

Effects of pollen load, parasitoids and the environment on pre-dispersal seed predation in the cleistogamous *Ruellia nudiflora*

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Abstract Few studies have simultaneously addressed the effects of biotic and abiotic factors on pre-dispersal seed predation (PSP). Plant–seed predator interactions may be influenced by natural enemies and pollinators (the latter through changes in fruit or seed traits), and the activity of pre-dispersal seed predators and their natural enemies may both be affected by the abiotic environment. Additionally, in the case of cleistogamous plants with fruit dimorphism, PSP may be biased towards larger and more seeded chasmogamous (CH) fruits [relative to the smaller cleistogamous (CL) fruits], and the effects of biotic and abiotic factors may be contingent upon this fruit dimorphism. We studied PSP in the cleistogamous *Ruellia nudiflora* using a split-plot experimental design and asked the following: (1) is PSP biased towards CH fruits and is there an effect of pollen load on PSP? (2) Do parasitoids influence PSP and is their effect influenced by pollen load or fruit type? And (3) do light and water availability modify PSP and parasitoid effects? PSP was higher for CH relative to CL fruits, and under low water availability it was lower for pollen-

supplemented CH fruits relative to open-pollinated CH fruits. Parasitoids were not influenced by abiotic conditions, but their negative effect on PSP was stronger for pollen-supplemented CH fruits. Overall, we show that fruit dimorphism, abiotic factors and natural enemies affect PSP, and that these effects can be non-additive.

Keywords Cleistogamy · Fruit dimorphism · Seed predation · Shade · Watering

Introduction

Pre-dispersal seed predation (PSP) has been shown to not only affect the performance of individual plants and populations (Crawley 1992; Louda 1982), but also plant species composition and ecosystem function (reviewed by Kolb et al. 2007). Pre-dispersal seed predators are also a strong selective force acting on flower, fruit and seed traits (Gómez and Zamora 1994; Ehrlén et al. 2002; Cariveau et al. 2004; Whitney and Stanton 2004; Kolb and Ehrlén 2010), as well as on the evolution and maintenance of plant sexual dimorphisms when attack is consistently biased towards plant traits associated with a specific gender within a population (Marshall and Ganders 2001; Ashman 2002). However, little is known about the ecological and evolutionary consequences of PSP in plants that present floral dimorphisms within each individual such as cleistogamous species.

Dimorphic cleistogamy (hereafter “cleistogamy”) is an intra-plant floral dimorphism present in several angiosperm species (693 species in 50 families) and is relatively widespread in some families (9 % of Violaceae and 5 % of Poaceae; Campbell et al. 1983; Culley and Klooster 2007). In cleistogamous species, each plant produces closed,

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obligately self-pollinated cleistogamous (CL) flowers, and open, potentially out-crossed chasmogamous (CH) flowers (Culley and Klooster 2007). This dimorphism may influence the behaviour of seed predators because fruits developing from CH flowers are usually larger and more seeded than fruits from CL flowers, presumably representing a more attractive resource for herbivores. Accordingly, previous studies have shown that adult females use oviposition cues, which correlate with the survival of the predator's progeny such as fruit or seed traits (e.g. chemical composition, size, toughness; Díaz-Fleischer and Aluja 2003; Östergård et al. 2007; Sharma and Amritphale 2008). In addition to differences in size and number of seeds between CH and CL fruits, CH flowers in the same plant may receive pollen loads of a variable size, which may be at least partly due to pollinator activity (Herrera 2000; Cariveau et al. 2004). CH flowers receiving larger pollen loads usually obtain more resources from the plant and produce more or larger seeds (Knight et al. 2005, 2006), and thus represent a more suitable resource for seed predators.

Aside from fruit or seed traits, PSP may be influenced by abiotic conditions as well as natural enemies of seed predators. For example, increased PSP under humid and shaded conditions has been reported in a number of studies (Traveset 1991; Leimu et al. 2002; Kolb and Ehrlén 2010), and seed predator-mediated selection on plant traits has been shown to depend on environmental context (Kolb and Ehrlén 2010). In addition, parasitoids are among the most important natural enemies of invertebrate herbivores (Price 1984; Morris 1991; Hawkins et al. 1997) and can have substantial negative impacts on PSP, which may in turn positively affect plant reproduction (e.g. Gómez and Zamora 1994; Abdala-Roberts et al. 2010). Despite the evidence gathered thus far, previous studies have not addressed how abiotic factors such as water and light availability interact with fruit dimorphism and pollinator effects (via changes in pollen load) to shape variation in PSP. Moreover, the third trophic level may also respond to these factors, adding another layer of complexity to the understanding of PSP patterns. Addressing the interactive effects of biotic (e.g. pollinators and parasitoids) and abiotic factors on PSP is even more challenging in cleistogamous species because these factors may interact with fruit type (CH or CL). For instance, pollinators affect the pollen load size of CH flowers (but not CL), and fruits developed from flowers receiving larger pollen loads may be not only more attractive for the seed predator but also indirectly affect parasitoids by influencing the opportunity of finding an herbivore. However, these relationships may vary depending on the environmental context if the activity of pollinators, seed predators or parasitoids is influenced by the abiotic environment (Fig. 1).

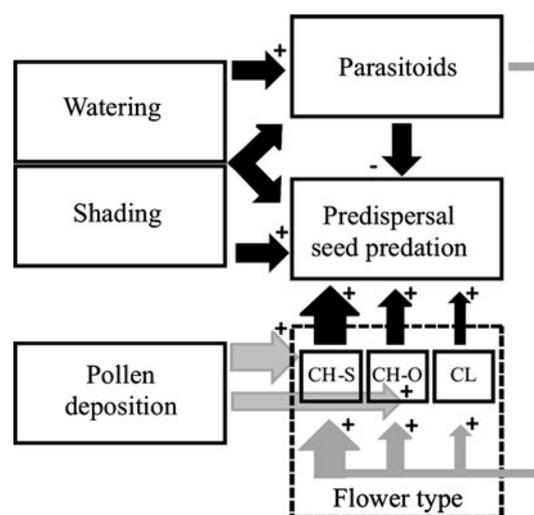


Fig. 1 Conceptual diagram representing the hypothesized effects of shading, watering, pollen supplementation (*Pollen deposition*), parasitoids and flower type [open-pollinated (*CH-O*) and pollen-supplemented chasmogamous (*CH-S*) and cleistogamous flowers (*CL*)] on pre-dispersal seed predation associated with *Ruellia nudiflora*. Arrow thickness and sign represent the expected strength and direction (positive or negative) of the effect, respectively. Black arrows indicate effects on pre-dispersal seed predation and grey arrows effects on biomass of fruits or seeds; see text for details of predation

Like other cleistogamous plants, *Ruellia nudiflora* (Acanthaceae) exhibits a fruit dimorphism: CL fruits are smaller, produce fewer seeds and are usually located at a more basal position on the plant than are CH fruits (Standley et al. 1974). Seeds from both fruit types are consumed by larvae of a noctuid moth, which in turn is attacked by several species of parasitoids (Abdala-Roberts et al. 2010). Based on an experimental manipulation of biotic and abiotic factors, in this work we address the effects of pollen load size, parasitoids, shading and watering on PSP associated with CH and CL fruits in *R. nudiflora*. First, we predicted that PSP would be CH-biased because these fruits are presumably more attractive for ovipositing female moths (Fig. 1). Second, we tested the hypothesis that CH fruits produced by larger pollen loads allocate more resources to seeds (Knight et al. 2005, 2006) with this resulting in a greater likelihood of seed predator attack (Fig. 1). Third, we assessed the effect of parasitoids on PSP as well as if this effect was contingent upon fruit type and pollen load. Because parasitoids track seed predator abundance, and based on the predictions of higher PSP for CH fruits, we also expected that parasitoid frequency and top-down effects would be greater on CH fruits relative to CL fruits, especially in CH fruits receiving additional pollen (Fig. 1). Finally, we addressed the influence of water and light availability on PSP, as well as the effects of fruit type and pollen load on seed predators and

parasitoids were contingent upon these abiotic factors (Fig. 1). Although for other cleistogamous species shade and water stress have been shown to reduce CH flower number and increase CL flower production (Culley and Klooster 2007), a recent study indicated that watering and shading do not influence the number of CH or CL flowers produced by *R. nudiflora* (Munguía-Rosas et al. 2012). Thus, we expected abiotic effects on PSP not to act via changes in relative allocation to CH and CL structures, but rather by directly influencing habitat choice or activity level of seed predators or parasitoids (Fig. 1). Similarly, we hypothesized that parasitoids would negatively influence PSP through a direct effect on seed predators (Fig. 1).

Overall, in examining the effects of plant traits (fruit dimorphism), pollen load, the abiotic environment, and the third trophic level, this study provides an integrative and novel evaluation of multiple factors non-additively shaping PSP patterns. In doing so, this work advances our understanding of the relative importance and potential for non-additive effects of plant traits, abiotic and biotic factors on plant–seed predator interactions.

Materials and methods

Study system

The study site was located 4 km east of Molas, in the municipality of Merida in north-central Yucatan, Mexico (20°49′51″N, 89°36′44.2″W, 10 m a.s.l.). The climate at this site is warm subhumid with summer rains, and the mean annual rainfall and temperature are 850 mm and 26.2 °C, respectively (Chico-Ponce de León 1999). Vegetation is represented by a heavily disturbed tropical dry forest with discontinuous canopy, for which dominant tree species are *Bursera simaruba* (Burseraceae) and *Lysiloma latisiliquum* (Fabaceae). *Gymnopodium floribundum* (Polygonaceae); the herb *Parmentiera millspaughiana* (Bignoniaceae) dominates the understory (Flores and Espejel 1994).

R. nudiflora is a perennial herb which is distributed from Texas to the Caribbean (Long 1977), reproduces mainly by seed (Munguía-Rosas, unpublished data) and has a dimorphic cleistogamous reproductive system (sensu Culley and Klooster 2007): CL flowers are reduced in size, with corollas that do not open and are obligately self-pollinated, whilst CH flowers are larger, do open, and can be either self- or cross-pollinated (Abdala-Roberts et al. 2009). Although CH flowers are self-compatible, self-pollinated CH flowers have a lower probability [$38 \pm 4\%$ (hereafter mean ± 1 SE), $n = 15$ plants] of setting fruit relative to those receiving allogamous pollen ($78 \pm 4\%$, $n = 10$ plants; Abdala-Roberts et al., unpublished data).

Pollinators are generalist bees and butterflies, which are abundant and usually briefly visit a few flowers per plant (Munguía-Rosas, unpublished data). Previous research has shown that the inbreeding coefficient of established reproductive adult plants of *R. nudiflora* corresponds to an outcrossing rate of 0.99 ± 0.01 in the study area, suggesting that selfed offspring are produced infrequently or that they are outperformed by out-crossed progeny during early life stages (Marrufo 2011). CL flowers and fruits are produced mainly at the beginning (April–June) and at the end (October–December) of the reproductive season, whilst CH flowers are produced mostly during the middle of the reproductive season (June–September); hence, CH and CL phenologies exhibit limited overlap throughout the reproductive season (Munguía-Rosas et al. 2012). There is no evidence that phenology and pollinator or seed predator abundance are linked at the study population given that pollinator and seed predator availability remains relatively constant throughout *R. nudiflora*'s reproductive season (Munguía-Rosas, unpublished data). Fruits are dry capsules that are dispersed ballistically; CL fruits are typically smaller (16.61 ± 0.22 mg, $n = 1,089$ fruits), fewer seeded (8.14 ± 0.07 seeds, $n = 1,089$ fruits) and located at a more basal position on the plant than CH fruits (28.7 ± 0.46 mg; 11.51 ± 0.17 seeds, $n = 806$ fruits). Similarly, CL seeds are smaller (mean seed mass, 1.95 ± 0.04 mg, $n = 1,089$ fruits) than CH seeds (2.6 ± 0.04 mg, $n = 806$ fruits). A plant may produce from 60 to 80 CH fruits and from 80 to 100 CL fruits throughout a reproductive season. During this study, the proportion of CH ($47 \pm 1.76\%$) and CL ($53 \pm 1.76\%$) fruits produced per plant was slightly skewed for CL ($n = 271$ plants).

In the Yucatan Peninsula, *R. nudiflora* is found in habitats with different light availability, soil fertility and biotic conditions (Ortegón-Campos et al. 2009; Ortegón-Campos 2010). Environmental factors such as shade and water availability do not appear to affect the production of CH and CL fruits [CH fruit and CL fruit production for plants, open, non-watered plots, 82.84 ± 11.84 and 106.54 ± 8.06 ; shaded, non-watered plots, 71.93 ± 8.35 and 95.11 ± 9.89 ; open, watered plots, 76.27 ± 7.26 and 93.68 ± 5.96 ; and shaded, watered plots, 68.98 ± 7.59 and 88.44 ± 9.68 ; effects of shading ($F_{1,10} = 0.99$ – 1.39 ; $P = 0.26$ – 0.34) and watering ($F_{1,20} = 0.66$ – 3.53 ; $P = 0.07$ – 0.42) on number of CH and CL fruits were not statistically significant, see Munguía-Rosas et al. (2012) for details]. Larvae of an unidentified noctuid moth consume seeds previous to fruit dehiscence; adult females oviposit on recently formed fruits but oviposition cues are unknown. Typically, a single larva feeds inside a given fruit and leaves the fruit through an exit hole (Abdala-Roberts et al. 2010). For the examined fruits, we observed that larvae frequently damaged individual seeds to a

variable extent. Preliminary data ($n = 271$ plants) indicate that, on average, $43 \pm 1.25\%$ of the fruits produced per plant are attacked by this seed predator at the study site. Larvae of this moth are in turn attacked by up to seven species of parasitic wasps, some of which belong to the Braconidae (e.g., *Bracon* sp., *Chelonus* sp.) and Chalcididae (Abdala-Roberts et al. 2010).

Experimental design and treatments

In early April 2010, we randomly placed 11 main plots (2×2 m) throughout an area of ca. 2 ha where *R. nudiflora* naturally occurred. The shortest distance between any given pair of plots was 3.5 m. When plots were established, plants had not started reproducing; for this reason, the number of reproductive plants subsequently available per plot was highly variable. We used cotton thread to divide each main plot into two 2×1 -m sub-plots, one of which was shaded with a nylon mesh placed 90 cm above the forest floor and supported with four thin wood columns (2 cm diameter); this mesh reduced ca. 50 % of ambient photosynthetic photon flux density. The long side (2 m) of adjacent shaded and open sub-plots within each main plot was oriented from east to west, such that shading was projected toward the west in the morning and toward the east in the afternoon thus causing a minimal shading of the contiguous open sub-plot. Flower visitors and seed predators moved freely beneath the mesh, and across open and shaded sub-plots. Once again, we used cotton thread to divide each sub-plot into two, 1×1 -m sub-sub-plots (hereafter “minor plots”), two of them (one shaded and one open) were watered twice a week up to soil field capacity (ca. 5 L per minor plot every time) from 10 April 2010 to 22 October 2010. We started watering 1 month before the rainy season began and finished watering 1 month after the end of the rainy season. All 5 L of water was added to the centre of each minor plot, and visual inspection indicated that water did not reach contiguous plots, likely due to good water drainage conditions of the soil (i.e. high permeability and porosity; Munguía-Rosas et al. 2012). We tagged all reproductive plants (from one to seven plants) per minor plot. Because new *R. nudiflora* recruits do not reproduce during the first year of life, and to facilitate data collection, these young plants and individuals of other species were removed from all 11 main plots. In total, we tagged 271 plants: 56 plants in the control group (open and non-watered minor plots), 72 plants were shaded but not watered, 74 plants were shaded and watered, and 69 plants were watered but not shaded.

From early June to mid September 2010, we performed pollen supplementation on haphazardly chosen CH flowers from 1000 to 1100 hours using one pollen donor per flower, with the donor being located outside the

experimental plots. Pollen was placed gently on receptive stigmas of non-emasculated flowers until the stigma was visibly covered with pollen. We used only one pollen donor to standardize the number of donors per plant and because CH flower availability was limited outside the plots. We performed pollen supplementation of CH flowers for all 271 plants used in this study. In order to obtain a large enough sample size, a high proportion (about 80 %) of CH flowers available per plant on a given day were pollen supplemented once a week. In total, 835 CH flowers were pollen-supplemented during the experiment. By increasing pollen load size and presumably also pollen quality (i.e. an increase in the number of donors by one), this manipulation was aimed at enhancing pollination success relative to open-pollinated flowers. There was no evidence of pollen limitation in this population, i.e. fruits developed from open-pollinated flowers (hereafter “CH-O fruits”) and fruits developed from pollen-supplemented flowers (hereafter “CH-S fruits”) did not differ in seed number ($F_{1,1032} = 0.34$, $P = 0.78$) based on a generalized mixed-effects model with Poisson error distribution, where fruit type (CH-O, CH-S) was the fixed factor and the hierarchical arrangement of plots the random factors (see “Statistical analyses” below). Supplemented flowers were tagged but not bagged in order to avoid disruption of pollinator behaviour.

Pre-dispersal seed predation

We conducted weekly harvests of all available ripe fruits (CH-O, CH-S, and CL) per plant during the reproductive season (15.24 ± 0.79 fruits were collected per plant) and stored each fruit in individual bags for further examination in the laboratory. Whilst the sample of CH-S fruits was represented by cohorts of fruits from weekly hand-pollination, the sample of CH-O fruits was based on a continuous fruit production taking place throughout the week. Therefore, the number of CH-O fruits recorded was larger than the number of CH-S fruits sampled despite the fact that we pollinated 80 % of the flowers available per plant at each weekly sampling date. Each fruit was weighed, examined externally and internally for direct (larvae, damage in seeds) and indirect (frass, parasitoids or exit holes) evidence of PSP.

Parasitoid incidence

We assessed parasitoid incidence by examining fruits with signs of seed predation (see previous section). We dissected fruits and examined them carefully under the light microscope in search of parasitoid specimens (larvae or adults) or cocoons. We also searched for parasitoids inside bags where the fruits were kept because parasitoids

frequently exit the fruit a few days after samples are stored. Parasitoids and their cocoons are very small (ca. 1.5 mm) and their weight is negligible (less than 0.001 mg) relative to the weight of a seed (2.51 ± 0.05 mg, $n = 1,895$ seeds). In our experience, early death of seed predators rarely happens (<1 % of examined fruits) and first- and second-instar larvae are attacked by several of the parasitoid species; therefore, early caterpillar death likely did not bias parasitoid incidence.

Statistical analyses

We expressed PSP as the amount of seed biomass consumed by seed predators. Because we could only calculate the current biomass of each damaged fruit, we estimated the consumed biomass by subtracting current fruit weight from the mean biomass of undamaged fruits. To be conservative and select the appropriate undamaged fruit mean weight to estimate seed predation, we assessed differences in weight among undamaged fruits of each type (based on a posteriori contrasts) in the different shading and watering treatments using a linear mixed-effects model where fruit weight was the response variable and fruit type was an explanatory fixed factor (three levels: CL, CH-O and CH-S); the hierarchical structure of the experimental design (nested organization of plants, minor plots, subplots in main plots) was accounted for in the random portion of the model. Results from this model showed that differences in weight among fruit types were statistically significant ($F_{2,1906} = 301.26$, $P < 0.01$) and, according to a posteriori contrasts, CH-O (28.74 ± 0.45 mg) and CH-S (30.26 ± 1.09 mg) fruits differed ($t = 20$, 22 ; $P < 0.01$ in both cases) in weight relative to CL fruits (16.61 ± 0.22 mg), but did not differ themselves ($t = 1.67$; $P = 0.09$). In only 4 % of the cases, slightly damaged fruits weighed more than the mean weight of undamaged fruits, and were thus considered as undamaged in the analysis. The weight of undamaged fruits was not affected by shading ($F_{1,10} = 1.21$, $P = 0.3$) or watering ($F_{1,21} = 0.27$, $P = 0.8$), which justified pooling undamaged fruits from both treatments to calculate the amount of consumed biomass by the seed predator. We cannot rule out the possibility that seed predators discriminated against undamaged fruits due to their potentially smaller size, but this would lead to a conservative estimate of PSP (i.e. a smaller difference in biomass between damaged fruits and the mean of undamaged fruits). We also calculated the number of seeds consumed by subtracting the number of seeds (only seeds with more than 75 % of intact biomass) from the mean seed number of undamaged fruits for each fruit type; however, the model using the number of consumed seeds did not differ qualitatively from that using consumed seed biomass, and we therefore only show

results for the latter. Furthermore, we consider that estimating seed predation in terms of consumed seed biomass is more accurate than a quantification of seed number because many seeds were only partially eaten and it is known that partial seed damage reduces, but does not preclude, seed survival (Vallejo-Marín et al. 2006). In most cases, we did not find seed predator larvae alive inside fruits; instead, we found indirect evidence of their presence (frass, remains of larva) which had a negligible weight (up to 0.01 mg).

We used the above-mentioned estimation of PSP per fruit as the response variable in a linear mixed-effects model where fruit type (three levels: CH-O, CH-S and CL), shading (two levels: open and shaded sub-plots), watering (two levels: watered and non-watered minor plots), and all two-way and three-way interactions were included as fixed effects. The hierarchical structure of the split-split-plot design (with plant being the smallest hierarchical level) was considered in the random portion of the model to account for pseudoreplication (Pinheiro and Bates 2000). To evaluate the overall effect of PSP on reproductive output at the plant level, we also performed a model testing for effects on PSP in terms of the proportion of seed mass consumed by seed predators per plant [sum of seed mass lost in all fruits collected per plant divided by an estimation of total seed mass prior to predation (i.e. number of fruits multiplied by the mean seed mass of undamaged fruits)]. Because PSP at the plant level was expressed as a proportion, we fit PSP to a generalized linear mixed-effects model with binomial error and logit link function where shading, watering and the degree of cleistogamy (a covariate) were included in the fixed portion of the model. The degree of cleistogamy is the proportion of CL fruits produced per plant (relative to the total, i.e. both CL and CH, fruits produced). The random portion of the model was as described for the previous model except that the plant was not included as an independent variable. We fitted the per fruit and plant-level PSP models with the lme and glmmPQL functions, respectively. The lme routine belongs to the nlme library and glmmPQL routine belongs to the MASS library, both in R 2.10.1 software (R Development Core Team 2009). Linear mixed-effects and generalized linear mixed-effects models were fitted using maximum likelihood (Pinheiro and Bates 2000) and penalized quasi-likelihood methods (Venables and Ripley 2002), respectively. We used a posteriori contrast to assess differences among types of fruit and we inspected residual distribution to assess model fit (Crawley 2007). When interactions among main effects were significant, we assessed differences between means of treatment levels for each main effect at a time (simple main effects).

To assess how experimental factors affected parasitoids, we used the incidence of parasitoids (presence or absence)

Table 1 Results from a linear mixed-effects model analysis assessing the effects of fruit type, shade and water availability on pre-dispersal seed predation in *Ruellia nudiflora*

Source of variation	Statistics	<i>P</i> value
Fruit type	$F_{2,3706} = 71.134$	<0.001
Shade	$F_{1,10} = 6.946$	0.025
Watering	$F_{1,20} = 8.994$	0.007
Shade × watering	$F_{1,20} = 0.165$	0.688
Fruit type × watering	$F_{2,3706} = 6.051$	0.002
Fruit type × shade	$F_{2,3706} = 2.837$	0.066
Shade × water × fruit type	$F_{2,3706} = 2.161$	0.1154

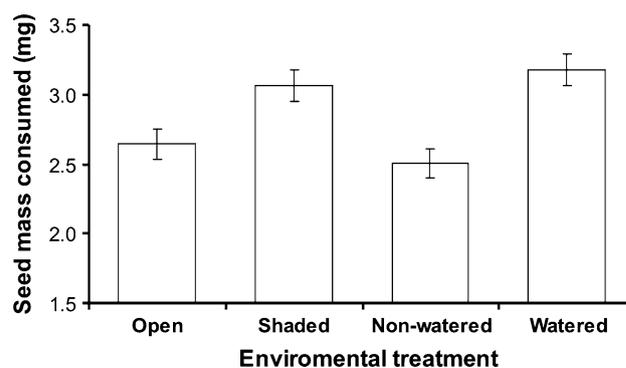
Pre-dispersal seed predation was estimated by subtracting the mass of a given damaged fruit from the mean mass of undamaged fruits of the same type. The effect of plant was included in the model to control for pseudoreplication when testing for the effect of fruit type. Only fixed effects are shown

in damaged fruits as the response variable in a mixed-effects generalized linear model with binomial error distribution and logit link function where the fixed effects were: fruit type, shade and watering. To assess if parasitoid incidence is correlated with seed predation level (likely a surrogate of seed predator abundance) we included per fruit PSP in the model as a covariate (estimated as described earlier). We considered all but the fourth-order interaction among fixed factors to facilitate matrix convergence. Finally, again based only on damaged fruits, we examined the effect of parasitoid presence on PSP and if this effect was contingent upon fruit type using a linear mixed-effects model where the response variable was PSP and the explanatory fixed-effects factors were parasitoid incidence, fruit type and their interaction. Random effects in these two models were as described before. The first model (parasitoid incidence model) was fitted with the `glmmPQL` function whilst the second model (parasitoid effects on PSP model) was fitted with the `lme` function. Models were fitted with the methods and software previously described. As for PSP models, differences among fruit types were examined with a posteriori contrasts and simple main effects were examined when an interaction term was significant.

Results

Pre-dispersal seed predation

PSP differed among fruit types (Table 1). The greatest per fruit PSP was observed for CH–O fruits (3.88 ± 0.15 mg, $n = 1,667$), followed by CH–S fruits (2.77 ± 0.28 , $n = 412$), and finally CL fruits, which showed the lowest mean value (1.97 ± 0.07 mg, $n = 1,906$). Both shading and watering had positive effects on PSP (Table 1); mean

**Fig. 2** Effect of shading and watering on *R. nudiflora* pre-dispersal seed predation. Bars represent the mean \pm SE ($n = 3,985$ fruits)

per fruit PSP in open sub-plots ($n = 2,013$ fruits) was 0.43 mg lower than in shaded sub-plots ($n = 1,972$ fruits), whilst mean per fruit PSP for non-watered minor plots ($n = 2,086$ fruits) was 0.61 mg lower than in watered minor plots ($n = 1,899$ fruits) (Fig. 2). Shading and watering effects on PSP did not interact significantly (Table 1; Fig. 2). Nonetheless, fruit type interacted significantly with the watering treatment (Table 1) because the magnitude of increase in PSP (Δ) due to watering was larger and statistically significant only for CH–S fruits ($\Delta = 1.75$ mg; watered minor plots, $n = 192$ fruits; non-watered minor plots, $n = 220$ fruits; $F_{1,21} = 4.46$, $P = 0.04$). Differences between watering treatments were not significant for CH–O fruits ($\Delta = 1.06$ mg; watered minor plots, $n = 901$ fruits; non-watered minor plots, $n = 766$ fruits; $F_{1,21} = 3.02$, $P = 0.09$) or CL fruits ($\Delta = 0.03$ mg; watered minor plots, $n = 993$ fruits; non-watered minor plots, $n = 913$ fruits; $F_{1,21} = 0.15$, $P = 0.70$) (Fig. 3).

At the plant level, PSP was also affected by shading ($F_{1,10} = 5.51$, $P = 0.04$) and watering ($F_{1,21} = 12.21$, $P < 0.01$), but not by the degree of cleistogamy ($F_{1,226} = 0.22$, $P = 0.63$). Specifically, percent of seed

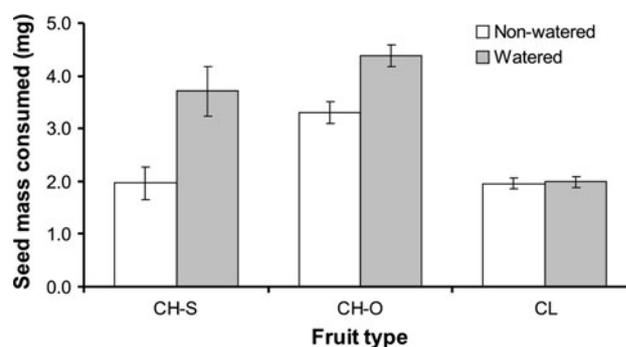
**Fig. 3** Effect of watering treatment on *R. nudiflora* pre-dispersal seed predation in fruits produced by different pollination treatments: CH–O, CH–S and CL flowers. Bars represent the mean \pm SE ($n = 3,985$ fruits). For abbreviations, see Fig. 1

Table 2 Results from a generalized linear mixed-effects model assessing the effect of fruit type, shading, watering and pre-dispersal seed predation on the incidence of parasitoids in *R. nudiflora*

Source of variation	Statistics	<i>P</i> value
Fruit type	$F_{1,1546} = 32.385$	0.002
Shade	$F_{1,10} = 3.925$	0.076
Watering	$F_{1,20} = 1.835$	0.191
Seed predation	$F_{1,1546} = 25.267$	<0.001
Fruit type × shade	$F_{2,1546} = 0.393$	0.675
Fruit type × watering	$F_{2,1546} = 0.432$	0.649
Fruit type × seed predation	$F_{2,1546} = 2.252$	0.105
Shade × watering	$F_{1,20} = 0.021$	0.887
Watering × seed predation	$F_{1,1546} = 2.633$	0.105
Shade × seed predation	$F_{1,1546} = 0.232$	0.630
Fruit type × shade × water	$F_{2,1546} = 0.075$	0.927
Fruit type × water × seed predation	$F_{2,1546} = 0.749$	0.473
Fruit type × shade × seed predation	$F_{2,1546} = 0.183$	0.873
Water × shade × seed predation	$F_{1,1546} = 0.538$	0.463

Pre-dispersal seed predation was estimated as the amount of seed biomass consumed by seed predators. The plant was considered as the smallest plot in the random portion of the model to account for pseudoreplication when testing for the fruit type effect. Only fixed effects are shown

biomass eaten by seed predators at the plant level was greater in plants from shaded sub-plots (15.2 ± 1.01 %, $n = 146$ plants) than in plants of open sub-plots (12.3 ± 0.79 %, $n = 125$ plants), whilst plants in watered (14.8 ± 0.93 %, $n = 143$ plants) minor plots showed greater PSP than plants in non-watered (12.7 ± 0.92 %, $n = 128$ plants) minor plots.

Parasitoid incidence

Based only on damaged fruits, we found that parasitoid incidence differed significantly among fruit types. Parasitoids were present in 43 % of the CH-O fruits ($n = 861$), 36 % of the CH-S fruits ($n = 817$), and 33 % of the CL fruits ($n = 141$). However, a posteriori contrasts indicated that only CH-O and CL fruits differed significantly. Parasitoid incidence in damaged fruits was negatively correlated with per fruit PSP (coefficient = -0.06 , $Z = 2.66$, $P = 0.01$). No other factor or interaction included in the model was statistically significant (Table 2).

Pre-dispersal seed predation of damaged fruits differed significantly among all three fruit types (Table 3), with CH-S fruits being the most heavily attacked (8.11 ± 0.02 mg, $n = 141$ fruits), followed by CH-O fruits (7.52 ± 0.004 mg, $n = 861$ fruits), and finally CL fruits (4.61 ± 0.002 mg, $n = 817$ fruits). We found a significant effect of parasitoids on PSP (Table 3): PSP was lower when parasitoids were present (5.5 ± 0.004 mg,

Table 3 Results from a linear mixed-effects model assessing the effect of fruit type and parasitoid incidence on pre-dispersal seed predation in *R. nudiflora*

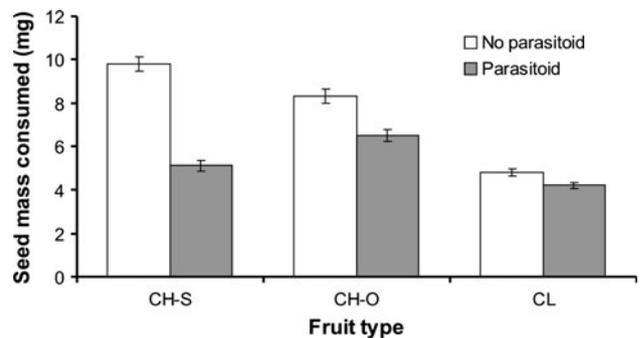
Source of variation	Statistics	<i>P</i> value
Fruit type	$F_{2,1559} = 79.945$	<0.001
Parasitoid incidence	$F_{1,1559} = 32.094$	<0.001
Fruit type × parasitoid incidence	$F_{2,1559} = 7.153$	0.008

Plant was considered as the smallest plot in the random portion of the model to avoid pseudoreplication. Only fixed effects are shown

$n = 691$ fruits) relative to when they were absent (6.73 ± 0.003 mg, $n = 1,128$ fruits). Moreover, the effects of fruit type and parasitoid incidence interacted significantly (Table 3). Specifically, the difference in PSP between fruits with and without parasitoids was much larger for CH-S fruits ($\Delta = 4.68$ mg; $n = 50$ fruits with parasitoid; $n = 91$ fruits without parasitoid; $F_{1,45} = 15$; $P < 0.01$), followed by CH-O fruits ($\Delta = 1.82$ mg; $n = 371$ fruits with parasitoid; $n = 490$ fruits without parasitoid; $F_{1,666} = 8.5$; $P < 0.01$), and finally CL fruits which showed the smallest reduction in PSP by parasitoids ($\Delta = 0.59$ mg; $n = 270$ fruits with parasitoid; $n = 547$ fruits without parasitoid; $F_{1,586} = 6.05$; $P = 0.01$) (Fig. 4).

Discussion

In this study, we have shown that the intensity of PSP on the cleistogamous *Ruellia nudiflora* is contingent on fruit type, and is also influenced by biotic (parasitoids and pollinators) and abiotic environmental factors (water and light availability), some of which non-additively shaped plant–seed predator interactions. Our study thus highlights the importance of approaching PSP from a multifactorial perspective where the outcomes of this interaction are influenced not only by the biotic and abiotic environment, but also by variation in plant traits determined by a

**Fig. 4** Effect of parasitoid on pre-dispersal seed predation in fruits produced from different pollination treatments: CH-O, CH-S and CL flowers. Bars represent the mean \pm SE ($n = 1,846$ fruits). For abbreviations, see Fig. 1

reproductive dimorphism, which characterizes cleistogamous plant species.

Independent and non-additive effects of fruit type and the environment on PSP

Although CH fruits are a less predictable resource than CL fruits (because production of the former is more temporally constrained and opportunistic) and CH flowers in cleistogamous plants typically have lower fruit-set than CL flowers (Schoen and Lloyd 1984; Oakley et al. 2007), here we found that CH fruits suffered from greater PSP than CL. This result is likely due to the fact that CH fruits in *Ruellia nudiflora* (as in many other cleistogamous plant species; Campbell et al. 1983; Goodwillie et al. 2005; Culley and Klooster 2007) are larger and have more seeds than CL fruits. The inherent larger size and seed number of CH fruits may represent greater resource availability for the offspring of seed predators and potentially lead to higher larval survival (Östergård et al. 2007). One issue we cannot discard, however, is that the more apical position of CH fruits in *R. nudiflora* may have also influenced the observed pattern of CH-biased attack. Accordingly, CH fruits are more apparent and thus presumably more easily detected by ovipositing females. Another alternative is that PSP is higher for CH fruits because more seed biomass is available for the herbivore in these fruits and this leads to an increase in their consumption; further experiments controlling for fruit size and type would be necessary to address this possibility. Confounding effects between fruit type and phenology are also possible owing to the fact that CH and CL output show limited phenological overlap. However, we suspect that phenology is likely of secondary importance relative to fruit type because there are other potential resources available year-round for the herbivore, as well as because CH fruit production is much more temporally constrained and less reliable than CL fruit production (the latter is true also for other cleistogamous species; Oakley et al. 2007). Regarding environmental variables, it appears that shaded and humid microhabitats are more favourable for *R. nudiflora*'s pre-dispersal seed predators, which agrees with findings from previous studies (Traveset 1991; Kolb and Ehrlén 2010). Indeed, abiotic factors represent a major determinant of insect survival and, in turn, of PSP; for example, about 50 % of bruchid eggs laid on pods of *Acacia farnesiana* died due to heat and desiccation in a tropical deciduous forest in Costa Rica (Traveset 1991). In our study area, an extended dry season of 6 months takes place every year and extreme temperatures of up to 43 °C have been frequently recorded at the study site during the reproductive season of *R. nudiflora*. Therefore, higher mortality of seed predator larvae leading to a reduction in PSP under hotter and drier conditions is

likely a relevant factor in our study system. Previous work with other cleistogamous species has shown that shade and water negatively and positively influence the production of CH and CL flowers, respectively (Culley and Klooster 2007), but this seems not to be the case for *R. nudiflora* in our study. Shading (but no watering) appears to only affect the phenology of CL fruits in this plant (earlier production), whilst the total number of structures (and thus the proportion) produced remain constant (Munguía-Rosas et al. 2012). Hence, although we cannot rule out completely an indirect effect of the abiotic environment on PSP through changes in reproductive allocation because reproductive output by fruit type did vary to some extent between treatments, it is likely that the observed effects of shade and watering on PSP represent mostly a direct effect of the abiotic environment on seed predator abundance through changes in habitat choice or activity level.

Interestingly, fruit type and watering effects on PSP were not additive. The magnitude of increase in PSP due to watering was greater for CH-S relative to CH-O and CL fruits. This result could be interpreted as CH-S fruits escaping from PSP to a greater extent—relative to the other fruit types—under drier conditions. Nonetheless, such an escape advantage by CH-S fruits disappears when water availability is high because watering led to an overall increase in PSP across all fruit types. We suggest that faster ripening of CH-S fruits under dry conditions might confer an advantage in terms of escape from seed predators. More rapid development of fruits receiving larger pollen loads has been reported for other species (e.g. Winsor et al. 1987; Dogteron et al. 2000), but the speed of fruit development in relation to pollen load in *R. nudiflora* remains to be quantified.

We found that watering and shading also had positive effects on PSP at the plant level, which is consistent with results on a per fruit basis. However, PSP at the plant level is not explained by among-plant variation in the degree of cleistogamy, suggesting that this variation was not large enough to influence significantly variation in PSP at the plant level.

Effects of fruit type and the environment on parasitoids, and non-additive effects of parasitoids and fruit-type on PSP

As for PSP, parasitoid incidence was also CH-biased and the likelihood of parasitoid occurrence was greatest for CH-O fruits which were also the most frequently attacked by seed predators; contrary to our expectations, however (see Fig. 1), pollen supplementation, shade and watering did not lead to a greater likelihood of parasitoid presence. PSP was reduced in fruits with parasitoids relative to fruits without parasitoids, suggesting that parasitoids effectively reduce seed predation

in *R. nudiflora*. Accordingly, a previous survey of the study species found that parasitoids “saved” 4–14 % of seeds on average ($n = 21$ populations) regardless of the type of fruit (Abdala-Roberts et al. 2010). In this study, however, we further discriminated by fruit type and found that the amount of seed biomass saved by parasitoids was larger in CH fruits (10.5 %) than in CL fruits (3 %). Moreover, although they occurred more frequently in CH–O fruits, parasitoids saved more seed biomass in CH–S fruits (15 %) than in the former fruit type (6 %). This adds a layer of complexity to variation in third trophic-level effects on producers as they illustrate that the intensity of species-level trophic cascades (sensu Polis et al. 2000) can be influenced by the plant’s reproductive system (in this case via fruit dimorphism) as well as pollen load; the latter finding may also be suggestive of indirect effects of pollinators on plants through their effects on fruit traits influencing not only seed predators but also parasitoids. Accordingly, it is possible that parasitoids caused a greater reduction of PSP in CH–S fruits because pollen supplementation modified fruit traits which influence parasitoid foraging. For instance, the detection of seed predators inside fruits by parasitoids is determined by plant release of volatile infochemicals upon damage (Dicke and Sabelis 1988; Dicke and Vet 1999); hence, CH–S fruits may release these volatiles earlier upon damage, or release a greater total amount presumably eliciting a stronger indirect defence. This hypothesis, however, remains to be formally tested.

Conclusion

Overall, our findings underline the importance of plant fruit dimorphisms, particularly those present in cleistogamous species, in shaping plant–seed predator interactions. In turn, CH-biased PSP may have important consequences for the plant’s mating system (selfing vs. out-crossing) and therefore, the genetic background of the progeny by selectively favouring obligately self-pollinated (CL) progeny. Another important generality from our findings is that the magnitude of seed predator effects is also contingent upon the abiotic environment, particularly water and light availability (in this case, likely through changes in seed predator foraging behaviour or habitat choice). Furthermore, in showing that parasitoid effects on seed predators, and in turn on plants, are contingent upon pollen load size (taken as a surrogate of pollinator service), this study uncover a previously unexplored relationship of ecological effects among seed predators, parasitoids and pollinators.

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