 2010). Also, in this area, *R. nudiflora* leaves are mainly attacked by the larvae of several species of Lepidoptera, of which the generalists *Anartia jatrophae* L. and *Siproeta stelenes* L. (Nymphalidae) are the folivores with the most negative effect on fruit production (Ortegón-Campos et al. 2009).

Experimental design

The study was carried out from May to December 2013 in a plant nursery located on the CINVESTAV campus in the city of Merida, Yucatan. About 600 CL fruits were collected from 120 different adult plants from a wild population on campus. Only CL fruits were collected because they are far more abundant than CH fruit (Munguía-Rosas et al. 2013a, b) and also because this allowed us to control for the source of pollen as all CL fruits are sired via autonomous self-pollination, while CH fruits are sired either via outcrossing or selfing (Abdala-Roberts et al. 2014), which could increase the variance associated with the error. Also, mother plants were selected to be as

similar as possible (similar size, floral display and number of leaves) to reduce bias due to maternal effects. About 4800 seeds were obtained from all of the fruits, pooled and sown in germination trays filled with soil from the site plus gravel (1:1) to improve drainage. In early June 2013, a cohort of 340 apparently healthy seedlings with one pair of true leaves and of similar size were transplanted to individual plastic pots (1.5 L) filled with the same substrate used during the germination process. Transplanted plants were acclimatised for a couple of months, during which 106 plants died. In early August 2013, we started the experiment with the remaining 234 plants, and all the plants produced flowers and fruits during their first year. Over the course of the experiment, plants were watered as needed and any other plant species that germinated in the experimental pots were removed weekly. We applied two different treatments to experimental plants in a random full factorial design: artificial defoliation and manual pollen supplementation (enhancement of the pollination environment). The defoliation factor had two levels: removal of 50 % of the leaf lamina from all the leaves of each plant using scissors ($n = 114$ plants) and a control group (no defoliation, $n = 120$ plants); the defoliation treatment was also applied to new leaves that appeared over the course of the experiment. We did not see any folivores on the experimental plants during the experiment, and thus assumed that no additional leaf tissue was removed from experimental plants apart from that which we removed experimentally. Half of the plants in the defoliation and control groups were assigned to a second treatment of enhancement of the pollination environment ($n = 117$), and the other half were used as controls ($n = 117$). So, the final distribution of plant sample size per treatment combination was as follows: no treatment (control + control) = 60, defoliation only (control + defoliation) = 57, pollination environment enhancement only (pollination enhancement + control) = 60 and combination of the two treatments (pollination enhancement + defoliation) = 57 (Fig. 1). The pollination enhancement treatment was aimed at enhancing the natural pollination service; therefore, the stigma of every CH flower produced by experimental plants was supplemented with pollen from a different plant until the stigma was apparently saturated; pollen donors belonged to a natural population adjacent to the nursery

(ca. 500–800 m away). Pollen was supplemented daily at midday (2–4 h before flower senescence). As the plant nursery was fully exposed to the sun, we often observed several flower visitors (*A. mellifera* and other un-identified native bee species) on the flowers of the experimental and control plants during the experiment. Plants receiving the same treatment were put together to expedite treatment application and data collection; however, there was no greater spatial plant separation between treatments than within a treatment, so we considered the whole group of plants—regardless of the treatment—as a single common garden (Fig. 1).

From August to December 2013, we counted all open CH flowers weekly, as well as the mature CH and CL fruits produced per plant. CH flowers remain on the plant for less than 1 day (6 h) and mature CH and CL fruits for less than 1 week (6 days) which eliminated the possibility of overestimation. Individual plant survival (alive vs. dead) was recorded at the same time we recorded the number of reproductive structures. CL flowers were not recorded because these cannot be differentiated from early CH flower buds; in contrast, CH and CL fruits are easily distinguished because, when ripe, CH fruits are larger

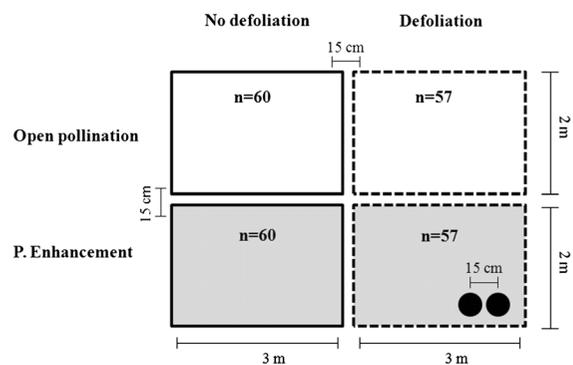


Fig. 1 Experimental design used to assess the effect of two treatments: Defoliation [two levels: control (No defoliation) and 50 % artificial defoliation (Defoliation)], and pollination environment enhancement [two levels: control (Open pollination) and hand pollen supplementation (P. Enhancement)]. Plant groups receiving defoliation treatments are represented by dashed-line rectangles, and the non-defoliated groups are rectangles with continuous lines. White rectangles are open-pollinated plant groups, and gray rectangles are pollen-supplemented groups. Sample size per treatment combination is shown inside each rectangle. The size of the experimental groups and distance between experimental groups and between pots (black circles) are also shown

Table 1 Results of the log linear models fitted to assess the effects of pollination environment enhancement (P. Enhancement), 50 % artificial defoliation (Defoliation) and their interaction (P. Enhancement \times Defoliation) on the production of chasmogamous flowers (CH flower), chasmogamous fruit (CH fruit) and cleistogamous fruit (CL fruit)

Source of variation	Reproductive structure		
	CH flower	CH fruit	CL fruit
P. Enhancement	$F = 0.08$	$F = 0.11$	$F = 0.12$
Defoliation	$F = 0.51$	$F = 5.48^*$	$F = 3.99^*$
P. Enhancement \times Defoliation	$F = 0.51$	$F = 8.72^{**}$	$F = 0.33$

A separate model was fitted per structure (three models in total). For all F tests, the degrees of freedom of the numerator and denominator were 1 and 230, respectively

* $P < 0.05$, ** $P < 0.01$

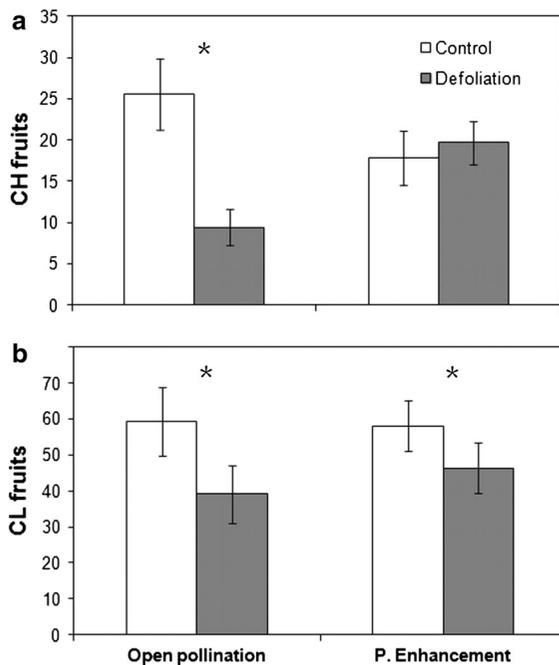


Fig. 2 Chasmogamous (CH) fruit (a) and cleistogamous (CL) fruit (b) production by *Ruellia nudiflora* plants under a defoliation treatment [Control vs. 50 % defoliation (Defoliation)] and a pollination environment enhancement treatment [Open pollination vs. Pollen supplementation (P. Enhancement)]. The experimental design was full factorial. Bars show the mean ± 1 standard error. The asterisk indicates statistically significant differences

than CL fruits and the style only remains on CH fruits (Munguía-Rosas et al. 2012, 2013b).

Statistical analysis

The total number of open CH flowers, CH fruits and CL fruits was fitted to a generalised linear model with

a quasi-Poisson error distribution and log link function (Crawley 2013), where the explanatory variables were defoliation (two levels), enhancement of pollination environment (two levels) and their interaction. A separate model was fitted per reproductive structure (CH flowers, CH and CL fruits), 3 different models in total. The effect of defoliation, pollination environment enhancement and their interaction on individual plant survival at the end of experiment (alive vs. dead) was addressed with a generalised linear model with a binomial error distribution and a logit link function (Crawley 2013). We also ran a survival analysis to assess the effect of defoliation, pollen supplementation and their interaction on time to plant death (number of weeks each plant survived) (Hosmer et al. 2008; Crawley 2013). The survival analysis assumed a non-constant hazard, allowed data censoring and had a Weibull error distribution (Hosmer et al. 2008; Crawley 2013). All statistical analyses were run in R software, version 2.14.0 (R Development Core Team 2011). For the survival analysis, the “survival” package for R was used.

Results

Reproductive structures

There was no effect of pollination environment enhancement, defoliation or their interaction on CH flower production (Table 1). Pollination environment enhancement alone did not affect the production of CH fruit, but defoliation and the pollination environment enhancement \times defoliation interaction did (Table 1), that is, defoliation (9.40 ± 2.23 fruit) significantly

reduces CH fruit production relative to the control group (25.55 ± 4.31 fruit) in open-pollinated plants; however, defoliated plants (19.65 ± 2.67 fruit) did not differ from the control plants (17.88 ± 3.29 fruit) when the pollination environment was enhanced (Fig. 2a). Mean CL fruit production was negatively affected by defoliation (control = 58.72 ± 5.90 , treatment = 42.56 ± 5.39), but not by the pollination environment enhancement treatment or by the pollination environment enhancement \times defoliation interaction (Table 1; Fig. 2b).

Survival

Final survival was statistically lower in plants with defoliation (19 %) than in the control plants (31 %) ($\chi^2_1 = 4.61$, $P = 0.03$). Neither enhancement of the pollination environment ($\chi^2_1 = 1.90$, $P = 0.17$) nor the pollination environment enhancement \times defoliation interaction had a statistically significant effect

on survival ($\chi^2_1 = 2.91$, $P = 0.09$) (Fig. 3a). However, the results of the survival analysis revealed that time to death is significantly affected by the interaction between enhancement of the pollination environment and defoliation ($\chi^2_1 = 4.03$, $P = 0.04$), that is, plants in the enhanced pollination environment lasted longer but only when no defoliation treatment had been applied (control = 15.51 ± 0.54 weeks, defoliated = 13.94 ± 0.58 weeks; Fig. 3b). The enhancement of the pollination environment ($\chi^2_1 = 2.12$, $P = 0.14$) and defoliation ($\chi^2_1 = 3.41$, $P = 0.07$) treatments alone did not have any effect on time to death (Fig. 3b).

Discussion

Our results clearly show that, as expected, the effects of defoliation and enhancement of pollination environment on CH fruit production are non-additive. In contrast, the negative effect of leaf herbivory on CL fruit was lower than for CH fruit, and this effect was not influenced by enhancing the pollination environment. Flower production was not affected by any of the experimental treatments. Surprisingly, there is no evidence of any reproductive cost for plants receiving pollen supplementation even when they have lost 50 % of their photosynthetic area; on the contrary, plants in an enhanced pollination environment may have increased survival if they are not defoliated.

Our results show that defoliation has a statistically significant detrimental effect on CH and CL fruits in open-pollinated plants. However, this effect was far greater for CH fruit (63.21 %) than for CL fruit (33.89 %). The differential impact of herbivory on CH versus CL can be explained as it is expected that photosynthate reduction would have a greater impact on the structure with higher cost of maintenance (i.e., CH fruit and seeds). This may also explain why we did not detect any significant effect of herbivory on CH flower production. Although CH flowers can be energetically more expensive than CL flowers, the energetic cost of both flower types is far lower than the maintenance cost for fruit and seeds since the flowers (CH and CL) only live for a few hours, while the fruit and developing seeds demand gradually increasing resources over several days. Although it was not feasible to count CL flowers, a negative effect of

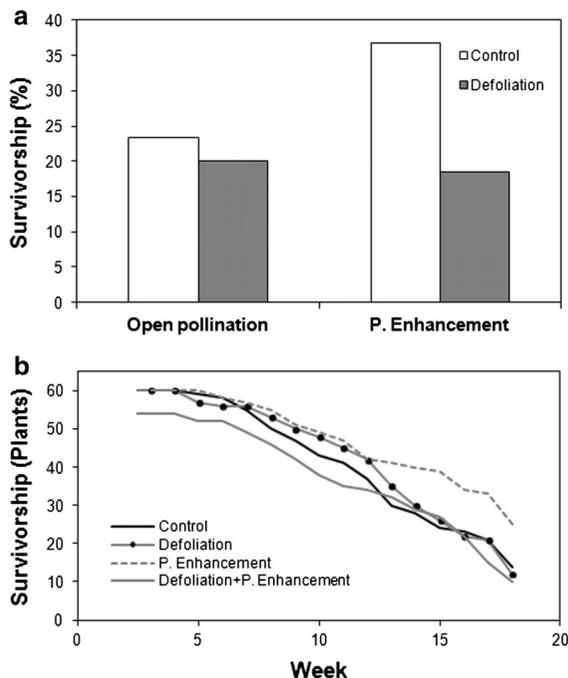


Fig. 3 Total (a) and weekly (b) survival of *Ruellia nudiflora* under experimental conditions. Two treatments were applied: defoliation [Control vs. 50 % defoliation (Defoliation)] and pollination environment enhancement [Control vs. Pollen supplementation (P. Enhancement)]. The experiment was full factorial. Bars show the percent of plants that survived to the end of the experiment (a), and the number of plants that survived each week (b)

herbivory on these flowers is unlikely because they are even cheaper in terms of resources than CH flowers are (Oakley et al. 2007). As we did not detect any effect of the treatments on CH flower production, the observed effects on CH fruit production may also suggest that similar effects on CH fruit set can be expected.

Previous studies have also identified a greater sensitivity of CH reproductive structures to leaf herbivory; in fact, these studies have shown that, in addition to reducing CH structure production, herbivory can increase the production of CL structures (Steets and Ashman 2004; Schutzenhofer 2007). Some authors have mentioned that folivores may have a role in mating systems, i.e., as defoliated plants produce more CL structures, folivores are indirectly increasing the selfing rate (Steets and Ashman 2004; Schutzenhofer 2007; revised by Carr and Eubanks 2014). Although our results for open-pollinated plants may support this idea to some extent, the fact that pollination environment enhancement cancelled out the detrimental effects of herbivory seen in open-pollinated plants (Fig. 2) makes us think that the impact of folivores on plant mating systems is dependent on the pollination environment, that is, folivores may have some detrimental effects on the production of potentially out-crossed seeds, but this effect is negligible when the pollination environment is optimal. We suspect that the mechanism underlying the interactive response to defoliation and pollination environment enhancement in *R. nudiflora* is an increase in photosynthetic efficiency triggered by pollen supplementation, which has been previously reported in *Primula veris* L. (Lehtilä and Syrjänen 1995). The fact that selective biomass allocation to CH fruit and seeds following pollen supplementation has been reported in *R. nudiflora* reinforces this suspicion (Munguía-Rosas et al. 2012). Plants with monomorphic and heteromorphic reproductive structures are both exposed to complex biotic environments; however, our results suggest that heteromorphic species, such as CL plants, have a more complex response to their biotic environment because floral morphs respond differentially to the same biotic variables and these effects may not be additive.

Plant survival, measured as a dichotomous variable at the end of the experiment, was only negatively affected by defoliation (Fig. 3a). However, a non-additive effect of the pollination environment and defoliation was recorded when survival was measured

as time to death (Fig. 3b), that is, enhancing the pollination environments increased time to death but only when plants had not been defoliated. It is possible that compensatory mechanisms (i.e., increased photosynthetic rate triggered by pollen supplementation) are also involved in cancelling the negative effects of leaf herbivory on plant survival as suggested by previous studies (Fuji and Kennedy 1985; Lehtilä and Syrjänen 1995). On the other hand, the fact that pollen-supplemented plants—even the defoliated ones—did not die more quickly also suggests that there is no cost of reproduction; rather, an enhanced pollination environment may increase plant survival if there is no defoliation. The absence of any cost of reproduction or even a negative cost (positive association between reproductive effort and survival/growth) has been reported in some plant species (revised by Obeso 2002), which is explained by compensatory effects (e.g. increased photosynthetic rate) or because there is no real resource limitation (Obeso 2002). It is also possible that a reproductive cost may appear the following year; however, mortality in *R. nudiflora* was as high as 75 % during the first year; therefore, one would expect to detect any cost of reproduction at the time of the first reproductive event in this plant species. The high mortality exhibited by *R. nudiflora* during the experiment is not surprising because the life history traits of this plant species (pioneer habit, short generation time, high fecundity and high growth rates) are compatible with an *r*-selected strategy, for which mortality rate is typically high (Pianka 1970). As we used only CL-derived progeny, we cannot discount the possibility that high mortality is at least partially influenced by the inbreeding produced by the obligate selfing seen in CL flowers. We also recognize that, although it offers a likely explanation, the compensatory mechanism proposed in this study needs further evaluation and a different compensatory mechanism cannot be ruled out.

In conclusion, the effect of folivores and the pollination environment on fruit yield in plants with sexual heteromorphism (specifically, *R. nudiflora*) is dependent on the floral morph. These effects are non-additive, but only for CH fruit. Therefore, the previous suggestion of an indirect influence of folivores on the mating system of CL plants (via negative effects on CH flower production; Steets and Ashman 2004; Steets et al. 2006; Schutzenhofer 2007) may be offset in pollinator-rich environments. Thus, a multiple

biotic interaction approach, in which the floral morph is explicitly considered, is needed to reach a better understanding of the role of the biotic environment in the reproductive success and mating system of plants with sexual heteromorphisms.

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Conflict of interest The authors declare that they have no conflict of interest.

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