Test of biotic and abiotic correlates of latitudinal variation in defences in the perennial herb *Ruellia nudiflora*

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Summary

1. Geographic variation in abiotic factors and species interactions is widespread and is hypothesized to generate concomitant patterns of species trait variation. For example, higher rates of herbivory at lower latitudes are thought to select for increased plant defences, although latitudinal variation in defences may also be influenced directly by abiotic factors and indirectly by predators and parasitoids reducing herbivore pressure.

2. We measured defences of the herb *Ruellia nudiflora* among 30 populations spanning a latitudinal gradient from northern Yucatan to southern Belize that vary substantially in leaf herbivory (four-fold), seed herbivory (25-fold) and seed herbivore parasitism (14-fold). These surveyed populations span one-third of the species' latitudinal distribution (5° of latitude), the entire precipitation gradient of its distribution, and one-third of the temperature gradient of its distribution. Our prior work showed that leaf herbivory decreased with latitude and that seed herbivory increased with latitude. Here, we measured leaf trichome density and leaf and seed phenolics and tested whether latitudinal variation in climate, herbivory and parasitism explained latitudinal variation in these defensive traits.

3. Patterns of variation in leaf trichomes fully supported predictions, with trichome density increasing with a parallel increase in herbivory towards lower latitudes. While seed phenolics were positively associated with herbivory, and seed herbivory tended to increase with latitude, the predicted (positive) association between latitude and defence was not detectable. There was no detectable association between parasitoids and seed defences. In addition, the association between leaf herbivory and phenolics was weak, and leaf phenolics were not associated with latitude. Importantly, variation in the abiotic environment was associated with plant defence, indicating that abiotic factors can play a major role in shaping plant defences, independently of herbivory.

4. *Synthesis.* Latitudinal variation in abiotic factors may drive concomitant patterns of variation in plant defences, independently of herbivory. Collectively, these findings highlight the need for assessing geographic variation in plant defences from a multi-factorial perspective, testing for the simultaneous influence of biotic and abiotic factors.

Key-words: climate, herbivory, intraspecific variation, latitudinal variation, parasitism, plant defences, plant–herbivore interactions

Introduction

Geographic variation in species interactions is ubiquitous (Mooney & Tillberg 2005; Thompson 2005; Gripenberg & Roslin 2007), and is frequently structured along biotic and/or abiotic gradients (Schemske *et al.* 2009; Maron, Baer & Angert 2014). For example, previous work has shown that species interactions become stronger towards the equator and this is expected to result in stronger selection on species traits (Dobzhansky 1950; Pianka 1966; Schemske 2002; Schemske

et al. 2009). For plant–herbivore interactions, herbivore damage and abundance are thought to be generally higher at lower latitudes (Coley & Aide 1991; Pennings *et al.* 2009; Schemske *et al.* 2009), and increased herbivore pressure should in turn select for higher investment in plant defences (Pennings *et al.* 2009; Marquis, Ricklefs & Abdala-Roberts 2012). Accordingly, previous studies have shown that both aquatic and terrestrial plant species from tropical regions exhibit higher levels of chemical defences than their temperate counterparts (Levin & York 1976; Hay & Fennical 1988; Coley & Aide 1991; Bolser & Hay 1996; Rasmann & Agrawal 2011; Pearse & Hipp 2012; Moreira *et al.* 2014), and feeding bioassays under controlled conditions with marine (Bolser & Hay 1996) and salt-marsh (Pennings, Siska & Bertness 2001) species have reported that herbivores feed less on plants from lower latitudes. Despite these findings, recent syntheses indicate that most studies (> 60%) have not found a relationship between latitude and herbivory (Moles *et al.* 2011a), and few (16%) have found higher levels of plant defence at lower latitudes (Moles *et al.* 2011a,b).

Several factors may account for inconsistencies in the relationship between latitude and plant defences and thus help explain different patterns of geographic variation in plant traits. First, herbivore damage is commonly pooled across herbivore species or guilds with different life-histories which do not respond in the same way to biotic or abiotic conditions and may therefore exhibit different herbivory-latitude relationships (for exceptions see Pennings *et al.* 2009; Salazar & Marquis 2012; Kim 2014; Anstett, Naujokaitis-Lewis & Johnson 2014). Correspondingly, variation in defences among plant tissues has been frequently overlooked and may respond to varying levels of damage imposed by different herbivore guilds or species (Rasmann & Agrawal 2011; Moreira *et al.* 2014). Likewise, to date most work has been biased towards measuring plant defences in leaves (e.g. studies in Moles *et al.* 2011a), ignoring reproductive and underground tissues. Second, most studies have not evaluated the relative influence of biotic and abiotic factors (but see Castillo *et al.* 2013), despite the fact that many chemical (e.g. phenolics) and physical (e.g. trichomes, spines) defences respond not only to herbivore pressure, but could also be adaptive within the context of tolerance to climatic conditions (Reich & Oleksyn 2004; Pearse & Hipp 2012; Moreira *et al.* 2014; Pratt *et al.* 2014), and resource availability (Coley, Bryant & Chapin 1985; Johnson & Rasmann 2011). Accordingly, recent work has shown that geographic variation in plant defences can be strongly associated with variation in abiotic conditions, independently of variation in herbivore pressure (Pearse & Hipp 2012; Moreira *et al.* 2014). Similarly resource availability may also be an important factor influencing geographic gradients in plant defences (Johnson & Rasmann 2011; Moreira *et al.* 2014). For example, if plants at lower latitudes have more resources and grow faster, they may invest less in defence based on growth-defence trade-offs (Coley, Bryant & Chapin 1985) despite increases in herbivory. Third, despite evidence showing that top-down control of herbivores by predators and parasitoids is stronger at lower latitudes (Coley

& Aide 1991; Pemberton 1998; Novotny *et al.* 1999; Stireman *et al.* 2005), most work has ignored the indirect effects of variation in carnivore top-down effects on plant defences (Dyer & Coley 2002; Björkman, Berggren & Bylund 2011; Johnson & Rasmann 2011). While measurements of herbivory reported in previous studies account for the effects of natural enemies (i.e. net effects of herbivores in the presence of predation and parasitism), by not measuring predation or parasitism, most past studies cannot assess the independent effects of the third trophic level on geographic variation in herbivory and (indirectly) plant defences.

Although many studies on geographic variation in plant defences have performed comparisons among species across broad spatial scales (e.g. Rasmann & Agrawal 2011; Marquis, Ricklefs & Abdala-Roberts 2012; Pearse & Hipp 2012; Moreira *et al.* 2014), a parallel line of research has centred on evaluating geographic patterns of plant intra-specific variation (Endler 1977; Hall *et al.* 2007; Woods *et al.* 2012; Lehndal & Ágren 2015). Whereas interspecific comparisons have served to uncover macro-evolutionary patterns in plant defences occurring at broad geographic scales (Agrawal 2011; Moreira *et al.* 2014), intra-specific studies provide a direct evaluation of how plant traits evolve along biotic and abiotic gradients (Woods *et al.* 2012; Pratt & Mooney 2013), and in doing so contribute to understanding the micro-evolutionary dynamics that underlie macro-evolutionary patterns. Although intra-specific comparisons by necessity span smaller latitudinal ranges, species' distributions may nevertheless span and adapt to significant biotic and abiotic clines. For example, Pratt *et al.* (Pratt & Mooney 2013; Pratt *et al.* 2014) report latitudinal variation for chemical defences, growth rate and reproduction for populations of *Artemisia californica* along a 5° latitudinal gradient along the coast of California (USA). Likewise, other studies have similarly found intra-specific latitudinal clines in plant life-history traits (Woods *et al.* 2012), and anti-herbivore defences (Pennings & Silliman 2005; Salgado & Pennings 2005; Pratt *et al.* 2014; Lehndal & Ágren 2015; see also Pellissier *et al.* 2014 for examples of elevation gradients). These findings thus emphasize the importance of addressing plant intra-specific variation along biotic and abiotic gradients in order to understand the underlying mechanisms driving macro-ecological patterns.

We tested for latitudinal variation in defences for different tissues of the perennial herb *Ruellia nudiflora* (Engelm. and Gray) Urb. (Acanthaceae) by sampling 30 populations of this species distributed along a 5° latitudinal transect extending from northern Yucatan (Mexico) to southern Belize, covering one-third of the species' latitudinal distribution. The sampled transect exhibits a fourfold precipitation gradient and 2 °C variation in mean temperature, which represent the entirety and one-third of the precipitation and temperature gradient of *R. nudiflora*'s distribution, respectively, and is therefore relevant for addressing the effects of these climatic factors on geographic variation in plant defences within this species. We previously reported that leaf herbivory decreased and seed herbivory increased with latitude, while parasitism of seed herbivores was variable but unrelated to latitude (Fig. 1a–c;
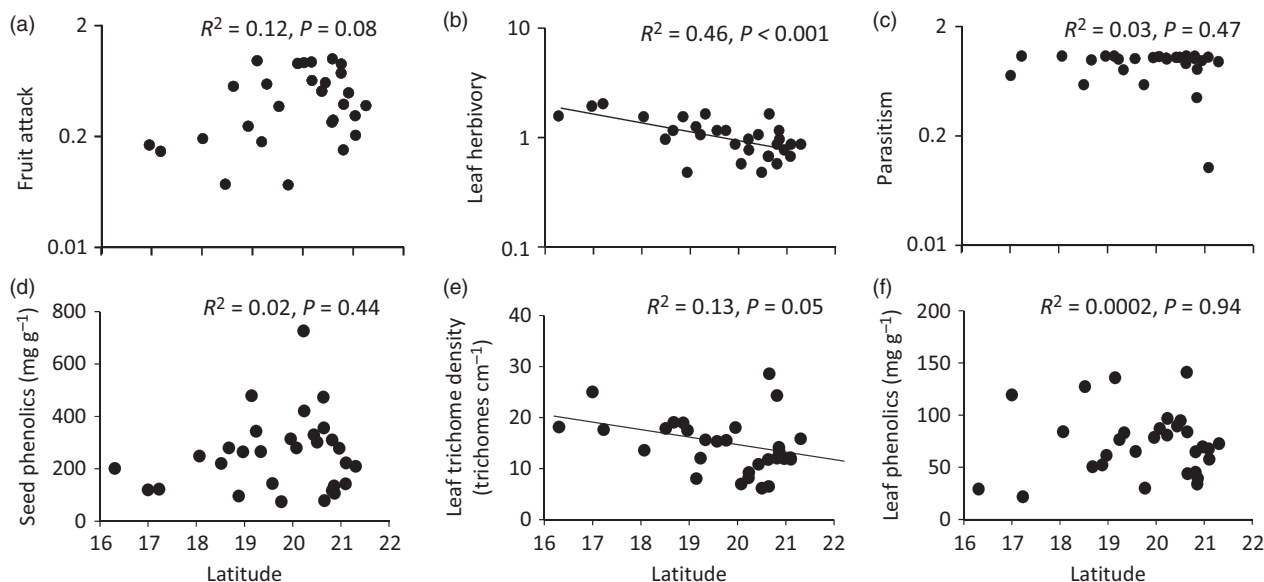
Fig. 1. Relationship between latitude and (a) the proportion of fruits attacked by seed-eating caterpillars (*Tripudia* sp.), (b) the amount of leaf area consumed by insect leaf chewers, (c) the proportion of parasitized seed-eating caterpillars, (d) total phenolics in seeds, (e) total phenolics in leaves and (f) leaf trichome density for *Ruellia nudiflora* populations sampled from northern Yucatan (Mexico) to southern Belize (*N* = 28 populations for seed herbivory, *N* = 30 for leaf herbivory and defensive traits). Leaf herbivory values are based upon visual estimates of leaf area consumed (see Materials and Methods). For fruit attack and parasitism we plot original values on a logit scale, whereas for leaf herbivory values are plotted on a log-scale (modified from Moreira *et al.* 2015). R^2 values and *P*-values in these cases come from simple regressions using transformed data. Seed phenolics, leaf phenolics and trichome density are plotted on the original scale and statistics are based upon untransformed data. In all cases, circles represent population means. Fruit attack, leaf herbivory and parasitism data are from: Moreira *et al.* (2015). Latitudinal variation in herbivory: Influences of climatic drivers, herbivore identity and natural enemies. Oikos. 144, 1444–1452.

Moreira *et al.* 2015). In the present study, we quantified plant defences based upon tissue samples collected at the same time as surveys of herbivory and parasitism in Moreira *et al.* (2015). We tested whether plant defences in seeds (phenolic compounds) and leaves (phenolics and trichomes) varied along this latitudinal gradient, and whether such variation was associated with biotic interactions (herbivory, herbivore parasitism) and the abiotic environment (climate). Specifically, we asked: (i) Is there a latitudinal gradient in *Ruellia nudiflora* anti-herbivore defences, and does this gradient vary across types of tissue? (ii) Is there an association of climatic factors (temperature, precipitation) and biotic interactions (herbivory, parasitism) with any such latitudinal variation in plant defences? In so doing, this study uniquely evaluates the independent and combined effects of climatic and biotic factors underlying geographic variation in plant defensive traits.

Materials and methods

NATURAL HISTORY

Ruellia nudiflora is distributed from southern Texas (U.S.) to northern Honduras (Long 1977; Tripp 2007), and grows in open, disturbed habitats such as roadsides or edges of secondary forest which vary greatly in abiotic conditions such as precipitation and temperature (Ortegón-Campos *et al.* 2009). It is self-compatible and produces chasmogamous (CH) flowers that have an open corolla which are visited by insect pollinators, and cleistogamous (CL) flowers that do not open, have reduced corollas and self-pollinate obligately. CH flower

production occurs during July and August and overlaps with CL flowering (Munguía-Rosas *et al.* 2012). Fruits are dry and dehiscent and seeds disperse balistically. Leaves have unbranched, glandular trichomes which are most abundant on the ventral portion of the leaf blade (L. Abdala-Roberts pers. obs.) and in other plant species have been shown to confer resistance against biotic (herbivores; Pollard & Briggs 1984; Valverde, Fornoni & Núñez-Farfán 2001; Castillo *et al.* 2013) and abiotic factors (reducing water loss; Woodman & Fernandes 1991; Hare & Elle 2001).

Both types of fruit are attacked by larvae of a noctuid moth (*Tripudia* sp., Lepidoptera: Noctuidae) which specializes on the genus *Ruellia* (Pogue 2009), feeding on seeds prior to fruit dehiscence (Abdala-Roberts *et al.* 2010; Abdala-Roberts & Mooney 2013, 2014). Seed-eating caterpillars are in turn attacked by parasitic wasps belonging to Braconidae (four species), Ichneumonidae (one species) and Pteromalidae (two species), as well as one fly species belonging to Tachinidae (Abdala-Roberts *et al.* 2010). On a per-fruit basis, parasitism reduces consumption by the seed herbivore and thus results in an indirect positive effect on plant fitness (Abdala-Roberts *et al.* 2010). There is substantial population-level variation in the proportion of fruits attacked by the seed herbivore and in the proportion of parasitized seed herbivore larvae (Abdala-Roberts *et al.* 2010; Cuautle & Parra-Tabla 2014), and such patterns remain relatively constant across years (Moreira *et al.* 2015). Seed herbivory and thus parasitism concentrate at the peak of reproduction which takes place from mid-July to early August across all of the populations we have sampled previously in the Yucatan Peninsula (Abdala-Roberts *et al.* 2010; Moreira *et al.* 2015). In addition, we previously showed, based upon the same plant populations sampled in the present study, that latitude was associated with decreases in leaf herbivory and increases in seed herbivory, but unassociated with seed herbivore parasitism (Moreira *et al.* 2015).

Leaves of *R. nudiflora* are attacked by several species of generalist caterpillars (Lepidoptera). Previous surveys of multiple *R. nudiflora* populations in the Yucatan Peninsula (Mexico) showed that *Anartia jatrophae* and *Siproeta stelenes* (Lepidoptera: Nymphalidae) are the most common leaf herbivores on this plant and consume the most leaf area (Ortegón-Campos *et al.* 2009). These species are dietary generalists, feeding on members of Acanthaceae, Scrophulariaceae and Verbenaceae (DeVries 1987; Lederhouse *et al.* 1992). Previous work has shown that most leaf herbivory on *R. nudiflora* occurs during the rainy season (July–September), tends to be highest during the first third of the rainy season (July–August), and such patterns remain relatively consistent among populations (Ortegón-Campos *et al.* 2009; Abdala-Roberts *et al.* 2010).

FIELD SAMPLING

We surveyed 30 *R. nudiflora* populations distributed from northern Yucatan (Mexico) to southern Belize spanning five degrees in latitude from 16°N to 21°N (ca. 900 km.; Table S1 in Supporting Information). The sampled transect spans one-third of the latitudinal distribution range of *R. nudiflora* (14°N to 29°N; Tripp 2007), and covers the entire precipitation gradient and one-third of the temperature gradient experienced by this plant throughout its distribution range. From north to south along our latitudinal gradient, there was more than fourfold increase in precipitation (700–2900 mm per year), a 20% decrease in coefficient of variation in precipitation (among months), and a decrease of 2 °C in mean annual temperature (from 26 to 24 °C).

Herbivory and parasitism

As described in Moreira *et al.* (2015), herbivory, seed herbivory, and parasitism associated with the seed herbivore were surveyed across all populations at the peak of fruit production (July 2013). Ten plants were selected per population, and for each plant we visually estimated whole-plant leaf damage by insect leaf chewers using the following scale: 0 = undamaged; 1 = 0–25% of leaf area consumed; 2 = 25–50% leaf area consumed; 3 = 50–75% leaf area consumed; 4 = > 75% leaf area consumed (Moreira *et al.* 2015). Although this approach resulted in a rather coarse estimate of leaf herbivory, we were still able to detect ample variation among populations (fourfold), indicating that this method was satisfactory and if anything resulted in a conservative test of variation among populations (Moreira *et al.* 2015). For 28 of the 30 populations, we also estimated seed herbivory and parasitism by collecting 10 mature fruits from each of 10 plants within each population (different set of plants than those used to estimate leaf damage). Fruits were dissected and, based upon caterpillar and parasitic wasp presence, we estimated the proportion of fruits attacked by the seed herbivore (number of attacked fruits/total number of fruits collected per plant) and the proportion of seed herbivore-attacked fruits with parasitoid (number of attacked fruits with parasitoid/number of fruits with a seed herbivore). As with previous work in this system (Abdala-Roberts *et al.* 2010), these surveys revealed substantial variation among populations in seed herbivory (up to 25-fold), leaf herbivory (fourfold) and parasitism associated with the seed herbivore (14-fold) (Moreira *et al.* 2015). In addition, parasitism is positively associated with seed herbivory ($R^2 = 0.29$, $P = 0.006$; data from this study), suggesting positive density-dependent attack by parasitoids as well as the potential for indirect effects on seed defences (and plant fitness) via reduced seed herbivory (Fig. S1).

Conducting a single measurement of herbivory could lead to a latitudinal bias if the growing season and thus the duration of exposure to herbivory co-varies (decreases) with latitude (Pennings, Siska & Bertness 2001). In this case, such bias is unlikely as the latitudinal transect was relatively narrow. In addition, by measuring trophic interactions during the portion of the rainy season when leaf damage and fruit attack (and parasitism) are highest (July–August; Ortegón-Campos *et al.* 2009; Abdala-Roberts *et al.* 2010) our sampling scheme further reduced a bias in estimates of herbivory as we were able to measure damage at the peak of interaction strength for all of the populations sampled.

Leaf and fruit sampling for quantification of defences

In July 2013 we collected 10 immature CH fruits per plant for chemical analyses for the same 30 populations. These plants were a subset of the 10 plants used to record leaf damage per population and were sampled at the same time as herbivory and parasitism were measured. During sampling, we took special care of exclusively collecting undamaged fruits. We only present results for chemical analyses of immature CH fruits because immature CL fruits were rare in most populations. In addition, from the same plants we collected three fully expanded leaves with no evidence of damage. Both fruit and leaf samples were placed in plastic bags, kept in cooler with ice at 5 °C, and then transported to the laboratory for quantification of phenolic compounds (seeds and leaves) and estimation of trichome density (leaves). By sampling undamaged tissues our goal was to reduce variation in defences caused by site-specific induction of plant defences. However, sampling undamaged leaves and fruits does not eliminate induction as induced plant responses may be systemic; therefore, it is possible that the sampled plants were induced at some level and induction could be contributing to observed levels of plant defences. In addition, sampling undamaged tissues may also lead to biases in the estimation of chemical defences if those tissues are more highly defended.

QUANTIFICATION OF PLANT DEFENCES

For each of 244 plants [8.31 ± 0.36 plants per population (mean ± SE)], we estimated the density of glandular trichomes on the ventral side of leaf blades by taking two 3-cm^2 disks from one leaf, and counting all trichomes present on each disk under a stereoscopic microscope. One disk came from the distal portion of the leaf and the other from the basal portion; these two values were then averaged per plant for statistical analyses. Only a single leaf was measured as careful inspection suggested relatively low leaf-to-leaf variation within individual plants (L. Abdala-Roberts, pers. obs.). In addition, for a subset of four to five plants within each population [4.6 ± 0.15 for seeds, 4.83 ± 0.07 for leaves (population mean ± SE)], we collected leaves and immature fruits to quantify the concentration of phenolic-based compounds. We chose phenolics because they are widely recognized as herbivore feeding deterrents across many plant taxa (Marquis 1992; Salminen & Karonen 2011; Mithöfer & Boland 2012). In addition, other studies have shown that the production of phenolic compounds may also be influenced by resource availability and abiotic conditions (e.g. light availability; Close *et al.* 2003; Abdala-Roberts *et al.* 2014). Although we did not quantify the extent to which phenolics and trichomes provide resistance against herbivores in *R. nudiflora*, significant associations (positive or negative) are strongly suggestive of a role of such traits in mediating plant–herbivore interactions. Phenolic compounds in the seeds and leaves were extracted and analysed as described by Moreira, Zas & Sampedro (2012). Briefly, ca. 1 g. of oven-dried plant

material (45 °C to constant weight) was finely ground in liquid nitrogen and ca. 300 mg were used for extraction with aqueous methanol (1:1 vol:vol) after sonication (15 min). Samples were analysed by HPLC using a Grace C18 reversed phase column (3 μm, 150 × 4.6 mm; Grace Davison Discovery Science, Columbia, MD, USA) and an YL9100 instrument with diode array detection (YL Instrument Co., Anyang, Korea). The 15 μL injection was eluted at a constant flow of 0.7 mL min^{-1} with a gradient of acetonitrile and 0.25% phosphoric acid in water as follows: from 80% to 50% water in 5 min, then form 50% to 30% in 5 min, and kept at 30% for 7 min, and a final step from 30% to 5% in 4 min, followed by 5 min of equilibration time. Peaks were detected by a diode array detector at 270 nm (for hydrolizable tannins), 320 nm (for ferrulic acid derivates), 370 nm (for flavonoids) and 500 nm (for anthocyanins). Absorbance spectra were recorded from 200 to 900 nm. Peaks showing a characteristic absorption band of phenolics (Mabry, Markham & Thomas 1970) were recorded. Concentrations were calculated by using a standard curve that related peak areas to known gallic acid (for hydrolizable tannins), caffeic acid (for caffeic acid derivatives), quercitin (for flavonoids) and cyanidin (for anthocyanins) concentrations using 270 nm absorbance.

GEOGRAPHIC AND CLIMATIC VARIABLES

The latitude and longitude of each *R. nudiflora* population were determined using a Global Positioning System device (Garmin, Olathe, KS, USA). To characterize the climatic conditions present at each population site, we used a subset of the BioClim climate variables (available at: http://www.worldclim.org/), specifically: BIO1 (annual mean temperature, °C), BIO4 (temperature seasonality, expressed as the standard deviation of temperature among months*100), BIO5 (maximum temperature of the warmest month, °C), BIO6 (minimum temperature of the coldest month, °C), BIO12 (annual precipitation, mm), BIO13 (precipitation of the wettest month, mm), BIO14 (precipitation of the driest month, mm), BIO15 (precipitation seasonality, expressed as standard deviation of precipitation across months). The procedures used to calculate these variables are fully described in Hijmans *et al.* (2005).

STATISTICAL ANALYSES: BIOTIC AND ABIOTIC CORRELATE OF LATITUDINAL VARIATION IN DEFENCES

First, to assess the presence of latitudinal variation in plant defences (Rasmann & Agrawal 2011; Moreira *et al.* 2014), we performed population-level simple regressions between latitude and each defensive trait (i.e. mean values across plants within each population), namely: total phenolics (sums across compound classes) for seeds, total phenolics for leaves and leaf trichome density. In addition, to explore whether there were differences in the patterns exhibited by different classes of phenolic compounds, we conducted regressions separately for caffeic acid derivatives, gallic acid derivatives (i.e. hydrolyzable tannins), flavonoids, and anthocyanins and present these results in Supporting Information (see Tables S2 and S3).

Second, to investigate the influence of biotic and abiotic correlates of latitudinal variation in plant defences, we performed population-level multiple regressions where climatic variables (temperature, precipitation), herbivory (leaf damage or proportion of attacked fruits for leaf and seed phenolics, respectively, calculated as above) and parasitism (proportion of parasitized seed herbivore larvae for seed defences, calculated as above) were predictors of total phenolics in seeds, total phenolics in leaves and leaf trichome density. These analyses also included

the effect of latitude in order to test whether there were residual effects after accounting for biotic and abiotic factors that covary with latitude. Similarly, we also conducted multiple regressions separately for each class of phenolic compounds and these results are presented as Supporting Information (Table S4). These separate analyses of each group of compounds raises concerns about Type I error inflation (four classes of compounds across two types of tissue), and some of the phenolic groups were correlated. Therefore, whenever significant (uncorrected) effects were observed in such analyses these were interpreted based upon Bonferroni-adjusted *P*-values (Table S4).

Plant defence-herbivory relationships are dynamic and likely involve ecological and evolutionary feedbacks. Therefore, the causality of (positive or negative) associations between defence and herbivory may be difficult to determine. Herbivore diet breadth may be particularly relevant in this regard. For dietary specialists herbivores (i.e. the seed herbivore attacking *R. nudiflora*) we expect co-evolutionary dynamics between the plant and the herbivore where the relationship between rates of herbivory and plant defences may vary, with a positive relationship indicating that herbivory drives defences (Agrawal *et al.* 2012; Castillo *et al.* 2013), and a negative relationship indicating that defence drives herbivory (Bernays & Chapman 1994). In contrast, for dietary generalist herbivores (i.e. leaf-chewers feeding on *R. nudiflora*), herbivore abundance (and thus herbivore pressure) is driven by a larger resource pool, and herbivore pressure should thus drive plant defence evolution (Castillo *et al.* 2014). Accordingly, ascribing causality in herbivory-defence relationships is uncertain.

In the above tests for the association between defence and climate, assessing the separate effects of all eight climatic variables would make the regression models unnecessarily complex. To simplify this analysis, we summarized these climatic variables by conducting two principal component analyses (PCA) using PROC FACTOR (rotation = varimax) in SAS 9.2 (SAS Institute 2008, Cary NC), one for temperature variables and one for precipitation variables. In each case, climatic variables were summarized with the first principal component. The first principal component explained 79% of the variance in the four temperature variables across populations ('PC temperature' hereafter), and was positively related to annual mean temperature, temperature seasonality, and maximum temperature of the warmest month, and negatively related to minimum temperature of the coldest month. Similarly, the first principal component explained 77% of the variance in the four precipitation variables across populations ('PC precipitation' hereafter), and was positively related to annual precipitation, precipitation of the wettest month and precipitation of the driest month. The standardized *z*-scores of the first principal component (PC hereafter) of each analysis were used in the multiple regression analyses. Latitude was significantly negatively associated with PC precipitation ($R^2 = 0.61$, $P < 0.001$) but not to PC temperature ($R^2 = 0.04$, $P = 0.319$), and PC temperature was unrelated to PC precipitation ($R^2 = 0.07$, $P = 0.168$).

Throughout the results section, we present means and SE as descriptive statistics. All regressions were conducted with PROC REG in SAS 9.2. To visualize the effect of a significant predictor on a given defensive trait after accounting for all other factors in the multiple regression models, we constructed partial residual plots (Larsen & McCleary 1972). For these graphs, the residuals from a multiple regression where a defensive trait is predicted by all factors except the predictor of interest are plotted against the residuals from a multiple regression where the predictor of interest is defined as the response variable of all the other factors. The slope of this relationship is the partial correlation coefficient for the predictor of interest (Larsen & McCleary 1972).

Results

EFFECT OF LATITUDE ON PLANT DEFENCES

Phenolic compounds

We found 7.7-fold population variation in the total concentration of phenolics (mean across tissues ranged from 52.09 ± 8.46 mg g^{-1} to 403.10 ± 113.97 mg g^{-1}), whereas examining each tissue separately there was 6.5-fold population variation in leaves (21.69 ± 1.76 to 141.04 ± 2.51 mg g^{-1}) and 9.8-fold variation in seeds (74.37 ± 4.62 to 725.55 ± 52.69 mg g^{-1}). Across both tissues, caffeic acid derivatives were by far the most abundant class of phenolic compounds, followed by hydrolyzable tannins, flavonoids and anthocyanins (Table S2). There was an overall positive association between total concentration of leaf and seed phenolics ($R^2 = 0.32$, $P = 0.001$), but seeds exhibited a 3.5-fold higher concentration of phenolics than leaves (Table S2). This difference was consistent across all classes of phenolic compounds except hydrolysable tannins which were present at a higher concentration in leaves (Table S2).

Results from univariate regression analyses indicated no association between latitude and total phenolics in either seeds or leaves (Table 1a; Fig. 1d,f). Similarly, there were no significant relationships between seed or leaf phenolics and latitude when running analyses separately for each class of compound (Table S3).

Trichomes

Leaf trichome density also varied extensively and exhibited up to threefold variation across populations (range: 17.87 ± 1.99 to 54.08 ± 7.72 trichomes cm^{-2}). In this case, simple linear regression indicated a significant association between trichomes and latitude where trichome density increased with decreasing latitude (Table 1a; Fig. 1e).

BIOTIC AND ABIOTIC FACTORS UNDERLYING LATITUDINAL VARIATION IN PLANT DEFENCES

Phenolic compounds in seeds

After accounting for biotic and abiotic correlates of latitude, multiple regression analyses indicated a positive relationship between fruit attack and total seed phenolics (Table 1b; Fig. 2a), but no significant associations between seed phenolics and latitude, parasitism or climatic variables (Table 1b; Fig. 2d,g). Analyses by class of phenolic compound showed that caffeic acid derivatives, the most abundant class of phenolic compound in seeds, tended to be positively associated with fruit attack (marginally significant; Table S4), whereas for hydrolysable tannins we found significant negative associations with latitude and precipitation (Table S4).

Phenolic compounds in leaves

Multiple regression revealed a marginally significant negative association between leaf damage and total leaf phenolics (Table 1b; Fig. 2b), significant negative associations of precipitation and latitude with total leaf phenolics (Table 1b; Fig. 2h), but no effect of temperature (Table 1b; Fig. 2e). This residual influence of latitude occurred because the effects of precipitation and latitude cancelled each other in the univariate regression as these two predictors were both negatively associated with leaf phenolics but were negatively correlated themselves (see Materials and Methods). Therefore, an effect of latitude emerged after precipitation was accounted

Table 1. Results from simple regressions testing for the effect of latitude alone (a), and multiple regressions testing for the effects of latitude, biotic (herbivory, parasitism) and abiotic (temperature, precipitation) factors (b) on the total concentration (mg g^{-1}) of phenolics in seeds and leaves, and on leaf trichome density (trichomes cm^{-2}) in *Ruellia nudiflora*

Predictor(s) (a) Simple regressions	Total seed phenolics*		Total leaf phenolics*		Leaf trichomes	
	β	R^2 (*P*-value)	β	R^2 (*P*-value)	β	R^2 (*P*-value)
Latitude	15.86	0.021 (0.44)	0.31	0.0002 (0.94)	**−1.46**	**0.13 (0.05)**
	Model $R^2 = 0.422$		Model $R^2 = 0.276$		Model $R^2 = 0.401$	
(b) Multiple regressions	β	r^2 (*P*-value)	β	r^2 (*P*-value)	β	r^2 (*P*-value)
Fruit attack	**0.16**	**0.16 (0.05)**	–	–	–	–
Parasitism	0.01	0.00 (0.99)	–	–	–	–
Leaf damage	–	–	−27.99	0.10 (0.10)	**7.22**	**0.23 (0.01)**
Temperature	0.09	0.09 (0.15)	−5.35	0.03 (0.30)	*1.51*	*0.11 (0.09)*
Precipitation	0.08	0.08 (0.16)	**−18.30**	**0.16 (0.04)**	0.52	0.05 (0.70)
Latitude	0.06	0.06 (0.26)	**−17.62**	**0.18 (0.02)**	0.69	0.01 (0.57)

R^2, coefficient of determination; β, slope estimator; r^2, partial correlation coefficient.
Significant ($P \leq 0.05$) and marginally significant ($0.05 < P < 0.10$) effects are in bold and italics, respectively.
*Simple and multiple regressions were also conducted separately for each of four classes of phenolic compounds (caffeic acid derivatives, hydrolysable tannins, flavonoids and anthocyanins). These results are presented in Tables S3 and S4 as Supporting Information.
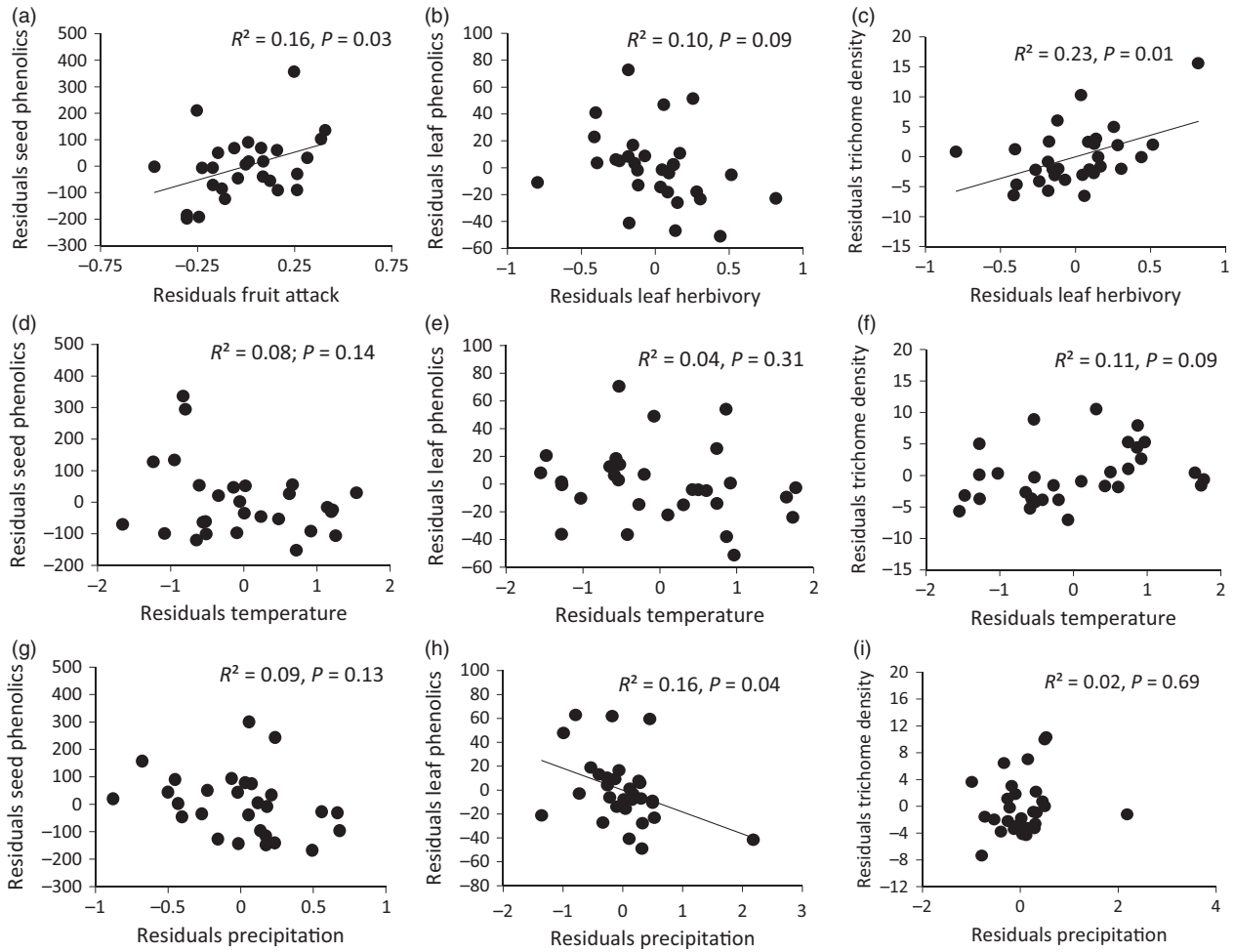
Fig. 2. Partial residual plots showing the relationships between the residuals from a multiple regression where a given defensive trait of *Ruellia nudiflora* is predicted by all factors except the factor of interest and the residuals from a multiple regression where the factor of interest is the response variable of the predictors in the former model. For example, panel A depicts the relationship between the residuals from a multiple regression where total seed phenolics is predicted by all factors except fruit attack (i.e. temperature, precipitation and latitude) ('residuals seed phenolics' on the Y axis) and the residuals from a multiple regression where fruit attack is the response variable predicted by the same factors in the former model (temperature, precipitation and latitude) ('residuals fruit attack' on the X axis). Each row of panels depicts the relationship between a given predictor (herbivory: a–c; temperature: d–f; precipitation: g–i) and each of the three plant defensive traits measured. Predicted relationships come from simple linear regressions and the slope of each relationship is the partial correlation coefficient (r^2) for the effect of the predictor (X axis) on a given plant trait (Y axis) (r^2 values are reported in Table 1). We do not show plots for seed herbivore parasitism as this predictor was not associated with any of the measured plant traits. Circles represent plant population means. Precipitation and temperature values represent *z*-scores from the first axis of two principal components analyses summarizing a suite of relevant precipitation- and temperature-related variables (see Statistical analyses).

for in the multiple regression. Analyses by compound class showed similar results for caffeic acid derivatives (Table S4), the dominant class of phenolic compound likely driving results for total leaf phenolics, as well as hydrolysable tannins (Table S4).

Trichomes

The observed association between latitude and leaf trichome density (Fig. 1e) became non-significant after accounting for biotic and abiotic factors, where leaf herbivory and temperature explained most of the latitudinal variation in this trait (Table 1b). Specifically, multiple regression indicated a significant positive association between trichome density and leaf

herbivory (Table 1b; Fig. 2c), a marginally significant positive association with temperature (Table 1b; Fig. 2f), but no effect of precipitation (Table 1b; Fig. 2i).

Discussion

OVERVIEW

Substantial population variation in *R. nudiflora* herbivory (fourfold in leaf herbivory and 25-fold in seed herbivory across 5° latitude; Moreira *et al.* 2015) was significantly associated with variation in plant defences. Patterns of variation in leaf trichomes fully supported predictions, with trichome density increasing in concordance with a parallel increase in her-

bivory towards lower latitudes. Similarly, seed phenolics were positively associated with herbivory, but seed herbivory tended to increase with latitude (Moreira *et al.* 2015) and the predicted (positive) association between latitude and defence was not detectable. Contrary with predictions, the association between herbivory and leaf phenolics was weak (marginally significant). In addition, although populations varied 14-fold in seed herbivore parasitism and parasitism was associated with seed herbivory, there was no association of parasitoids with seed defences. Finally, variation in the abiotic environment (fourfold in precipitation and 2 °C in mean annual temperature) was also associated with plant defence, indicating that abiotic factors can play a major role in shaping plant defences, independently of herbivory. Temperature tended to be positively associated (marginally significantly) with trichome density but did not affect seed or leaf phenolics, while precipitation was inversely correlated with leaf phenolics but did not affect seed phenolics or trichomes. Also, having accounted for herbivory and climatic variation, leaf phenolics but not seed phenolics or trichome density, were associated with and decreased with latitude.

EFFECT OF BIOTIC INTERACTIONS ON GEOGRAPHIC VARIATION IN PLANT DEFENCES

There was evidence in support of the expectation of higher defences at lower latitudes for some but not all of the defensive traits measured in *R. nudiflora*. Leaf trichome density was positively associated with leaf herbivory (for similar example see Castillo *et al.* 2013), and latitudinal variation in herbivory (increasing damage with decreasing latitude; Moreira *et al.* 2015) resulted in a concomitant latitudinal gradient in trichome density. This finding agrees with previous work showing that latitudinal variation in herbivory drives patterns of intra-specific latitudinal variation in defensive traits for several species of salt marsh plants (Salgado & Pennings 2005; Pennings *et al.* 2009). In contrast, leaf phenolics and seed phenolics were associated with herbivory (albeit weakly in the former case) but not in a latitudinal manner. Leaf herbivory and fruit attack both varied latitudinally (albeit weakly and contrary with predictions in the latter case, i.e. increase in attack with latitude; Moreira *et al.* 2015), but latitudinal variation in leaf herbivory and seed herbivory did not result in concomitant gradients in leaf and seed phenolics, respectively. These findings highlight the fact that latitudinal gradients in herbivory are not always associated with corresponding patterns of plant defence, as there may be other underlying factors driving geographic variation in plant defences (Johnson & Rasmann 2011). It is also possible that seed and leaf phenolics only partially integrate the defence syndrome of *R. nudiflora*, thus calling for further work focusing on additional defensive traits. For instance, there might be specific compounds that are important for resistance against the seed herbivore (*Tripudia* sp.), a dietary specialist herbivore on *Ruellia* species.

The fact that the effects of latitude on plant defenses are indirect and mediated by herbivory could also make latitude-defence associations more difficult to detect. This may be especially the case when assessing intra-specific variation because latitudinal ranges (and thus gradients of herbivory) in these studies are typically smaller (relative to interspecific studies conducted at broader spatial scales) and heterogeneity driven by factors other than latitude may swamp the latitude signal. Thus, within a species range we might be able to detect how geographic variation in herbivory drives geographic variation in plant defences (i.e. the mechanistic basis for explaining higher defences at lower latitudes), but there may not be a large enough latitudinal gradient in herbivore pressure to detect the indirect effects of latitude on plant defence. At the same time, however, our results emphasize the importance of testing for intra-specific geographic associations between herbivory and plant defence, independent of latitude. Ultimately, such associations will allow us to understand finer scale ecological mechanisms occurring within the geographic ranges of individual species that contribute to broader scale macro-ecological patterns in plant defence investment (e.g. tropical versus temperate species).

It is important to note that although we exclusively sampled undamaged leaves and fruits, our estimates of defences do not differentiate between herbivore-induced and constitutive levels of plant defence investment. Therefore, some of the geographic patterns in plant defences observed could result from induced responses to herbivory during the current growing season and/or constitutive levels of plant defence investment (Salgado & Pennings 2005). Future work measuring tissue-specific inducibility will clearly contribute to understanding the contribution of induced defences to herbivory-defence relationships in this study system. In addition, our study did not address tolerance, an important component of plant defence against herbivores. For instance, Lehndal & Ågren (2015) recently reported that gradients in climatic variables and herbivory drive latitudinal differences in tolerance and resistance in the perennial herb *Lythrum salicaria*. Reduced herbivore pressure at higher latitudes results in lower resistance in northern populations of this plant, whereas shorter growing seasons at higher latitudes favour earlier plant development, larger plants receive more damage at the time of herbivore emergence, and this in turn favours higher tolerance in northern populations. Therefore, an explicit consideration of induced versus constitutive defences as well as tolerance to herbivory will contribute to a better understanding of how biotic and abiotic factors drive geographic variation in plant defensive mechanisms, and such aspects deserve increased attention in future work.

Despite the expectation of an indirect effect of parasitoids on plant defence (Björkman, Berggren & Bylund 2011; Johnson & Rasmann 2011), i.e. parasitoids weaken herbivore effects on plant defences and thus alter plant defence-latitude relationships driven by herbivory, we found no detectable association of parasitism with population variation in *R. nudiflora* seed chemical defences. This non-significant result occurred even though levels of parasitism associated with the seed herbivore were high (> 80% in most of the populations sampled; Moreira *et al.* 2015) and parasitism intensity is posi-

tively associated with seed herbivory. Furthermore, there was no evidence of a latitudinal gradient in parasitism (Moreira *et al.* 2015), and this would have precluded an effect of parasitoids on latitudinal variation in seed herbivory and seed defences. Although a prior study similarly found no latitudinal gradient in predation by insectivorous birds (Mooney *et al.* 2010), data on predation and parasitism by arthropods gathered thus far suggests stronger effects at lower latitudes (reviewed by Dyer & Coley 2002; Schemske *et al.* 2009). For example, Stireman *et al.* (2005) found higher rates of parasitism on caterpillar species at lower latitudes, and a recent study by Marczak *et al.* (2011) found latitudinal variation in herbivore abundance for a salt marsh plant was predominantly driven by predator effects. Although our results suggests that parasitoid effects are of secondary importance in this system, more work is needed to determine the underlying factors associated with latitudinal variation in third trophic-level effects in order to integrate tri-trophic interactions into theory on latitudinal variation in plant defences (Johnson & Rasmann 2011; Zhang & Adams 2011).

EFFECT OF CLIMATIC FACTORS ON GEOGRAPHIC VARIATION IN PLANT DEFENCES

Our results indicate that considering only herbivory is insufficient to explain geographic variation in plant defences. In particular, the fact that precipitation was negatively associated with leaf phenolics suggests that individuals of *R. nudiflora* at sites with lower water availability (and thus lower resource availability) are more highly defended, and is in keeping with the Resource Availability Hypothesis (Coley, Bryant & Chapin 1985). This hypothesis predicts that plants adapted to resource-poor and stressful habitats are relatively slow-growing, have low tolerance for herbivory, and as a consequence invest more heavily in defences (Coley, Bryant & Chapin 1985; reviewed by Endara & Coley 2011). Following from this finding and as pointed out by previous work, resource availability is probably an important factor influencing latitudinal gradients in plant defences and may explain departures from expectations depending if resources covary positively or negatively with latitude (Johnson & Rasmann 2011; Moreira *et al.* 2014). For example, all else being equal, plants at lower latitudes experience higher levels of herbivory but would invest less in defences than expected because they have more resources, grow faster, and presumably invest less in defences (Coley, Bryant & Chapin 1985). In our case, there was a negative effect of latitude on leaf phenolics (driven by some unmeasured factor), as well as a negative effect of precipitation, but these factors were negatively correlated themselves and thus exerted opposing effects which resulted in no overall effect of latitude on this trait.

We also found that, in addition to leaf herbivory, temperature was also an underlying factor associated (albeit more weakly) with *R. nudiflora* leaf trichome density. In this case, the fact that trichome density tended to be positively associated with temperature suggests that this trait plays a role in abiotic resistance by reducing leaf water loss due to evapo-

transpiration at sites with higher temperatures (see Woodman & Fernandes 1991; Hare & Elle 2001). It is important to note, however, that although this study spanned one-third of the temperature range of *R. nudiflora*, the lack of strong correlations between plant defences and temperature could have been due to insufficient variation in temperature across the studied populations. Accordingly, if we had sampled populations spanning the entire distribution range of *R. nudiflora* then significant correlates of temperature could have emerged. However, independently of this limitation, our results underscore the importance of simultaneously testing for influence of biotic and abiotic factors on geographic variation in plant traits, and also highlight that plant defences may respond to multiple abiotic drivers, independently of herbivore pressure (Pearse & Hipp 2012; Moreira *et al.* 2014; Rasmann, Pellissier & Alvarez 2014).

The fact that trichomes and leaf phenolics were associated with different climatic factors, in addition to herbivore pressure, whereas seed phenolics were not shows that the environmental correlates of geographic variation in plant defences vary among traits within a particular tissue as well as among plant tissues. Based on this, we suggest that addressing within-plant defence allocation patterns is an important consideration in order to improve our understanding of the underlying mechanisms associated with intra-specific geographic variation in plant defences. At the same time, measuring geographic variation in other chemical (e.g. alkaloids; Banko *et al.* 2002) and physical traits (e.g. toughness; Marquis, Ricklefs & Abdala-Roberts 2012) across tissues would be useful in order to determine the generality of tissue-specific patterns.

FUTURE DIRECTIONS

First, our findings emphasize that studies on latitudinal variation in plant defences should be more comprehensive in their assessment of underlying factors by testing for the simultaneous influence of biotic and abiotic drivers. These multi-factorial evaluations will provide insights into the relative importance and mechanisms by which biotic and abiotic forcing shape geographic variation in plant traits. Second, the relative importance of different factors (e.g. herbivory versus abiotic conditions) shaping geographic variation in plant defences is likely scale-dependent. For instance, large-scale studies addressing geographic variation in plant defences across many species incorporate latitudinal gradients in herbivory that are sufficiently large to detect associations between latitude and plant defences without accounting for factors operating at finer scales. However, at smaller spatial scales typical of studies assessing intra-specific variation we have that multiple factors (which do not strictly covary with latitude) may introduce enough variation that their importance becomes greater and the signal of latitude is lost. Thus, depending on the spatial scale of study, we emphasize that future work should not only test for effects of latitude *per se*, but also more broadly evaluate geographic variation in herbivory and determine which non-latitudinal factors drive vari-

ation in herbivory and plant defences. Third, a push towards more studies addressing intra-specific geographic variation in plant traits is needed as this provides the basis for understanding how plants evolve along biotic and abiotic gradients, and will contribute to uncover finer scale, micro-evolutionary processes underlying large-scale, macro-ecological patterns. Finally, more studies using 'phytometer' plants or reciprocal transplants (e.g. Pennings *et al.* 2009) as well as experimentally manipulating herbivore and natural enemy effects across different latitudes are necessary in order to disentangle the causality of herbivory-defence associations in space. In summary, we consider that current understanding of the biogeography of plant–herbivore interactions will move forward by addressing latitudinal variation in herbivory and plant defences from a multi-factorial perspective, as well as by assessing both intra- and interspecific variation at multiple scales in order to bridge species-specific dynamics with macro-ecological patterns.

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Data accessibility

Data files used for statistical analyses performed in this study are available at Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.qg7p3 (Abdala-Roberts *et al.* 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Geographical coordinates and climatic characteristics of *Ruellia nudiflora* populations.

Table S2. Concentration of different groups of phenolic compounds in seeds and leaves.

Table S3. Association between latitude and the concentration of different groups of phenolic compounds.

Table S4. Effects latitude, biotic and abiotic factors on the concentration of different groups of phenolic compounds.

Figure S1. Relationship between the proportion of fruits attacked by seed herbivore and the proportion of seed herbivores parasitized.