Soil fertility and parasitoids shape herbivore selection on plants

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Summary

1. Although plants and herbivores interact under varying soil resources and natural enemy effects, little is known about how these factors influence plant–herbivore interactions and shape the evolution of plant and herbivore traits. Here, we ask whether soil fertility and parasitoids shape selection on fruit number imposed by a seed predator (SP) on the perennial herb *Ruellia nudiflora*.

2. We used a common garden where half the plants of 14 genetic families were fertilized and recorded the abundance of cleistogamous (CL) fruits and seeds, SPs and parasitoids. We calculated relative fitness per family based on CL seed number under the following three scenarios: three trophic levels (accounting for SP and parasitoid effects), two trophic levels (accounting for SP but not parasitoid effects), and one trophic level (fitness in absence of SPs), and compared selection strength on fruit number between trophic scenarios and fertility environments.

3. In unfertilized conditions, SPs selected for increased CL fruit number, whereas parasitoids dampened (but did not eliminate) this selective impact. With fertilization, however, selection by SPs was reduced and unaffected by parasitoids.

4. *Synthesis*. Overall, we show that parasitoids can shape herbivore selection on plants, but that both herbivore and parasitoid selective impacts depend upon the abiotic environment. These findings underscore how linkages between abiotic factors and trophic complexity influence the ecological and evolutionary outcomes of species interactions.

Key-words: diffuse selection, functional response, parasitoids, plant–herbivore interactions, seed predation, soil fertility, tri-trophic interactions

Introduction

Over the last two decades, research on species interactions has shifted from the study of pairwise interactions to examining how the effects of one species on another are influenced by the matrix of interactions in which each species is embedded (Strauss & Irwin 2004). Much of the work on this subject has involved plants and insects, looking at how plants mediate interactions among herbivores, pollinators and carnivores (Strauss & Irwin 2004; Ohgushi 2005), how plant competition mediates plant–herbivore interactions (Hämback & Beckerman 2003; Agrawal 2004; Linhart *et al.* 2005) and how plants mediate herbivore–predator interactions (Fritz 1995; Hare 2002; Mooney & Singer 2012). In parallel to these ecological dynamics, it has also become increasingly clear that the evolutionary outcomes of species interactions are contingent upon the community context (Inouye & Stinchcombe 2001; Strauss, Sahli & Conner 2005; Walsh 2013).

Geographic variation in the intensity of ecological interactions suggests the presence of diffuse co-evolutionary dynamics (Zangerl & Berenbaum 2003; Siepielski & Benkman 2004; Thompson 2005), where selection among pairs of interacting species is modified by the community context (Strauss, Sahli & Conner 2005; Haloin & Strauss 2008). A handful of studies have tested directly for diffuse selection, particularly in the context of selection on plant resistance to multiple herbivores within a single site (Simms 1990; Iwao & Rausher 1997; Juenger & Bergelson 1998; Stinchcombe & Rausher 2001; see also Walsh 2013). From a mechanistic standpoint, it has been proposed that interaction modifications (*sensu* Wootton 1994), where one species modifies the function of the interaction between two other species, have important evolutionary implications and may lead to diffuse selection (Miller & Travis 1996; Inouye & Stinchcombe 2001; Strauss, Sahli & Conner 2005). However, context-dependent selection can also arise in the absence of interaction modifications when the recruitment responses of interacting species are nonlinear (for other examples, see Inouye & Stinchcombe 2001).

For instance, if the selective effect of a consumer on its resources varies as a function of resource density (i.e. density-dependent attack), then third parties (or other factors) altering resource density could alter consumer selection (Mooney & Singer 2012; Singer *et al.* 2012), even if the (non-linear) function describing resource–consumer interactions remains unchanged. As an example, Singer *et al.* (2012) demonstrated that the intensity of bird predation on caterpillars varied among eight host tree species based on caterpillar density, suggesting that selection by birds might vary among tree species even if the density-dependent functional response of birds to caterpillars was uniform.

Despite good examples of diffuse selection, we still know very little about how predators and the plant's resource environment alter herbivore selection on plants. For instance, there is ample evidence for carnivore indirect effects on plant biomass (Schmitz, Hambäck & Beckerman 2000) and fitness (Romero & Koricheva 2011), but to date, virtually no study has tested whether carnivores modify herbivore selection on plants (see Rudgers 2004; Rutter & Rausher 2004 for direct selection by predators on plants). Similarly, very few studies have looked at how abiotic forces such as soil or climatic factors shape the evolution of plant–herbivore interactions (Johnson & Stinchcombe 2007; Kolb & Ehrlén 2010). As a result, we have a limited understanding of how tri-trophic interactions and environmental effects shape the evolutionary outcomes of consumptive interactions (Mooney, Pratt & Singer 2012).

Ruellia nudiflora Engelm. and Gray Urb. (Acanthaceae) is a short-lived perennial herb found throughout southeast Mexico (Ortegon-Campos *et al.* 2012). It produces cleistogamous (CL) flowers, which do not open and self-pollinate obligately, and chasmogamous (CH) flowers, which do open and are visited by pollinators. Fruits from both types of flower are attacked by the larvae of a seed-eating moth, which in turn is attacked by several species of parasitoids (Abdala-Roberts *et al.* 2010). We previously documented the effects of plant genetic family and soil fertility on fruit production and investigated the mechanisms by which these two sources of variation in fruit number cascaded up the food chain (Abdala-Roberts & Mooney 2013). Here, we analyse additional data from the same experiment to determine whether parasitoids and soil fertility influence selection by the seed predator on *R. nudiflora* reproductive display size (Abdala-Roberts *et al.* 2014a). Young plants of this species reproduce almost entirely through CL reproduction (ca. 90% of fruit output; data from this study) and, in the present experiment, the few CH flowers produced were consumed by vertebrate herbivores, so selection by seed predators and parasitoids occurred solely through feeding on CL fruits. Specifically, we tested for seed predator selection on CL fruit number, and if such selection was altered by both parasitoids and soil fertility. In doing so, our aim was to understand the dual influence of consumers and resources on plant trait evolution, and we interpret our findings with respect to the shape of the relationships (i.e. functional or recruitment responses) describing plant-seed predator and seed predator–parasitoid interactions.

To our knowledge, this study offers the first test for carnivore effects on herbivore selective impacts and further evaluates the potential for the plant resource environment to influence the selective outcomes of multitrophic interactions.

Materials and methods

Ruellia nudiflora is a short-lived (two- to three-year life span), self-compatible, perennial herb distributed from southwestern USA to southeast Mexico, and in Yucatan (Mexico), it grows across a wide range of climatic and soil conditions (Ortegon-Campos *et al.* 2012). Chasmogamous (CH) flowers have an open purple corolla [2.33 ± 0.04 (mean ± SE) cm in diameter] and are visited by insect pollinators, whereas cleistogamous (CL) flowers do not open, have reduced pale yellow corollas (0.2–0.3 cm in diameter), and self-pollinate obligately; CH flowers also frequently self-pollinate autonomously upon corolla dehiscence when anthers come into contact with the stigma (Abdala-Roberts *et al.* 2014b). In addition, CH flowers are found on vertical inflorescence stalks that are 20–30 cm in height, while CL flowers are usually produced on lateral inflorescences at the base of the plant. The peak of CH flower production is during July and August, and CL flower output is usually greater and spans over a longer period of time (Munguía-Rosas *et al.* 2012). Fruits are dry and dehiscent, with CH and CL fruits producing a mean of 13.27 ± 0.16 (S. E.) and 9.44 ± 0.02 seeds, respectively (data from this study). Finally, reproduction is strongly CL-biased during the first year of life (as observed in this study), a common pattern in cleistogamous species (Culley & Klooster 2007).

Both CH and CL fruits are attacked by larvae of a single, as-yet-unidentified species of noctuid moth (Lepidoptera: Noctuidae) that feeds on seeds prior to fruit dehiscence. Adult female moths oviposit on recently pollinated flowers, and except when parasitized, a single larva grows inside a developing fruit and usually consumes all the seeds within a given fruit [$95 \pm 0.10\%$ (mean ± SE) of seeds consumed per attacked fruit; Abdala-Roberts & Mooney 2013]. Previous work has shown differences among plant genotypes in seed predator attack and in plant traits influencing seed predation (e.g. reproductive display size; Abdala-Roberts & Mooney 2013). In addition, seed predators show a nonlinear, saturating functional response as estimated by a quadratic regression model where both fruit number ($P < 0.0001$) and fruit number^2 ($P = 0.0004$) are significant predictors of seed predator abundance (seed predator number = 1.28*fruit number + 0.0091*fruit number^2 − 10.58; Fig. 1a) (Abdala-Roberts & Mooney 2013). As a result, increasing fruit output reduces the proportion of fruits attacked per plant (i.e. negative density-dependent attack; Fig. 1a, inset) (Abdala-Roberts & Mooney 2013). In turn, seed predator larvae are attacked by seven species of parasitic wasps belonging to Braconidae (four species), Ichneumonidae (one) and Pteromalidae (two), as well as one fly species of Tachinidae. Parasitoids stop or reduce seed predator feeding, thus having an indirect positive effect on plant fitness by 'rescuing' seeds from seed predator consumption (Abdala-Roberts *et al.* 2010). Data from this experiment indicate that attack by the most abundant parasitoid at the study site (*Bracon* sp.) is density-independent (Fig. 1b, inset), that is, the proportion of parasitized herbivores is unrelated to the number of fruits attacked by the seed predator (Abdala-Roberts & Mooney 2013).

EXPERIMENTAL SET-UP

We established a common garden field experiment at a site located 4.6 km east of the town of Molas, Yucatan, Mexico (20°29′10″ N,
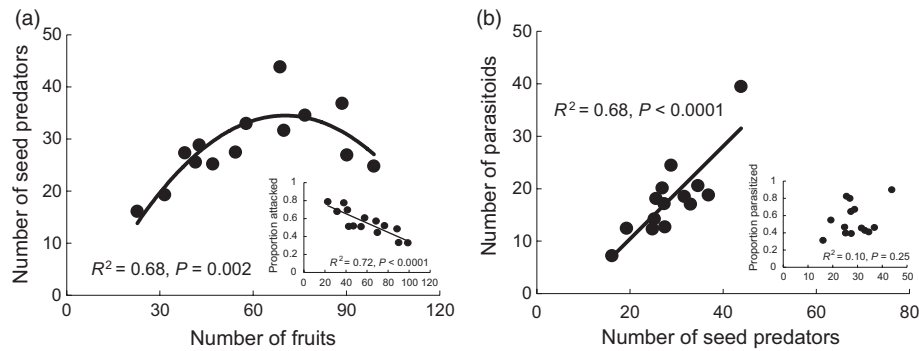
Fig. 1. Bivariate plots using plant family means to describe the functional responses exhibited by a noctuid seed predator and its parasitoids associated to the perennial herb *Ruellia nudiflora* (modified from Abdala-Roberts & Mooney 2013). Panel 'a' shows the relationship between cleistogamous fruit number and seed predator number (i.e. the number of fruits with a seed predator), while panel 'b' shows the relationship between seed predator and parasitoid number (i.e. the number of attacked fruits with a parasitoid). Insets indicate the proportion of fruits attacked by the seed predator as a function of fruit number (panel 'a'), as well as the proportion of seed predator larvae that were parasitized (i.e. proportion of attacked fruits with a parasitoid) as a function of seed predator number (panel 'b').

89°59′75″ W), to factorially test for the effects of *R. nudiflora* plant family (14 maternal lines) and soil fertilization (fertilized or unfertilized) on tri-trophic interactions among plants, seed predators and parasitoids (Abdala-Roberts & Mooney 2013). Initially, CH fruits were collected from 14 mother plants at a population located 10 km away from the common garden. All the seeds collected from a given plant were considered a maternal family composed of a mixture of full- and half-sibs. Two months after seed germination, 20 seedlings per family were transplanted to the common garden ($n = 280$), where distances among rows and among plants within rows were 0.5 m and plant positions were randomized throughout the plot. Mortality was low throughout the experiment (<5%), and sample size was 17.73 ± 0.41 (range: 16–20) plants per family at the end of the experiment.

For the soil fertility treatment, we randomly selected half of the plants of each family and watered them twice with 40 mL of a solution (Ferticoral, Impulsora Agroquímica del Sureste, Mexico) containing N (20%), P (30%) and K (10%), at a concentration of 2 g per litre. This level of fertilization released plants from nutrient limitation sufficiently to cause a 1.5-fold increase in leaf production [not fertilized (NF) = 8.13 ± 0.45 leaves; fertilized (F) = 12.40 ± 0.55 leaves] (Abdala-Roberts & Mooney 2013), which falls within the range of fertilization effect sizes for plant biomass reported by previous studies in terrestrial ecosystems (Gruner *et al.* 2008). This fertilization effect size is also comparable to variation in plant growth among *R. nudiflora* populations, as a 1.4-fold to 1.5-fold difference in above-ground biomass was observed for plants grown with contrasting soil conditions from sites where this species naturally occurs (Ortegon-Campos *et al.* 2012). Specifically, plants from two populations (neither one used in this study) were grown in two naturally occurring soils, with a 1.5-fold difference in plant growth between soil types for one population [mean in soil A = 4.79 ± 0.63 g, mean ($\pm$SE) in soil B = 3.21 ± 2.15 g] and a 1.4-fold difference for another population (soil A = 2.88 ± 0.36 g; soil B = 4.08 ± 0.76 g; Ortegon-Campos *et al.* 2012). During fertilizer application, we took special care to minimize run-off to neighbouring plants; run-off was also reduced by growing plants at the centre of a depression at each planting point. Fertilization did not influence seed predator (F = 59.1 ± 2.3%; NF = 55.4 ± 2.6% of fruits attacked) or parasitoid (F = 54.9 ± 2.7%; NF = 54.5 ± 3.2% of seed predators parasitized) attack, measured as the proportion of attacked fruits and the proportion of attacked fruits with parasitoids (i.e. proportion of parasitized seed predator larvae), respectively, but did modify seed predator

and parasitoid recruitment responses (Abdala-Roberts & Mooney 2013).

From September 2011 to December 2011, we conducted weekly counts of CL fruits produced by each plant. We found up to three-fold differences among families for CL fruit output [mean ($\pm$SE): 33.50 ± 7.45 to 101.70 ± 10.35 fruits], which resulted in significant genotypic variation for this trait (Abdala-Roberts & Mooney 2013). Likewise, we found up to twofold differences among families in the proportion of fruits attacked by the seed predator (35.2 ± 4.2% to 75.1 ± 5.6%; $P = 0.005$) and in the proportion of attacked fruits with parasitoids (36.3 ± 9.7% to 77.1 ± 5.4%; $P = 0.12$) (Abdala-Roberts & Mooney 2013). On the other hand, CH flowers were consumed by vertebrates prior to fruit maturation (Abdala-Roberts & Mooney 2013) and – had they matured – CH fruits would have constituted only a small portion (<10%) of total fruit production. Such pattern, where reproduction is strongly skewed towards CL reproduction during the first year of life, is common in *R. nudiflora* as well as other cleistogamous species (Culley & Klooster 2007). Accordingly, only CL fruits were available to seed predators (and parasitoids), and only CL seed contributed to plant fitness during this experiment. Older plants invest similarly in CH and CL reproduction and produce close to twice as many fruits each year compared with first-year plants (Munguía-Rosas *et al.* 2012). Based on this, CL seed production for first-year plants typically represents between 20 and 30% of lifetime seed production depending on whether plants live three or two years, respectively. Although previous work has shown that CL and CH seeds have similar germination rates, the above percentages based upon seed output may be a moderate overestimation of the contribution of first-year CL reproduction to lifetime fitness considering that CL seedlings have lower performance than CH seedlings under high competition (E. Gutiérrez, unpublished data) or low light availability (Munguía-Rosas, Campos-Navarrete & Parra-Tabla 2013). Regardless of this consideration, however, first-year CL seed production is probably correlated with and represents an important component of lifetime fitness. CL fruits were sampled from each plant on a weekly basis from September 2011 to December 2011 and dissected to record seed number, and seed predator and parasitoid presence. Weekly sampling of fruits provided a precise measure of total fruit production (as well as insect attack) per plant because fruits take ca. 10 days to mature. During each visit, we collected all mature fruits per plant.

SEED PREDATOR SELECTION ON THE PLANT: EFFECTS OF PARASITOIDS AND SOIL FERTILITY

Plant fitness under different trophic scenarios

Although performing field manipulations of seed predator and parasitoid abundance is the ideal approach to test for their effects on plant fitness, experimentally excluding seed predators and parasitoids to test for their effects on plant fitness is not feasible in this study system. In particular, seed predators cannot be excluded without affecting pollinators, and parasitoids cannot be excluded without affecting seed predators and pollinators. Yet the nature of this system makes it possible to measure the impacts of seed predators and parasitoids without experimental manipulation. Unparasitized seed predators consume virtually all seeds in a fruit, whereas parasitized seed predators do not. The seeds remaining in a fruit following parasitoid attack (i.e. seed rescue) thus represent a precise measure of parasitoid indirect effects on plant fitness (in this study, $23 \pm 1.0\%$ of total seeds produced per plant were rescued by parasitoids). At the same time, undamaged fruit size is correlated with seed number (Pearson's $r = 0.70$, $P < 0.0001$, $n = 116$), and there is no detectable difference in the size of fruits attacked and not attacked by seed predators (Abdala-Roberts & Mooney 2013). Therefore, seed number in unattacked fruits can be used to estimate potential seed production (i.e. fitness) in the absence of seed consumption and thus the number of seeds lost to seed predators.

We calculated the fitness of each plant under three contrasting trophic scenarios. First, for each plant, we estimated potential seed number in the absence of seed predator effects (hereafter 'mono-trophic' scenario) by multiplying the number of CL fruits produced (i.e. based on the sum of weekly counts) by the corresponding plant family mean for seed number per unattacked fruit, specific to each fertilization level. Secondly, fitness in the presence of seed predators alone (hereafter 'bi-trophic' scenario) was estimated by assuming that all seeds rescued by parasitoids were instead lost to seed predation (potential seed number – seeds consumed assuming no seed rescue); results did not change qualitatively after accounting for rare cases of remaining seeds in fruits with unparasitized herbivores (i.e. 5% of the cases); thus, for simplicity, we assumed that all seeds were consumed in attacked fruits without parasitoid. Finally, the observed number of seeds per plant (direct counts from collected fruits) represented a measure of fitness in the presence of both seed predators and parasitoids (hereafter 'tri-trophic' scenario). Previous work has shown that seed predator and parasitoid attack vary greatly across *R. nudiflora* populations (*in situ* measurements ranging from 5–60% to 0–65%, respectively, $n = 21$ populations; Abdala-Roberts *et al.* 2010). Therefore, these trophic treatments provide a realistic representation of the range of spatial variation in tri-trophic interaction strength, including populations where both seed predation and parasitism are low (mono-trophic scenario), seed predation is high but parasitism is low (bi-trophic scenario), as well as cases where parasitism is high (tri-trophic scenario) (Abdala-Roberts *et al.* 2010).

Our measure of seed predation may overestimate seed predator impacts on plant fitness if plants reduce resource investment in attacked fruits, possibly in turn increasing investment in unattacked fruits. There are two potential mechanisms for this: first, maturation of attacked fruits might be halted via selective fruit abortion (e.g. Östergård, Hambäck & Ehrlén 2007); secondly, attacked fruits might produce fewer or smaller seeds. We offer two lines of evidence that suggest that resource reallocation effects were weak or absent in this system. First, while *R. nudiflora* has been shown to abort fruits under some conditions (e.g. due to low pollen loads or low light

availability; Abdala-Roberts *et al.* 2014b), we did not find evidence of fruit abortion for experimental plants at the field site [$n = 161$ flowers (and the fruits they set) followed throughout a one-month period; L. Abdala-Roberts, unpublished data]. Because some of these fruits were attacked, this suggests that seed predator damage (and/or other factors) did not induce selective fruit abortion. Secondly, fruit size of attacked and unattacked fruits is identical and this trait is correlated with seed number. Although it is possible that fruit elongation was not affected by seed predator damage while seed production and maturation were reduced, this provides at least partial evidence that resources were not shunted from attacked fruits as this could have caused a reduction in size of attacked fruits. Finally, for each family, we related the mean number of seeds per unattacked fruit for each plant to the proportion of attacked fruits per plant and in all cases found either no relationship (R^2 values ranging from 0.0006 to 0.15) or a negative relationship (four families, R^2 values ranging from 0.26 to 0.66). If resources had been diverted from damaged fruits, this would have resulted in a positive relationship where plants with a greater proportion of attacked fruits should have undamaged fruits with more seeds.

Relative fitness and selection under different trophic scenarios

We computed three estimates of relative fitness for each plant by dividing CL seed number for each trophic scenario by the overall (i.e. population) mean CL seed number specific to each scenario. Subsequently, we calculated plant family means for relative fitness specific to each trophic scenario and fertilization treatment. We then measured selection on CL fruit number by regressing family means for relative fitness onto CL fruit number and, using ANCOVA, then compared these slopes (i.e. selection gradients) among trophic scenarios within each fertility environment, as well as across fertility environments separately for each trophic scenario (see Statistical procedures ahead).

Seed number per fruit is relatively conserved and varies little among plant families (1.2-fold: mean $\pm$ SE: 8.64 ± 0.44 to 10.27 ± 0.30); thus, positive selection on fruit number was expected. Yet genotype variation in CL fruit number is large (threefold: 33.50 ± 7.45 to 101.70 ± 10.35; Abdala-Roberts & Mooney 2013), suggesting trade-offs between current and future CL fruit production or between fruit production and other fitness components (e.g. CH reproduction) maintain variation in CL fruit output (otherwise, such variation would be much smaller or absent as fruit number is strongly correlated with plant fitness). Accordingly, our aim was to investigate natural selection imposed by seed predators on CL fruit number and how this selection is modified by parasitoids and the resource environment.

Statistical procedures

We performed simple linear regression analyses with PROC REG in SAS ver. 9.1 (SAS Institute 2002) using plant family means to test for directional selection on CL fruit number. Because quadratic terms testing for disruptive or stabilizing selection were never significant ($0.07 \leq P \leq 0.98$), we present results for models assessing only directional selection. We regressed plant family means for relative fitness onto CL fruit number, separately for each trophic scenario (mono-, bi- and tri-trophic), both under low and high soil fertility. We used family means for all selection analyses because genotypic selection analysis avoids environmentally induced correlations between measured and unmeasured traits that can influence estimates of plant

fitness in phenotypic selection analyses (Rausher 1992; Iwao & Rausher 1997; Stinchcombe *et al.* 2002). It is important to note, however, that family-level replication was relatively low ($n = 14$), and while we were still able to detect treatment effects on patterns of linear selection (see Results), this may have prevented the detection of nonlinear patterns of selection. We calculated standardized selection gradients (β_σ) for each regression model by multiplying original slope values by the population standard deviation for fruit number (Lande & Arnold 1983; Stinchcombe 2005) and provide corresponding standard errors.

To test for differences in slope values (i.e. selection gradients) between trophic scenarios and levels of soil fertility, we first performed an ANCOVA model using the entire data set and modelling relative fitness as dependent upon CL fruit number, our experimental factors (trophic scenario and soil fertility), all two-way interactions, and the three-way interaction. We were particularly interested in the fruit number × fertility treatment and fruit number × trophic scenario interactions because these terms tested for an effect of soil resource environment and trophic complexity, respectively, on the selection gradient for CL fruit number. Accordingly, if this model yielded significant interactions, we proceeded to assess specific differences in selection between trophic scenarios within each fertility environment, as well as the effect of fertility on selection separately under each trophic scenario based upon a set of component ANCOVAs using subsets of the entire data set. First, we ran component ANCOVAs to compare pairs of slopes from each trophic scenario one at a time, separately within each level of soil fertility, for a total of six analyses (three pairwise combinations under each fertilization level). The test of difference in slopes between the mono- and bi-trophic scenarios assessed the selective effect of seed predators on CL fruit number, while the test of difference in slopes between the bi- and tri-trophic scenarios assessed the effect of parasitoids on selection by the seed predator. Secondly, we performed another set of component ANCOVAs (three) to compare the slopes of each trophic scenario across fertilization treatments. In this case, the test of difference in slopes across fertilization treatments for the bi-trophic scenario, for example, assessed the effect of soil fertility on selection by the seed predator.

Residuals from all regression and ANCOVA models were normally distributed ($P > 0.05$; Kolmogorov–Smirnov tests). For simplicity, we only report results for the interaction terms from the component ANCOVA models. The overall ANCOVA with the full data set as well as the component ANCOVAs testing for differences in selection gradients among pairs of trophic scenarios within each fertilization treatment were performed in SAS ver. 9.1 with PROC MIXED because these models included the effect of plant family, treated as random, to control for using the same plants within each family to compute fitness values for each trophic scenario within a given fertility environment. We did not test for the significance of the family effect since we were only interested in accounting for these repeated values per family in our analyses. ANCOVAs testing for differences in selection gradients across fertilization levels for each trophic scenario were conducted with PROC GLM. For the component ANCOVAs, the slope of each subset of the data (i.e. for a given trophic scenario) was used three times for comparison with slopes from other subsets: twice when comparing against slopes of other trophic scenarios within a given fertility environment and once when comparing with the slope of same trophic scenario across fertility environments. Accounting for these multiple comparisons yields a corrected *P*-value of 0.017 (0.05/3) as cut-off level for statistical significance (which is a conservative correction; García 2004). However, in all cases, statistical differences among slopes were strong (*P*-values ranging from 0.01 to <0.0001, see Results) and remained significant after *P*-value correction. Thus, we report results based upon uncorrected significance values.

Results

Under both fertilized and unfertilized environments, positive directional selection on CL fruit number was observed under all three trophic scenarios, being weakest for the mono-trophic scenario, intermediate for the tri-trophic scenario and strongest for the bi-trophic scenario (Table 1).

The overall ANCOVA based upon the entire data set revealed significant fruit number × fertility, fruit number × trophic scenario, and fruit number × fertility × trophic scenario interactions, indicating that patterns of selection on CL fruit number were altered by soil fertility and trophic complexity (Table 2). To assess differences in the strength of selection between levels of soil fertility and trophic scenarios, we used component ANCOVAs to compare changes in selection among trophic scenarios separately within each fertility environment, as well as changes in selection across fertility environments within each trophic scenario.

UNFERTILIZED ENVIRONMENT

In comparing the mono- and bi-trophic scenarios using ANCOVA, we found that accounting for seed predator effects caused a significant (86%, based upon comparison of standardized selection gradients) increase in the strength of directional selection, indicating that seed predators strengthened selection on fruit number (significant fruit number × trophic scenario term, mono- vs. bi-trophic comparison; $F_{1,12} = 42.97$, $P < 0.0001$; Table 1 and Fig. 2a), with the underlying mechanism being that this herbivore exhibits negative density-dependent recruitment to fruits (Fig. 1a; Abdala-Roberts & Mooney 2013). Importantly, the ANCOVA comparing the bi- and the tri-trophic selection gradients revealed that parasitoids dampened selection by seed predators on fruit number as the tri-trophic selection gradient was significantly (28%) weaker relative to the bi-trophic selection gradient ($F_{1,12} = 15.34$, $P = 0.002$; Fig. 2a). However, despite this parasitoid dampening effect, selection gradients for the mono- and tri-trophic

Table 1. Measurements of directional selection on *Ruellia nudiflora* cleistogamous fruit number under two soil fertility treatments (NF = not fertilized, F = fertilized), and based upon different trophic scenarios (trophic): mono-trophic: selection on plant traits assuming no seed predator or parasitoid effects on seed output; bi-trophic: selection accounting for seed predation but not seed rescue by parasitoids; tri-trophic: selection accounting for both seed predator and parasitoid effects. Both unstandardized (β) and standardized (β_σ) selection gradients and standard errors (SE) are reported. Model R^2 and *P*-values are also presented

Soil	Trophic	β (SE)	β_σ (SE)	R^2	P
NF	Mono	0.015 (0.0008)	0.65 (0.03)	0.96	<0.0001
	Bi	0.028 (0.002)	1.21 (0.08)	0.94	<0.0001
	Tri	0.022 (0.001)	0.95 (0.04)	0.94	<0.0001
F	Mono	0.014 (0.0008)	0.60 (0.03)	0.95	<0.0001
	Bi	0.020 (0.002)	0.86 (0.08)	0.89	<0.0001
	Tri	0.019 (0.001)	0.82 (0.04)	0.94	<0.0001

Table 2. Results from ancova models based upon the entire data set testing for changes in the strength of directional selection of *Ruellia nudiflora* cleistogamous fruit number across trophic scenarios ('mono-trophic', 'bi-trophic' and 'tri-trophic') and levels of soil fertility (fertilized and unfertilized). Significant results are shown in bold

Source	d.f.	F	P
Fruit number	1,59	582.04	**<0.0001**
Trophic scenario (T)	2,59	27.52	**<0.0001**
Fertility treatment (F)	1,59	0.23	0.68
T × fruit number	2,59	35.29	**<0.0001**
F × fruit number	1,59	4.92	**0.03**
T × F	2,59	0.87	0.42
T × F × fruit number	2,59	4.26	**0.01**

scenarios still differed significantly (ANCOVA: $F_{1,12} = 34.68$, $P < 0.0001$; Fig. 2a).

FERTILIZED ENVIRONMENT

As for unfertilized conditions, ANCOVA indicated that directional selection on fruit number under the fertilized environment became (43%) stronger once the effect of seed predators was accounted for (bi-trophic scenario) relative to selection under the mono-trophic scenario (significant fruit number × trophic scenario term, mono- vs. bi-trophic comparison: $F_{1,12} = 7.97$, $P = 0.01$). However, contrary to unfertilized conditions, we found no difference in selection gradients between the bi- and tri-trophic scenarios (ANCOVA: $F_{1,12} = 0.71$, $P = 0.41$; Fig. 2b), indicating that parasitoids did not influence selection by the seed predator. In addition, the difference in selection gradients between the mono- and tri-trophic scenario was significant (ANCOVA: $F_{1,12} = 12.05$, $P = 0.004$; Fig. 2b).

CROSS-ENVIRONMENT COMPARISON

We found that the mono-trophic selection gradient did not change across soil fertility environments (ANCOVA:

non-significant fertilization × fruit number interaction, mono-trophic scenario; $F_{1,24} = 0.26$, $P = 0.61$; Fig. 2a,b). However, fertilization did cause a significant (40%) reduction in the strength of the bi-trophic selection gradient (ANCOVA: $F_{1,24} = 6.52$, $P = 0.01$; Fig. 2a,b). This latter result could have been due, at least in part, to reduced variation in fruit number among families under fertilized conditions (fertilized: threefold; unfertilized: fivefold; significant family effect observed only under unfertilized conditions; Abdala-Roberts & Mooney 2013) which limited seed predator selective impacts on this trait. Fertilization also weakened the strength of selection under the tri-trophic scenario (ANCOVA: $F_{1,24} = 7.40$, $P = 0.01$), presumably as a consequence of weakened selection by the seed predator.

Discussion

Despite the large body of literature showing that carnivores indirectly influence plant biomass (Shurin *et al.* 2002; Mooney *et al.* 2010a,b) and fitness (Romero & Koricheva 2011), our study is among the few to test and find evidence for indirect evolutionary consequences of carnivores on species at lower trophic levels (Hare 2002; Walsh & Reznick 2008; see studies in Walsh 2013; Estes, Brashares & Power 2013). Specifically, we present the novel result that carnivore effects lead to diffuse selection by dampening herbivore selection for increased fruit number. In addition, conditions of high soil fertility dampened direct and indirect selection on fruit number by the seed predator and parasitoids, respectively. Although the selective effects of parasitoids and soil fertility on seed predator selection were relatively weak (compared to the selective effect of the seed predator on fruit number), these findings nonetheless underscore the relatively untested presumption that the evolutionary outcomes of species pairwise interactions are contingent upon the resource environment and multitrophic consumptive dynamics (Steinberg, Estes & Winter 1995; Johnson & Stinchcombe 2007; Kolb & Ehrlén 2010; Walsh 2013).
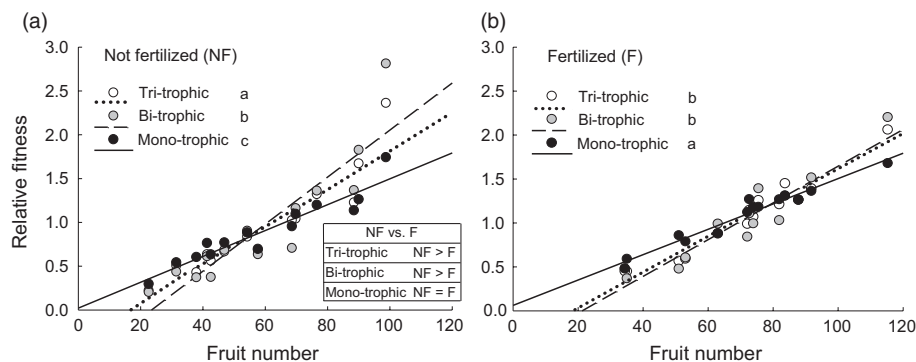

Fig. 2. Directional selection gradients on cleistogamous fruit number of *Ruellia nudiflora* under three trophic scenarios: in the absence of seed predator and parasitoid effects ('mono-trophic'), accounting for seed predator effects but not parasitoid seed rescue ('bi-trophic'), and accounting for both seed predator and parasitoid effects ('tri-trophic', i.e. observed fitness). Panels 'a' and 'b' show selection on fruit number under unfertilized (NF) and fertilized (F) soil conditions, respectively. For the legend in each panel, different letters indicate significant differences ($P < 0.05$) among trophic selection gradients within each level of fertilization. In addition, on the lower right corner of panel 'a', the 'NF vs. F' table indicates whether a selection gradient for a given trophic scenario was stronger, weaker or remained unchanged when moving from the unfertilized (NF, panel 'a') to the fertilized (F, panel 'b') soil environment.

FUNCTIONAL RESPONSES AND THE OUTCOME OF TRI-TROPHIC SELECTION ON FRUIT NUMBER

We found that the seed predator's negative density-dependent response, where the proportion of attacked fruits decreases with increasing fruit number (Fig. 1a, inset) (Abdala-Roberts & Mooney 2013), resulted in selection for increased *R. nudiflora* cleistogamous fruit number. In other words, because the rate of seed predator attack declined with fruit abundance, a greater proportion of fruits escaped attack for plant families with high fruit output relative those with low fruit output, and seed predators thus further increased positive selection on fruit number (see Leimu *et al.* 2002; Jones & Comita 2010). In a parallel, but opposing fashion, other studies such as that by Miller, Tenhumberg & Louda (2008) have shown that positive density-dependent attack by herbivores selectively favours smaller fruit output (see studies in Elzinga *et al.* 2007). Although consumer functional responses have traditionally received a great deal of attention in population ecology (Holling 1959; Abrams 1982), their evolutionary significance has received far less empirical attention (for theoretical work, see Abrams 2000). Our study thus provides empirical support for the linkage between consumer functional responses and the evolutionary consequences of consumptive interactions.

Parasitoids in turn weakened seed predator selection (i.e. the bi-trophic selection gradient) on fruit number, but this effect was relatively weak and not strong enough to eliminate seed predator selection. Importantly, we note that although parasitism rates were density-independent (Fig. 1b, inset; i.e. the proportion of parasitized herbivores remained constant with increasing herbivore abundance), the indirect benefit of parasitoids on plant fitness varied among families because of the herbivore's nonlinear recruitment response. Specifically, we observed that for plant families with small fruit output, herbivore attack rate was high and thus, the benefit of parasitism was large in terms of a proportional increase in plant fitness from seed rescue. In contrast, for plant families with high fruit output, herbivore attack rate was low and thus, the proportional fitness gain from parasitism was comparatively smaller. So, although parasitoid attack rate was constant across plant families (Fig. 1b), they nevertheless altered selection by the seed predator due to density dependence of the plant-seed predator interaction (Fig. 1a).

Our findings have important implications for understanding the evolutionary outcomes of multitrophic interactions, as well as predicting the occurrence and strength of diffuse selection. Over the last decade, changes in the function of pairwise species interactions through interaction modifications (*sensu* Wootton 1994) have been recognized as an important source of diffuse selection (Miller & Travis 1996; Inouye & Stinchcombe 2001; Mooney & Singer 2012). However, as shown here for tri-trophic dynamics, interaction strength may also change if species show nonlinear recruitment functions (Mooney & Singer 2012; Singer *et al.* 2012). In this case, carnivores need not to change the shape of plant–herbivore interactions (i.e. no interaction modification) but can still change herbivore selection when herbivore recruitment is nonlinear. We suggest that such patterns of diffuse selection may be common in tri-trophic systems, due to the prevalence of herbivore and predator nonlinear functional responses (e.g. Mooney & Singer 2012). More generally, the dynamics outlined here may occur in any indirect interaction where one or both of the component interactions are based upon a non-linear functional response, including (but not limited to) apparent competition (Holt & Lawton 1994), plant-mediated interactions among herbivores (Ohgushi, Craig & Price 2007), and herbivore-mediated interactions among plants (Hämback & Beckerman 2003). Accordingly, making an explicit distinction between functional response-based processes and other mechanisms leading to diffuse selection is fundamental in determining how and when species interactions lead to evolutionary change (Inouye & Stinchcombe 2001).

In finding that parasitoids dampened selection by the seed predator on *R. nudiflora* CL fruit number, this work provides novel evidence that carnivores can influence herbivore selection on plant traits (see Steinberg, Estes & Winter 1995). Whereas previous studies have shown selection on plant traits that facilitate carnivore recruitment (e.g. ant selection on extrafloral nectaries; Rudgers 2004; Rutter & Rausher 2004; see Hare 2002 for other examples), our study shows that carnivores can also indirectly shape plant trait evolution by altering selection imposed by herbivores. These findings suggest a currently untested, but perhaps more widespread mechanistic pathway through which carnivores influence plant evolution (Marquis & Whelan 1996; Hare 2002; Estes, Brashares & Power 2013). We argue that assessing the relative importance and prevalence of different mechanisms by which carnivores influence the evolution of plant traits will be an important step towards building a comprehensive theory on tri-trophic evolutionary dynamics.

SOIL FERTILITY INFLUENCES THE EVOLUTIONARY OUTCOMES OF TRI-TROPHIC INTERACTIONS

We found that the selective impacts of seed predator and parasitoid attack were contingent upon soil resources. Specifically, fertilization weakened direct selection imposed by the seed predator and indirect selection imposed by parasitoids on fruit number, possibly because of a reduction in phenotypic variation for fruit number among plant families. Accordingly, whereas previous research shows that plant–herbivore evolution is contingent upon abiotic conditions (Kolb & Ehrlén 2010; Salgado-Luarte & Gianoli 2012), here we move forward and show how both direct and indirect selections in a tri-trophic system are shaped by the resource environment. It is important to note, however, that the dampening effect of soil fertility was relatively weak and our evaluation of the selective effects of soil resource environment was based upon only two levels of soil fertility. Further work that evaluates patterns of selection across

multiple levels of soil fertility is needed to better assess the role of soil resources in shaping the evolutionary outcomes of multitrophic interactions associated with *R. nudiflora*.

RELEVANCE OF FINDINGS FOR PLANT LIFETIME FITNESS

We measured plant fitness based on cleistogamous seed output for young (first year) individuals primarily because plants exhibited very low investment in chasmogamous reproduction during the study season (Abdala-Roberts & Mooney 2013). This is a typical pattern observed for cleistogamous species such as *R. nudiflora* which invest mostly in cleistogamous reproduction during early life stages (Culley & Klooster 2007). Nonetheless, because we measured patterns of selection during a single growing season, it is not possible to predict effects on lifetime fitness, in part because selection on fruit output during 1 year may trade off with reproduction in subsequent years. Despite this limitation, however, for short-lived perennials such as *R. nudiflora*, selection acting upon young plants will likely have important consequences for lifetime fitness.

Conclusions

Because carnivores altered herbivore selection on plants, this study emphasizes the need for accounting for tri-trophic interactions to understand plant evolution (Marquis & Whelan 1996; Gassmann & Hare 2005; Mooney *et al.* 2010a; Mooney, Pratt & Singer 2012). Our results also suggest that explicitly assessing the shape of functional responses could complement current frameworks used to predict the evolutionary outcomes of direct and indirect selection from multi-species interactions (e.g. Miller & Travis 1996; Inouye & Stinchcombe 2001; Strauss, Sahli & Conner 2005). Specifically, we show for tri-trophic systems that carnivores do not have to change the strength of herbivore impacts on plants for diffuse selection to occur if herbivore recruitment is nonlinear. Concurrently, the observed influence of soil fertility on patterns of direct and indirect selections by the herbivore and parasitoids also calls for future studies controlling for and measuring the effects of the environment on tri-trophic evolutionary dynamics. As a whole, results from this study provide novel insight into the effects of carnivore top-down and resource bottom-up factors influencing the evolutionary outcomes of consumptive interactions.

Acknowledgements

Data accessibility

Data files used for statistical analyses performed in this study are available at DRYAD (Abdala-Roberts *et al.* 2014a).

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