

# Environmental control of reproductive phenology and the effect of pollen supplementation on resource allocation in the cleistogamous weed, *Ruellia nudiflora* (Acanthaceae)

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• **Background and Aims** Mixed reproductive strategies may have evolved as a response of plants to cope with environmental variation. One example of a mixed reproductive strategy is dimorphic cleistogamy, where a single plant produces closed, obligately self-pollinated (CL) flowers and open, potentially outcrossed (CH) flowers. Frequently, optimal environmental conditions favour production of more costly CH structures whilst economical and reliable CL structures are produced under less favourable conditions. In this study we explore (1) the effect of light and water on the reproductive phenology and (2) the effect of pollen supplementation on resource allocation to seeds in the cleistogamous weed *Ruellia nudiflora*.

• **Methods** Split-plot field experiments were carried out to assess the effect of shade (two levels: ambient light vs. a reduction of 50%) and watering (two levels: non-watered vs. watered) on the onset, end and duration of the production of three reproductive structures: CH flowers, CH fruit and CL fruit. We also looked at the effect of these environmental factors on biomass allocation to seeds (seed weight) from obligately self-pollinated flowers (CL), open-pollinated CH flowers and pollen-supplemented CH flowers.

• **Key Results** CH structures were produced for a briefer period and ended earlier under shaded conditions. These conditions also resulted in an earlier production of CL fruit. Shaded conditions also produced greater biomass allocation to CH seeds receiving extra pollen.

• **Conclusions** Sub-optimal (shaded) conditions resulted in a briefer production period of CH structures whilst these same conditions resulted in an earlier production of CL structures. However, under sub-optimal conditions, plants also allocated more resources to seeds sired from CH flowers receiving large pollen loads. Earlier production of reproductive structures and relatively larger seed might improve subsequent success of CL and pollen-supplemented CH seeds, respectively.

**Key words:** Cleistogamy, invasive plants, phenology, pollen supplementation, resource allocation, *Ruellia nudiflora*.

## INTRODUCTION

Mixed reproductive strategies in plants refers to the production of flowers which will result in progeny with contrasting genetic backgrounds (i.e. selfed vs. outcrossed), differential energetic investment or with variable morphology (Lloyd, 1984; Schoen and Lloyd, 1984; Venable, 1985; Goodwillie *et al.*, 2005). Plants with a mixed reproductive strategy have the benefits of different mating and reproductive avenues, and may have evolved to optimize fitness in highly variable environments (Schoen and Lloyd, 1984; Goodwillie *et al.*, 2005). Dimorphic cleistogamy (*sensu* Culley and Klooster, 2007) is an example of a mixed mating strategy where both obligately self-pollinated cleistogamous (CL) flowers and potentially outcrossed chasmogamous (CH) flowers are produced by a single plant. Since the production of CH and CL flowers is sensitive to different environmental cues (Culley and Klooster, 2007), dimorphic cleistogamy offers an excellent opportunity

to assess how plants with mixed reproductive strategies cope with contrasting biotic and abiotic conditions.

Previous research has shown that in cleistogamous plants, CH flowers are far more energetically expensive than CL flowers (mainly because CH flowers are larger than CL flowers, and only CH flowers produce rewards for pollinators) and the balance between CH and CL flower production is environmentally determined in several plant species (Campbell *et al.*, 1983; Culley and Klooster, 2007). Although there is evidence of the opposite (Mitchell-Olds and Waller, 1985; Berg and Redbo-Torstensson, 2000; Cheplick, 2006), most studies suggest that (owing to its reduced cost) sub-optimal conditions favour greater allocation to CL flowers whilst the production of CH flowers would be opportunistic and increase under optimal conditions (Campbell *et al.*, 1983; Culley and Klooster, 2007).

In order for cleistogamy to evolve, progeny from CH flowers must exhibit reproductive advantages different from progeny of CL flowers under different environmental conditions

(Schoen and Lloyd, 1984; Goodwillie *et al.*, 2005). CL flowers may be part of a bet-hedging strategy since these flowers are less costly than CH flowers and because CL flowers set seeds in the absence of pollinators and under disadvantageous environmental conditions (Oakley *et al.*, 2007). In this regard, the production of CL structures can be considered as a reproductive assurance strategy in cleistogamous plants. CL flowers may also promote local adaptations as both maternal sets of genes can be passed on to the progeny, purging deleterious alleles, though it could also lead to strong inbreeding depression (Schoen and Lloyd, 1984; Oakley *et al.*, 2007). On the other hand, CH flowers may have some benefits because genetically variable progeny could be favoured in spatially and temporally heterogeneous habitats (Culley and Klooster, 2007; Oakley *et al.*, 2007). Plants are not only able to reallocate resources between different types of flowers, but also some species allocate differentially among CH flowers as a response to the quantity of pollen they receive (Knight *et al.*, 2005, 2006). Pollen performance is influenced by pollen competitive environment, and the size of the pollen load is an important determinant of seed and fruit set (Niesenbaum, 1999). A more realistic picture of resource allocation in cleistogamous plants would emerge when the source of pollen and the size of the pollen load under a variety of environmental conditions are taken into account (Eckstein and Otte, 2005).

Some plant species alternate the production of CH and CL flowers during the same reproductive season, and some authors have suggested (without testing the hypothesis) that phenology might be indicative of optimal and sub-optimal conditions within a reproductive season (e.g. Sigrist and Sazima, 2002; but see Forrest and Thomson, 2008). Plant species with sequential production of CH and CL flowers may also regulate the production of CL fruit according to the reproductive success of CH flowers (Rebdo-Torstensson and Berg, 1995). Studies on the phenology of cleistogamous species have shown that either CH or CL flowers can be produced at first during the reproductive season and that the overlap in the production of CH and CL flowers is highly variable (Oakley *et al.*, 2007). Some studies have qualitatively addressed the sequence and the intensity in the production of the floral types (e.g. Jasieniuk and Lechowicz, 1987; Sigrist and Sazima, 2002; Imaizumi *et al.*, 2008) but we do not know of any quantitative study looking at the effect of environmental factors on temporal patterns in the production of CH and CL flowers or fruit under field conditions.

Cleistogamy is widespread in the genus *Ruellia* (Acanthaceae) (Long, 1977; Sell, 1977). However, relative to other genera (e.g. *Impatiens*, *Oxalis* and *Viola*), studies on cleistogamy in *Ruellia* are scarce (e.g. Sigrist and Sazima, 2002; De Souza-Lima *et al.*, 2005). In this genus, production of CH and CL flowers frequently shows little or no overlap and CL flowers occur mostly during the dry season (e.g. Sigrist and Sazima, 2002); therefore, *Ruellia* species are good models to test for the effect of environment on allocation patterns. In this study (1) we looked at the effect of two important environmental variables (light and water) on the reproductive phenology (production of CH flowers, CH fruit and CL fruit) of *Ruellia nudiflora*, a perennial herb with a long reproductive season (April–October); and (2) we

explored the potential effect of allogamous pollen supplementation on the biomass allocation to seeds under different environmental conditions. We predicted that optimal conditions (watered and non-light-limited sub-plots) not only favour greater investment (earlier, larger production and for longer) in CH flowers and CH fruit than CL, but also a selective allocation to those CH flowers receiving pollen supplementation. Alternatively, sub-optimal conditions (shaded, non-watered sub-plots) may also promote larger resource allocation to CH seeds from pollen-supplemented CH flowers because these may exhibit better performance under less favourable conditions than seeds from open-pollinated CH flowers.

## MATERIALS AND METHODS

### Study system

The study area is the locality of Molas, municipality of Mérida in central Yucatán, México (20°49'51"N, 89°36'44"W, 10 m a.s.l.). The climate is warm sub-humid with summer rains; mean annual rainfall and temperature are 850 mm and 26.2 °C, respectively (Chico-Ponce de León, 1999). Vegetation is a disturbed tropical dry forest. Dominant canopy species are *Bursera simaruba* (Bursaceae) and *Lysiloma latisiliquum* (Fabaceae), whilst *Gymnopodium floribundum* (Polygonaceae) and *Parmentiera millspaughiana* (Bignoniaceae) dominate the understorey (Flores and Espejel, 1994). *Ruellia nudiflora* (Fig. 1) is an invasive perennial herb that reproduces mainly by seeds (Munguía-Rosas, CINVSTAV-IPN, Mérida, México, unpubl. res.). This plant species is non-native to México; it presumably originates from southern Texas

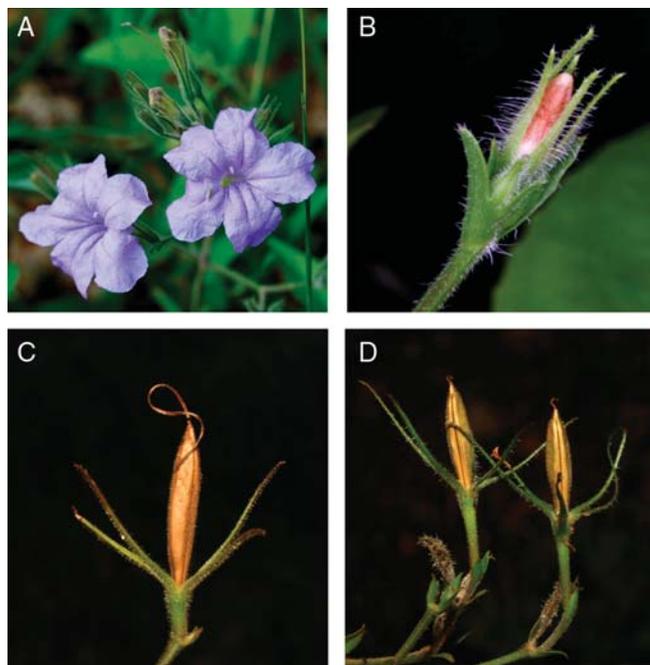


FIG. 1. Photographs of the reproductive structures of *Ruellia nudiflora*: (A) chasmogamous flowers, (B) cleistogamous flowers, (C) fruit from chasmogamous flowers, and (D) fruit from cleistogamous flowers.

(Turner, 1991), but currently it occurs from Texas to the Caribbean (Long, 1977).

*Ruellia nudiflora* has a dimorphic cleistogamous reproductive system (*sensu* Culley and Klooster, 2007). CL flowers are reduced, obligately self-pollinated and never open. CH flowers open for 1 d and can be either self-pollinated (delayed selfing has been reported) or outcrossed (Abdala-Roberts *et al.*, 2009). In the study area, the CH flowers are pollinated by some species of native and introduced bees (e.g. *Apis mellifera* and *Trigona fulviventris*) as well as butterflies (e.g. *Microtia elva*); there is no evidence of pollen limitation (Abdala-Roberts *et al.*, 2009). Fruits are dry capsules that disperse seeds ballistically. CL fruits are typically smaller, fewer seeded and more basal than CH fruits (Fig. 1).

*Ruellia nudiflora* can be considered as a sun plant as the net assimilation rate becomes saturated only at very high levels ( $1200 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) of instant photosynthetic photon flux density (PPFD); the light response curve is available online (Supplementary Data Fig. S1, available online). Seeds also have greater germination success under highly illuminated conditions (Cervera and Parra-Tabla, 2009), but it is unknown if shade affects any other aspect of the reproductive ecology of *R. nudiflora*.

### Phenology

In early April 2010, we placed 11 plots ( $2 \times 2 \text{ m}$ ) randomly in an area of about 2 ha where *R. nudiflora* occurs. At this stage plants had no reproductive structures, only a few leaves. The main plots were split into two sub-plots of similar size ( $2 \times 1 \text{ m}$ ; Fig. 2), one of them was shaded with a nylon cloth placed about 90 cm above the forest floor and supported with four thin columns (2 cm diameter). The cloth is neutral in terms of light, i.e. the red:far red ratio was not statistically different (paired *t*-test,  $t_8 = 1.299$ ,  $P = 0.225$ ,  $n = 10$ ) between an exposed location ( $0.871 \pm 0.004$ ; hereafter mean  $\pm$  s.e.) and under the cloth ( $0.864 \pm 0.012$ ). Under shaded conditions, ambient PPFD ( $1400\text{--}2000 \text{ mmol m}^{-2} \text{ s}^{-1}$ ; Cervera *et al.*, 2007) was reduced about 50%. The maximum photosynthetic rate for individuals receiving 50% of total ambient PPFD can become lower than  $9.06 \pm 0.24 \text{ mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ; that means that shaded sub-plots can lead to a 48% decrease in the net assimilation rate. In order to avoid shading of adjacent plots, shaded sub-plots were east–west oriented and the shortest distance between plots was 1.5 m. Flower visitors moved freely beneath the shade; also, open and shaded sub-plots did not differ in CH fruit production or mass (see the Results).

Shaded and open plots were again divided into two more sub-plots of similar size ( $1 \times 1 \text{ m}$ ); two of them (one shaded and one open) were watered twice a week until soil field capacity (about 5 L per sub-plot every time) from 10 April to 22 October 2010 (Fig. 2). Midday leaf water potential ( $\Psi_L$ ) was optimal ( $-0.39 \text{ MPa}$ ) during 88.2 and 55.6% of days for which the experiment lasted (196 d) in watered and non-watered sub-plots, respectively. Plants in sub-plots receiving only natural rainfall experienced some degree of water stress producing from moderate ( $\Psi_L$ :  $-0.76 \text{ MPa}$ ) to severe wilting ( $\Psi_L$ :  $-1.31 \text{ MPa}$ ) for 87 d, whereas plants in watered sub-plots were only slightly water stressed ( $\Psi_L$   $-0.53$ ) and for only 23 d

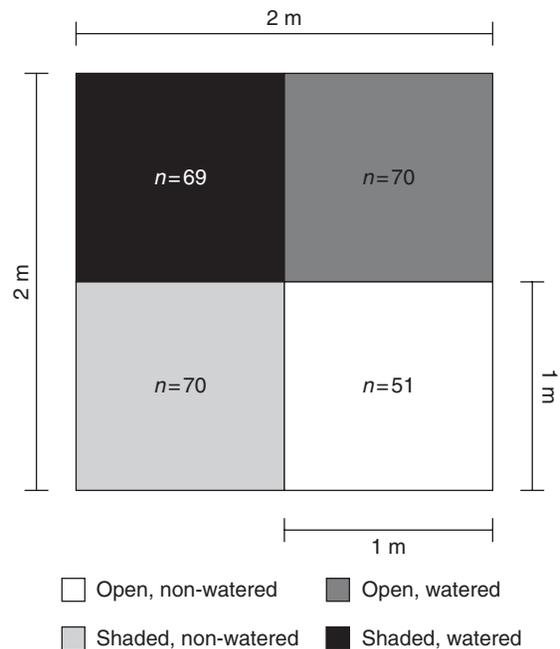


FIG. 2. Experimental split-plot design to assess the effect of shade (two levels: shaded vs. open) and watering (two levels: watered vs. non-watered) and its interaction on the reproductive phenology and resource allocation in *Ruellia nudiflora*. Each pattern shows a specific combination of treatments as shown in the key (control, open, non-watered). This plot was replicated 11 times; numbers inside sub-plots are the total number of plants per treatment.

(Supplementary Data Fig. S2). The terrain in the study area is flat, and the soil has excellent drainage; also, water was added at least 10 cm away from non-watered sub-plots. Therefore, water diffusion to adjacent sub-plots was unlikely. We tagged up to seven reproductive plants per sub-plot; excess plants and plants from different species were removed from experimental plots. In total we tagged 260 plants: 51 plants were in the control group (open and non-watered plots); 70 plants were shaded but not watered; 69 plants were shaded and watered; and 70 plants were watered but not shaded (Fig. 2). We counted all open CH flowers, CH fruit and CL fruit in the tagged plants once or twice a week until the growing season ended (17 December 2010). In some cases, plants were still bearing CL fruit at the end of the growing season; therefore, in these cases, the last count of CL structures was different from 0. We did not count CL flowers because, with our observation schedule, it was not possible to discriminate early CH flower buds from CL flowers and this may have produced a severe overestimation of CL flowers. CH and CL fruits are easily differentiated because CL fruits are smaller than CH fruits; also, in contrast to CL fruit, CH fruit usually bear remnants of the style (Fig. 1).

For the three different structures we counted (CH flowers, CH fruit and CL fruit) we recorded on a per-plant basis the following variables: production onset (day of the year on which we detected some reproductive structures for the first time), the end of production (the last time we saw the structures), duration of the production (the number of sampling days on which we saw the structures) and production of structures (the sum of the structures we counted during the growing season). As CH

flowers open for only 1 d, the counts of CH flowers are clearly underestimates of the total number of flowers produced; however, these are useful in relative terms for treatment comparison. Since the production start and end points are the times elapsed from a date of reference to an event, these data sets do not follow a normal distribution and were analysed with generalized mixed-effects models with gamma error distribution and reciprocal link function. We used generalized mixed-effects models instead of survival analyses because in the former we can specify a hierarchical error structure of the split-plot design; gamma models produce rather more conservative results than survival analysis with constant hazard (Crawley, 2002, 2007). Since the duration of production and the number of structures are numerical counted data, we analysed these data with generalized mixed-effects models with Poisson error distribution and logit link function. In all models the fixed effects were shade and watering treatments as well as their interaction. The hierarchical structure of plots was included in the random part of the model. Analyses were performed with the `glmmPQL` functions from the MASS library for R 2.10.1 software (R Development Core Team, 2009) which fits generalized mixed-effects models using the penalized quasi-likelihood method (Venables and Ripley, 2002).

#### *Pollen supplementation and biomass allocation*

We collected all mature CH and CL fruit once or twice a week from the same plants used for phenological recording as described above. Also, and with the same frequency, we performed pollen supplementation of about 80 % of available CH flowers in the 11 plots described in the previous section. Every flower received supplemental pollen from only one donor flower. Some plants producing more than one flower simultaneously received pollen from more than one plant; however, even in these cases each flower was supplemented with pollen from only one flower and one donor. Pollen was placed gently on receptive stigmas of non-emasculated flowers until stigma saturation before midday (1000–1100 h). We used only one pollen donor per flower to standardize the number of donors and because the availability of plants bearing CH flowers was limited. The petioles of supplemented flowers were tagged and the fruit were harvested when ripe. We did not protect supplemented flowers to avoid disturbance of pollinator foraging behaviour. We collected the fruit (open-pollinated CH, pollen-supplemented CH and CL fruit) which were then transported to the laboratory, weighed and dissected to count seeds.

As seed number and fruit weight are highly correlated traits ( $r_s = 0.72$ ,  $P < 0.01$ ,  $n = 2252$ ), we combined these variables, expressing biomass on a per-seed basis, dividing the fruit weight by the seed number. We did not find strong evidence of a seed size–number trade-off in the study species as correlation between seed size and number was extremely weak ( $r_s = -0.04$ ,  $P = 0.03$ ,  $n = 2252$ ). Allocation on a per-seed basis has more biological meaning than on a per-fruit basis because the former is correlated with progeny performance (Richardson and Stephenson, 1992; Bernard and Toft, 2007). Only non-damaged fruits were considered in data analyses because the biomass consumed by herbivores may bias the

results. Data from 2252 fruits were considered in the analysis: 553 in the control group (278 CL, 209 open-pollinated CH and 66 pollen-supplemented CH); 574 in the shaded treatment (266 CL, 211 open-pollinated CH and 97 pollen-supplemented CH); 574 in the watered treatment (283 CL, 227 open-pollinated CH and 64 pollen-supplemented CH); and 551 in the shaded and watered treatment (310 CL, 188 open-pollinated CH and 53 pollen-supplemented CH). Per-seed biomass (the response variable) was fitted to a linear mixed-effects model using the maximum likelihood method (Pinheiro and Bates, 2000) where the two treatments described above (shade and watering), type of fruit (CL as well as open-pollinated CH and pollen-supplemented CH) and all possible interactions were included as fixed factors. The random part of the model accounts for the hierarchical structure of the plots and repeated fruit collections from plants. The model was fitted with the `lme` function from the `nlme` library for R 2.10.1 software (R Development Core Team, 2009).

## RESULTS

### *Phenology*

The phenology of the three reproductive structures we considered in the study (CH flowers, CH fruit and CL fruit) were affected ( $P < 0.05$ ) by the shade, but the specific component of phenology affected varied (Table 1). The production of CL fruit started earlier in shaded plants (day 124 of the year: 4 May  $\pm$  1.32 d) than in plants in open sub-plots (day 130: 20 May  $\pm$  1.32 d) (Fig. 3A), the production of CH fruit ended earlier in shaded plants (day 264: 21 September  $\pm$  4.5 d) than in plants in open sub-plots (day 285: 12 October  $\pm$  4.04 d) (Fig. 3B) and, the duration in production of CH flowers was shorter in shaded plants (seen for 3.78  $\pm$  0.23 sampling days) than in plants in open sub-plots (5.12  $\pm$  0.34 sampling days) (Fig. 3C). However, the production of the three structures was not affected by any treatment (Fig. 3D). Watering and its interaction with shade treatment did not affect any phenological descriptor of any structure considered in this study (Table 1, Fig. 3).

### *Pollen supplementation and biomass allocation*

Fruit types differed significantly in per-seed mass: pollen-supplemented CH fruit had the heaviest seeds (3.00  $\pm$  0.32 mg), followed by open-pollinated CH fruit (2.59  $\pm$  0.05 mg), and CL fruit had the lightest seeds (2.16  $\pm$  0.05 mg) (Fig. 4). However, the interaction between type of fruit and shade treatment was significant (Table 2). The shade treatment produced a different effect on the three different types of fruit: this effect was negligible in CL fruit (open, 2.15  $\pm$  0.03 mg; shade, 2.19  $\pm$  0.08 mg) and open-pollinated CH fruit (open, 2.57  $\pm$  0.05 mg; shade, 2.62  $\pm$  0.08 mg); however, we found a large increase of the per-seed weight in pollen-supplemented CH fruit in plants under shaded conditions (open, 2.53  $\pm$  0.06 mg; shade, 3.41  $\pm$  0.50 mg) (Fig. 4). Watering treatment, the other second order interactions, as well as the third order interaction were all statistically non-significant (Table 2).

TABLE 1. Results of generalized mixed-effects linear models performed to assess the effect of shade (open vs. shaded), watering (watered vs. non-watered) and its interaction on the onset, end, duration and relative production of *R. nudiflora* chasmogamous flowers (Flowers), chamosgamous fruit and cleistogamous fruit (CH Fruit, CL Fruit)

Structure	Descriptor	Source of variation	Statistics	P-value
Flowers	Onset	Water	$F_{1,20} = 0.28$	0.60
		Shade	$F_{1,10} = 3.41$	0.09
		Water × shade	$F_{1,20} = 0.26$	0.61
	End	Water	$F_{1,20} = 1.36$	0.26
		Shade	$F_{1,10} = 4.27$	0.06
		Water × shade	$F_{1,20} = 0.77$	0.39
	Duration	Water	$F_{1,20} = 0.67$	0.42
		Shade	$F_{1,10} = 10.84$	<b>&lt;0.01</b>
		Water × shade	$F_{1,20} = 0.25$	0.62
Production	Water	$F_{1,20} = 0.27$	0.61	
	Shade	$F_{1,10} = 4.91$	0.05	
	Water × shade	$F_{1,20} = 0.35$	0.56	
CH Fruit	Onset	Water	$F_{1,20} = 0.48$	0.50
		Shade	$F_{1,10} = 4.26$	0.06
		Water × shade	$F_{1,20} = 0.26$	0.61
	End	Water	$F_{1,20} = 0.05$	0.98
		Shade	$F_{1,10} = 5.77$	<b>0.03</b>
		Water × shade	$F_{1,20} = 0.11$	0.75
	Duration	Water	$F_{1,20} = 0.95$	0.34
		Shade	$F_{1,10} = 4.87$	0.05
		Water × shade	$F_{1,20} = 0.089$	0.77
Production	Water	$F_{1,20} = 0.66$	0.42	
	Shade	$F_{1,10} = 0.99$	0.34	
	Water × shade	$F_{1,20} = 0.01$	0.93	
CL Fruit	Onset	Water	$F_{1,20} = 0.11$	0.74
		Shade	$F_{1,10} = 5.31$	<b>0.04</b>
		Water × shade	$F_{1,20} = 0.75$	0.39
	End	Water	$F_{1,20} = 3.77$	0.07
		Shade	$F_{1,10} = 1.14$	0.17
		Water × shade	$F_{1,20} = 0.34$	0.57
	Duration	Water	$F_{1,20} = 0.21$	0.65
		Shade	$F_{1,10} = 0.40$	0.54
		Water × shade	$F_{1,20} = 0.03$	0.96
Production	Water	$F_{1,20} = 3.53$	0.07	
	Shade	$F_{1,10} = 1.39$	0.26	
	Water × shade	$F_{1,20} = 0.34$	0.56	

The variables ‘onset’ and ‘end’ were fitted to gamma models, and the rest to Poisson models. Statistically significant differences are highlighted in bold.

DISCUSSION

Our results suggest environmental control of phenology and differential resource allocation depending on the amount of pollen received in the cleistogamous *R. nudiflora*. Under shaded conditions, production of CH flowers was for a shorter period and the production of CH fruit ended earlier, whilst shade induced an earlier start to CL fruit production. However, the total numbers of CH flowers and all fruit (CH and CL) were not affected by shade. Regarding biomass allocation to seeds, sub-optimal (shaded) conditions, but not unshaded conditions, favoured allocation to CH seeds receiving pollen supplementation. These results suggest that under sub-optimal conditions, not only are resources re-allocated to produce CL structures earlier, but also plants re-allocate

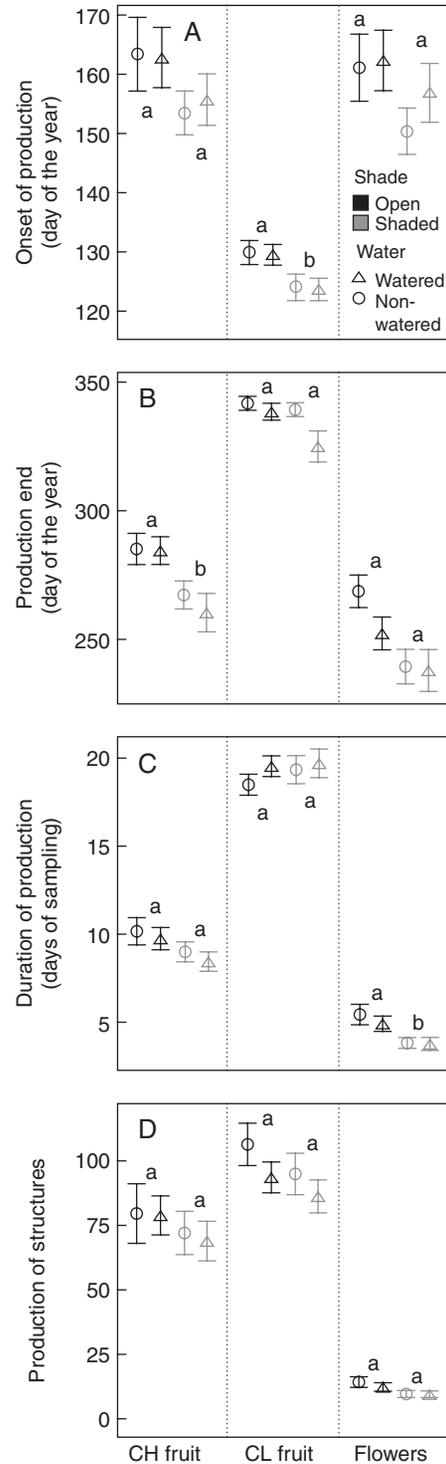


FIG. 3. Effect of shade and watering on the onset (A), end (B), duration (C) and production (D) of *R. nudiflora* chasmogamous flowers (Flowers), chamosgamous fruit (CH Fruit) and cleistogamous fruit (CL Fruit). The bars show the mean ± s.e.; different letters show statistical differences between shaded plants (shaded–watered, shaded–non-watered) and plants in open plots (open–non-watered, open–watered). Differences between watered and non-watered plants as well as the interactions were not statistically significant in all cases.

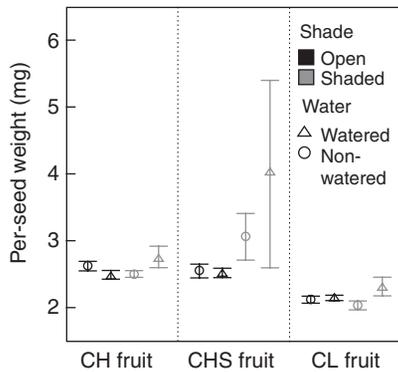


FIG. 4. Per-seed weight of *R. nudiflora* under field experimental conditions to assess the effect of two treatments: shade (shaded vs. open) and water supplementation (watered vs. non-watered) on biomass allocation to three different structures: cleistogamous fruit (CL Fruit), chasmogamous fruit (CH Fruit) and chasmogamous fruit supplemented with allogamous pollen (CHS Fruit). The bars show the mean  $\pm$  s.e. Per-seed weight was statistically different among structures, and the interaction treatment  $\times$  structure was also significant.

TABLE 2. Results of linear mixed-effects models to assess the effect of shade (shaded vs. open), watering (watered vs. non-watered) and three reproductive structures (chasmogamous flowers, chasmogamous fruit, cleistogamous fruit) on per seed mass

Source of variation	Statistics	P-value
Structure	$F_{2,1974} = 17.55$	<b>&lt;0.01</b>
Shade	$F_{1,10} = 1.69$	0.22
Water	$F_{1,20} = 2.13$	0.16
Structure $\times$ shade	$F_{2,1974} = 4.55$	<b>0.01</b>
Structure $\times$ water	$F_{2,1974} = 0.94$	0.39
Shade $\times$ water	$F_{1,20} = 2.67$	0.12
Structure $\times$ shade $\times$ water	$F_{2,1974} = 0.87$	0.42

Statistically significant differences are highlighted in bold.

biomass mainly to CH seeds that developed from CH flowers receiving larger pollen loads. Thus, the cleistogamous *R. nudiflora* responds to sub-optimal conditions with two contrasting strategies: earlier production of CL seeds and greater biomass allocation to presumably higher quality CH seeds.

Although we did not find any effect of shade or watering on the number of CH flowers, CH fruit and CL fruit, we did identify an effect of shade on the phenology of all these structures, though on different aspects. As a response to shade, the production of CH flowers was shortened and the production of CH fruit finished earlier relative to plants in open sub-plots. On the other hand, CL fruit (and, therefore, CL flowers) were produced earlier in plants under shaded conditions than in those plants in open sub-plots. In contrast to previous studies reporting that sub-optimal conditions increase the production of CL flowers relative to CH flowers (Culley and Klooster, 2007), our results have shown that *R. nudiflora* produces a similar number of CH and CL flowers or fruit regardless of environmental conditions; instead, temporal patterns of CH and CL flower and fruit production are differentially influenced by light availability. An earlier production of CL flowers

(and subsequently fruit) and a shorter period of production of CH flowers may be advantageous under shaded conditions even if the total number of flowers or fruit remains constant. *Ruellia nudiflora* starts its reproduction with CL flowers followed by production of CH flowers, and produces CL flowers once again at the end of the reproductive season. This sequence was not affected by water or light availability; therefore, an earlier production of CL flowers and fruit actually represents an earlier start of the reproductive season. Earlier flowering is usually positively correlated with reproductive success in plants (Munguía-Rosas et al., 2011) and this might be the case for our study species. On the other hand, a shorter period of production of CH flowers under shaded conditions, but in a similar quantity to those under more optimal conditions, may favour pollinator attraction, and therefore pollination success, because a larger floral display is presented. Our study also showed that *R. nudiflora* allocates more resources to flowers receiving larger pollen loads, and this could result in more vigorous and potentially more successful progeny in sub-optimal environments. This work reveals the importance of looking at fine scale changes in the phenology of CH and CL flowers and fruits using suitable quantitative methods. Up to now, previous studies looking at phenology of cleistogamous plants had focused only on the total production of CH and CL flowers or on qualitative temporal patterns (e.g. Jasieniuk and Lechowicz, 1987; Sigrist and Sazima, 2002; Imaizumi et al., 2008) and were unable to detect small variations in phenology in response to environmental variables.

It has been suggested that annual and perennial cleistogamous plants differ in their reproductive phenology (Oakley et al., 2007). According to these authors, in annuals CL structures are produced first (in some cases also at the end), followed by simultaneous production of CL and CH. In contrast, perennials usually sequentially produce CH and CL structures; in some cases, the production of CL structures occurs after CH but never before (Oakley et al., 2007). *Ruellia nudiflora* does not follow the pattern predicted for perennials: invariably CL structures are produced first, regardless of environmental conditions. Although some previous studies showed that soil moisture affects production of CH and CL flowers (e.g. Bell and Quinn, 1987), we did not identify any effect of watering on phenology or resource allocation. Our experiment was conducted under field conditions and therefore some limitations exist, such as the inability to avoid or reduce natural rainfall over specific sub-plots without disturbing light availability. Even so, we know that non-watered sub-plots were under sub-optimal or stressful conditions of water availability for a longer period of time than plants in watered sub-plots (Supplementary Data Fig. S2). However, we cannot rule out completely the possibility that the phenology of *R. nudiflora* may respond to an even stronger water deficit.

Sub-optimal (shaded) conditions affected not only the phenology of reproductive structures in *R. nudiflora*, but also patterns of resource allocation among CL fruit and CH fruit from flowers receiving larger pollen loads. Previous reviews have suggested that, in general, CL structures have priority over CH structures under sub-optimal conditions (e.g. Oakley et al., 2007). Under shaded conditions *R. nudiflora* plants allocated resources earlier to produce CL flowers and

fruits; however, the total number of these structures and the biomass allocated to CL seeds were not affected by shade or watering. Interestingly, our results suggest that, under sub-optimal conditions, those seeds sired from CH flowers receiving extra pollen may also be prioritized in cleistogamous plants. CL seeds, as previously suggested, may ensure reproduction under disadvantageous conditions (Campbell *et al.*, 1983; Culley and Klooster, 2007), but also, good-quality CH seeds may produce highly competitive seedlings under sub-optimal conditions. Sired seeds from larger pollen loads are not only heavier (Richardson and Stephenson, 1992), but their size is also positively correlated with performance in some species (Richardson and Stephenson, 1992; Bernard and Toft, 2007) including some cleistogamous species (e.g. Trapp and Hendrix, 1988; Berg and Redbo-Torstensson, 2000). Therefore, a superior performance of seeds sired from pollen-supplemented CH flowers is likely in *R. nudiflora*. Research in the study area has revealed that *R. nudiflora* has high genetic diversity (78% of polymorphic loci; Ortegón-Campos, 2010) and a high outcrossing rate ( $t_m = 0.9$ ; Marrufo, UADY, Mérida, México, unpubl. res.), which suggest that it is the size of the pollen load and not the origin (i.e. selfed vs. outcrossed) that is affecting preferential resource allocation to pollen-supplemented CH flowers. Possibly plants did not allocate more resources to pollen-supplemented CH flowers in open sub-plots because these plants are not resource limited and, therefore, these plants are able to allocate resources to CH flowers equally regardless of the size of the pollen load. It is unlikely that our results in terms of resource allocation (i.e. greater allocation to pollen-supplemented CH flowers under shaded conditions) would be an artefact introduced by limitation in pollinator service due to the shade cloth. First of all, the cloth was placed about 1 m from the soil, which allowed visitors to move freely beneath the cloth as well as fly over open sub-plots. Secondly, we did not identify any effect of shade on per-seed weight as a single factor, i.e. all non-manipulated CH flowers produced seeds of similar weight in open and shaded sub-plots (Fig. 2). Previous studies have documented that when applied to a sub-set of flowers, pollen supplementation is not indicative of pollen limitation; instead, it is useful to assess resource re-allocation (Parra-Tabla *et al.* 1998; Knight *et al.*, 2005, 2006).

In conclusion, our study suggests that temporal patterns in production of CH and CL structures are affected differentially by shade; CL structures are produced earlier under shaded conditions whilst CH structures were produced for shorter periods. However, in addition to an earlier production of CL structures, *R. nudiflora* also preferentially allocates resources to those CH flowers receiving larger pollen loads. Earlier production of CL seeds and potentially more vigorous CH seeds can be a mixed strategy to face unpredictable environments if earlier production of CL structures and the size of the CH seeds are both positively correlated with subsequent success of the resulting seedling from each seed type. If this is the case, CL progeny may provide cheap reproductive assurance if environmental conditions improve. Instead, CH seeds sired from large pollen loads may be highly competitive if sub-optimal conditions persist. Since *R. nudiflora* is an invasive weed in Mexico (Villaseñor and Espinosa-García, 2004; Cervera and

Parra-Tabla, 2009), our results may also be useful to understand the reproductive strategies allowing invasive species to colonize new habitats (Cheplick, 2006; Imaizumi *et al.*, 2008). Future studies looking at the effect of environment on resource allocation in any cleistogamous plant should also consider that the size of the pollen load may produce differential allocation patterns in CH seeds. It has been suggested that cleistogamy evolved as a response to environmental heterogeneity (Schoen and Lloyd, 1984); therefore, allocation to flowers, allocation to seeds and the performance of the progeny may produce different patterns under optimal and sub-optimal conditions.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Figure S1: light response curves for *Ruellia nudiflora* individuals acclimated to 50 and 90% of total ambient photosynthetic photon flux density (PPFD). Figure S2: number of days of indicated average midday leaf water potential during the period of the field experimental conditions for watered and non-watered individuals of *Ruellia nudiflora*.

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