

# Recent evolutionary divergence in a plant ring-species is not accompanied by floral phenology or pollinator shifts

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## Abstract

**Background and aims** – The study of factors and processes involved in evolutionary divergence can inform how biodiversity is generated and maintained. We evaluate shifts in phenology or in pollination systems as potential barriers to gene exchange and thus promoters of divergence at the population-species boundary in the plant ring-species *Euphorbia tithymaloides* in the Caribbean.

**Material and methods** – Combining collections-based and field-based observations and measurements, we evaluate evidence supporting that shifts in tempo of reproductive activity (floral phenology) or pollinator guilds (using visitation as a proxy) could be acting as mechanisms promoting divergence in *E. tithymaloides*. We focus on the geographic region where evolutionary divergence in this species has been documented: Greater and Lesser Antilles. Phenology data were derived from herbaria and online databases, for a total of 376 records across the Greater and Lesser Antilles. We quantified and characterized reward (nectar  $n = 13$  sites) and gathered visitation data using direct observation ( $n = 12$  sites) for a total of over 133 hours of observation/site.

**Key results** – The peak of floral activity of *E. tithymaloides* is in winter, when days are short (~late October–late May). Under natural conditions, plants in the Antilles produce up to 22.4  $\mu\text{L}$  of nectar, with mean sugar concentrations of ~46.5 °Brix that amount to up to 10.3 mg of total sugars, with no significant differences observed between plants of the Lesser and Greater Antilles. Hummingbirds are the main floral visitors of *E. tithymaloides* in both areas: Greater Antilles: 61%, Lesser Antilles: 85%, and network analyses support a floral visitor community turnover across islands/countries.

**Conclusion** – Evolutionary divergence in Caribbean *E. tithymaloides* along the Greater and Lesser Antilles is not accompanied by shifts in floral phenology or pollinator systems. Other factors, like pollinator turnover or pollinator-plant trait matching, might be at play. We outline hypotheses to this effect.

## Keywords

Caribbean, *Euphorbia*, gene flow, plant-pollinator interactions, reproductive isolation, speciation

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## INTRODUCTION

The study of factors and processes that involve evolutionary divergence can inform how lineages split, which is in turn an important player in speciation, a fundamental process in the generation of diversity (Coyne and Orr 2004; Butlin et al. 2010; Kulmuni et al. 2020). Biological systems undergoing active divergence or incipient speciation are therefore of great importance, as they offer unique

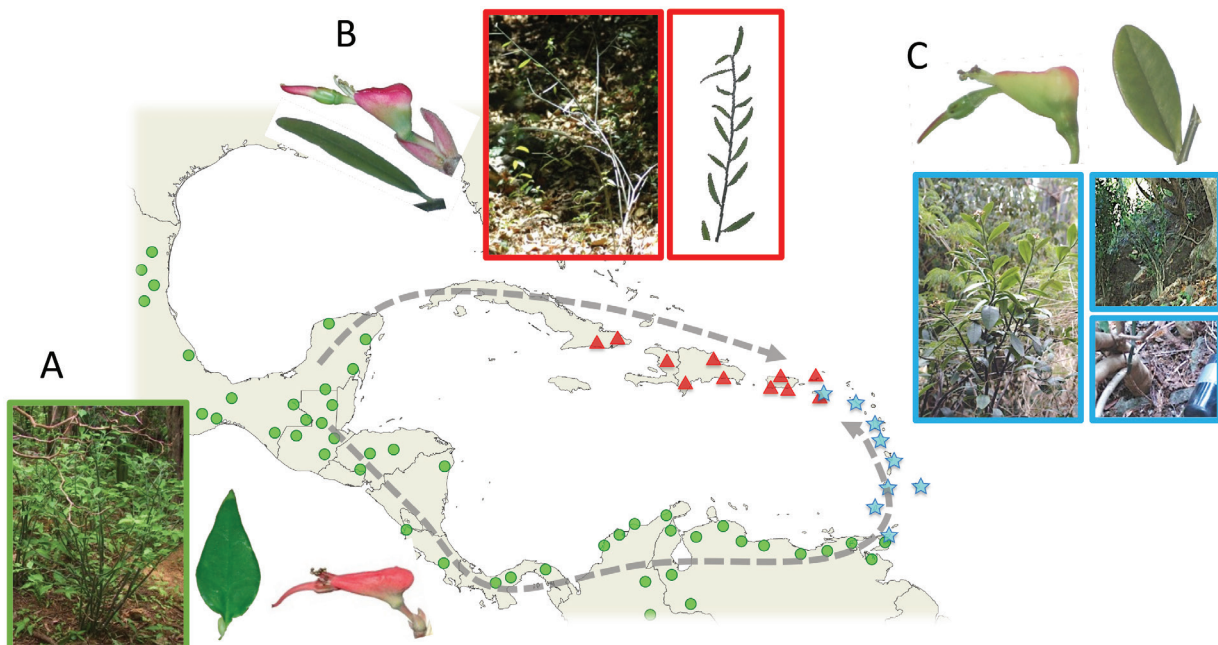
opportunities to the study of such processes and factors (Kulmuni et al. 2020).

In ring-species, evolutionary divergence occurs from an ancestral population along two fronts that conform to a circular distribution such that when the terminal populations at either end of the diverging fronts come together, barriers preventing homogenizing gene-flow are already in place so that the two fronts remain as distinct

(Irwin et al. 2001). The plant *Euphorbia tithymaloides* is an example of such system, having colonized the Caribbean Basin from Mexico/Guatemala along two expansion routes: one that travelled east through the Greater Antilles (GA front), and another that moved south through Central America, then east through the northern portion of South America, and then north, traversing the Lesser Antilles (LA front). Models of genetic variation and phylogenetic inference support evolutionary divergence in this system (Cacho and Baum 2012; Cacho et al. 2019), so that in the vicinity of the US Virgin Islands, close to the Anegada Passage, the terminal populations from either front (GA, LA) are the most distantly related and genetically distinct despite occurring in neighbouring geographic areas. Morphologically, the two terminal forms exhibit contrasting leaf morphologies (Jacobo-Arteaga et al. 2022) and habit, as well as some key differences in floral morphology (mainly size and pubescence) but also important similarities (i.e. floral shape; Fig. 1). Vegetatively, individuals from the GA front are small shrubs 0.8–3 m tall that divide at the base giving rise to somewhat delicate scandent branches (1–3 cm thick), and thin, linear and pubescent leaves (Dressler 1957). Individuals of the LA front are robust, self-supporting shrubs or even small treelets (also 1–3 m tall) with a single main stem that can be as wide as 6–9 cm at the base, and thick and glabrous leaves (Dressler 1957) (Fig. 1). Plants from the GA and LA fronts also differ in floral morphology, with those from the GA side presenting

smaller and somewhat pubescent cyathia (modified inflorescences of *Euphorbia*) that produce slightly less pollen (Cacho and José-Zacatula 2020) than those of the LA side, which are larger and always glabrous (Dressler 1957). Yet, plants from both fronts have evolved relatively shorter cyathia when compared to those from mainland populations, a case that was initially attributed to possible shared ancestry (Dressler 1957), but current genetic data and phylogenetic analyses support as a case of evolutionary convergence following divergence (Dressler 1957; Cacho et al. 2019). Genetic, phylogenetic, and population genetic analyses both based on a few markers (Cacho and Baum 2012) as well as at a genomic scale (Medina-Rodríguez 2022) support limited gene flow between populations of the GA and LA forms. This happens despite contrasting forms occurring in sufficiently close proximity for hummingbirds (hypothesized pollinators) to make their way between populations (Caribbean hummingbirds are known to move across islands, albeit not frequently; Lack 1973; Pulich 1968; Stimson 1944), and also for vegetative dispersal (Dressler 1957; N. Ivalú Cacho pers. obs.). Factors with the potential of driving divergence have not been studied in this system. In this work, we evaluate whether shifts in floral timing or in potential pollinators could be acting as such.

In plants, pre-zygotic factors such as shifts in floral phenology (Stiles 1975; Nuismer and Cunningham 2005; Brandenburg et al. 2009) and pollinator preferences (Fulton and Hodges 1999; Maad and Nilsson 2004) are



**Figure 1.** Evolutionary divergence along two fronts (dashed arrows) in *Euphorbia tithymaloides* L. in the Caribbean has given rise to two incipient insular lineages from mainland populations (panel A) that exhibit morphological, ecological, and genetic differences: one occurring in the Greater Antilles (GA lineage; panel B), and one in the Lesser Antilles (LA lineage; panel C). Map is simplified to show the geographic distribution of these three focal forms (Mainland, Greater Antillean, and Lesser Antillean). All photographs by N. Ivalú Cacho.

among some of the most important barriers promoting isolation among lineages (Coyne and Orr 2004; Wiens 2004). Differences in floral phenology can be effective in preventing breeding, and because they act early on, they can have a very strong contribution to reproductive isolation between diverging plant lineages at different evolutionary scales (Coyne and Orr 2004; Martin and Willis 2007; Martin et al. 2007; Osborne et al. 2020). In a population of mixed diploid and tetraploid *Chamerion angustifolium* (Onagraceae), opportunities for inter-cytotype mating were drastically reduced (by 47%) due to the combined effects of flowering asynchrony and insect foraging behaviour contributing to prezygotic mating isolation among cytotypes (Husband and Schemske 2000). Population level studies of local adaptation involving floral asynchrony show that floral phenology can evolve quite rapidly (Mitchell-Olds and Schmitt 2006; Franks et al. 2007), and it has been suggested that further divergence can lead to initial phenological differences to be strengthened by divergent selection (Chapurlat et al. 2020). In two closely related *Gymnadenia* orchid species that have been shown to introgress, flowering time and scent emission are under different selection regimes (Joffard et al. 2022), a scenario that is consistent with floral asynchrony playing an important role in maintaining species boundaries between closely related species (Chapurlat et al. 2020). Flowering time is under selection and phenology shifts mediate prezygotic reproductive isolation in *Metrosideros nervulosa* and *M. sclerocarpa* (Myrtaceae), two sister species that occur in sympatry (Osborne et al. 2020). At a broader scale, a study of plants representing nine genera (six families) where adaptation to harsh serpentine soils has evolved argues that phenological isolation plays a significant role across stages of adaptive divergence across clades (Sianta and Kay 2021), suggesting that these mechanisms operate more generally across plants.

The importance of pollinators in the generation and maintenance of plant diversity has long been recognized, Darwin being one of the first proponents of this idea (Darwin 1877; Stebbins 1970; Gentry 1982; Van der Niet and Johnson 2012). When interactions between plants and their pollinators are specialized, plants can present a set of traits that function in attraction and in improving efficiency of visitation and pollen transfer. As many as one fourth of the divergence events across angiosperms can be attributed to pollinator shifts (Van der Niet and Johnson 2012). At shallower phylogenetic scales, now classic studies (Schemske and Bradshaw 1999) as well as more recent ones (Temeles et al. 2016) continue to show that visitation patterns that influence pollen transfer can mediate reproductive isolation in plants. Experimental approaches suggest that changes in plant traits and mating systems (which would promote reproductive isolation) can rapidly follow changes in communities of pollinators (Gervasi and Schiestl 2017).

Reward offered by plants to their potential pollinators comes in the form of nectar or pollen, and its importance

mediating interactions of plants with their pollinators is well established (Armbruster 1993; Goldblatt and Manning 2006), to the point that reward has been used as a proxy to predict a plant's main group of pollinators (Baker and Baker 1990; Fenster et al. 2004; Zhao et al. 2016). The evolutionary ecology of pollination reward is complex, with factors as diverse as resources available to the plants, climatic fluctuations, and biotic interactions influencing reward simultaneously (Abrahamczyk et al. 2017). Yet one of the most consistent patterns substantiates the expectation that the energetic content of the reward offered by a plant is correlated to the energetic needs of its main pollinators (Heinrich and Raven 1972). There is still much to learn about the evolutionary ecology of nectar (Parachnowitsch et al. 2019), but many have successfully documented correlations between pollination systems and nectar characteristics (Perret et al. 2001; Nicolson 2002; Goldblatt and Manning 2006; Martén-Rodríguez et al. 2009; Bruneau 2014; Tavares et al. 2016; Tiedge and Lohaus 2017). In a phylogenetically informed study in *Iochroma* (Solanaceae) where reward, other floral traits, and visitation were quantified from observation and measurements in the field, reward was one the two attributes (the other being floral display) with direct association with pollinator preference, more so than traditionally invoked floral size and colour: plant species that produced larger quantities of nectar and larger displays were significantly more likely to be pollinated by hummingbirds than by insects (Smith et al. 2008a).

Here, we use a combination of collections-based and field-based observations and measurements to evaluate if there is evidence supporting that shifts in phenology or in floral visitors (a proxy for potential pollinators) could be acting as potential factors promoting or maintaining differentiation between the incipient lineages (GA and LA forms) of the slipper spurge *E. tithymaloides* L., a plant undergoing active evolutionary divergence in the Caribbean.

## METHODS

### Study system

We use the Caribbean slipper spurge (*Euphorbia tithymaloides* L., Euphorbiaceae) as a model to study early evolutionary divergence. These are moderately succulent shrubs that occur in seasonally dry forests and xeric scrublands throughout the Caribbean, with two incipient lineages that exhibit morphological (Dressler 1957; Cacho et al. 2019; Jacobo-Arteaga et al. 2022), ecological (Dressler 1957; Cacho and Baum 2012), and genetic divergence (Cacho and Baum 2012; Cacho et al. 2019). The morphological divergence between GA and LA plants is such that it has been recognized with subspecies status (*E. tithymaloides* subsp. *angustifolia*, GA form; *E. tithymaloides* subsp. *padifolia*, LA form; Dressler 1957). However, here we refer to them as 'forms' and

not ‘subspecies’ because we focus on factors involved in incipient lineage divergence rather than on taxonomy. Ecological differences among populations are subtle, and include the kind of forests they inhabit, with mainland populations occurring mostly on tropical but not coastal deciduous forests, populations from the Greater Antilles mostly inhabiting shaded understories of coastal forests even bordering mangroves, and those from the Lesser Antilles being most common in drier coastal shrub and scrublands, where they can be treelets or succulent leafless shrubs respectively, sometimes quite openly exposed to salt spray (Dressler 1957; N. Ivalú Cacho pers. obs.).

### Sampling

Because we are interested in detecting differences that have evolved in this system in incipient divergence, which might be subtle, we aimed at sampling sites focusing on the area of maximum divergence: the islands at either side of the Anegada Passage in the Caribbean, both in the Greater Antilles (GA) and the Lesser Antilles (LA). When possible, we included sites in the mainland, but our focus is on the region of maximum divergence and potential overlap.

In some areas, *E. tithymaloides* occurs in somewhat discontinuous patches that were considered together as a single site when besides being in close proximity, they shared habitat structure; when sites differed in canopy and vegetation structure, they were kept separate, as these variables can affect the behaviour of potential floral visitors. To this effect, insular habitats close to the coast of South America were considered Lesser Antilles (e.g. Curaçao), as they are under the effect of insularity and experience a rather different community of plants and potential visitors than do their continental counterparts. Despite efforts for a balanced sampling across areas, we have more sites in the Greater Antilles than in the Lesser Antilles (table S1 in Suppl. material 1, section 1) due to challenges to locate sites not dramatically altered by habitat loss or deterioration. It is not uncommon that forests where healthy populations had been documented are now replaced by development. In other cases, populations have shrunk (as documented per herbarium collections) or are relicts surrounded by real estate (N. Ivalú Cacho pers. obs.).

### Phenology data

We assembled a dataset with information derived from herbarium specimens, both from visiting herbaria and from digitized specimens available in digital repositories (see table S2 in Suppl. material 1, section 2), as well as from pictures of our own fieldwork and from others, available in iNaturalist ([www.inaturalist.org](http://www.inaturalist.org)). We selected images or specimens that had date and geographic information (geographic coordinates or a detailed description of locality that would allow manual georeferencing). For each specimen, we recorded day, month, and year of

collection, whether it was fertile or sterile, and for those that were fertile, we counted the total number of cyathia for that specimen. Because cyathia can take some time to achieve maturity (up to 2 weeks, personal observation) and sometimes can abort development, we only included cyathia in which the style was visible and had a healthy appearance based on size, thickness, and visibility of stigmatic surfaces, or had fruits at various stages of development. A total of 376 specimens met these criteria and were included in our study (Fig. 2A). Of these, 81 had locality data that did not allow for precise georeferencing but enough information to be assigned a geographic occurrence at a medium scale (island or country) and thus were included in our analyses.

Additionally, we tracked floral activity in 63 individuals (9 populations) along 12 months that are part of a living collection kept in a common garden, which only has representatives of mainland populations and the Lesser Antilles. Because a multitude of ecological and genetic factors can influence flowering, and because live collections do not represent the two areas we are focusing on (GA, LA) we decided to keep these data separate and only report it as supplementary information (Suppl. material 1, section 2).

### Potential shifts in pollination systems

We assessed two lines of evidence pertaining to pollination biology in this system: reward and visitation, both measured in field conditions. Together, these data inform about the pollination regimes experienced by plants of *E. tithymaloides* occurring in nature in our focal geographic areas.

#### Reward data (nectar)

We used standard methods to quantify and characterize nectar of *E. tithymaloides* across 13 natural sites in the Greater (n = 7) and Lesser Antilles (n = 4), and the mainland (n = 2). For details on study sites, see table S1 (Suppl. material 1, section 1). We measured nectar volume with calibrated capillary glass tubes and quantified its sugar content (percentage) with a temperature-compensated hand refractometer 0–32% sugar by volume (°Brix; VEE GEE Scientific BTX-1, QA Supplies, Norfolk, Virginia, U.S.A.) that is accurate to 0.2%. To access the chamber where nectar accumulates in the cyathia of *E. tithymaloides*, we pressed the glass capillary through the medial spur lobes that conform a ‘flap’ that closes shut the inflorescence compartment enclosing the nectar glands (Fig. 2B). We took a single sample for n = 6–37 cyathia per population (n cyathia/population: mean = 21.6, median = 22), for a total of n = 237 measurements (GA: n = 145, LA: n = 92). It has been reported that nectar volume and concentration in *E. tithymaloides* is finely regulated and can vary throughout the day (Veiga Blanco et al. 2013) so we made a point to collect samples from inflorescences that had been kept from being visited for 24 h and at similar times, to increase comparability of our

data. We set our sampling time around 1400 h because it increased our likelihood of sampling consistently across sites, some of which are of difficult access. To avoid nectar alteration from visitation (Perret et al. 2001; Dupont et al. 2004; Schmid et al. 2016), we bagged cyathia 24 h prior to our nectar measurements by placing microperforated biaxially-oriented polypropylene bags over cyathia (Fig. 2C); these bags allow both light and air exchange, so that the light or temperature regimes that our focal cyathia experienced are like those naturally experienced by plants (<https://amarapackaging.co.uk/>), and have been used as controls in studies evaluating materials specifically to make pollination control bags (Clifton-Brown et al. 2018). All data were collected directly in the field, and sugar total content (mg) was calculated as  $(\text{mg}) = (^\circ\text{Brix} \times \mu\text{L}) / 100$ , following previous studies (Ochoa-López et al. 2018).

Additionally, we tracked nectar volume and sugar content throughout 12 months in 19 individuals representing populations from the mainland and Lesser Antilles that are kept in a common garden. To avoid potential confounding issues derived from growing conditions (field vs. common garden) we analysed data separately and report common garden results in the supplement (Suppl. material 1, section 3).

### Visitation data

We collected visitation data from 12 natural populations representing the main areas of divergence in the Lesser Antilles ( $n = 4$ ) and Greater Antilles ( $n = 7$ ), as well as mainland areas ( $n = 1$ ; table S1 in Suppl. material 1, section 1; Fig. 2A, triangles), during 2–3 days per site.

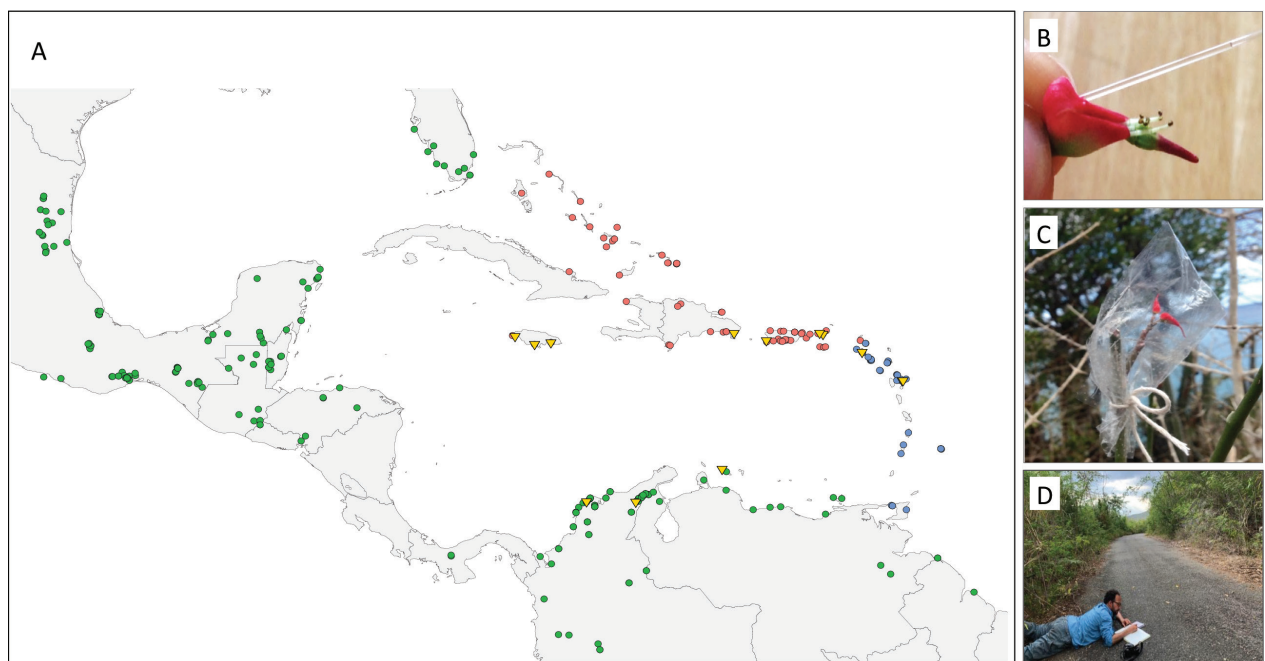
Upon arrival at a given location, we conducted a census and delimited areas for direct observation in a way that all or a significant portion of the population was represented. Then, within areas, we randomly selected focal plants for observation. Observation took place between 1300 and 1900 h on day 1, and from 0600 and 1300 h on day 2, with observation periods of about 1–2 h per observer that were staggered to maximize area and period of observation with the available observers (1–2 observers/site) given the delimited areas in each location. Observers positioned themselves at between 1 and 3 m from focal plants, which allowed observation while minimizing disturbance of potential visitors (Fig. 2D).

Data collection consisted of initial and final time of observation period, and for every visitation or sighting event (an appearance of a potential visitor in close proximity of a focal plant that does not lead to a visit): time at which event occurred (and duration, when possible), number of events, whether visits were short (likely quick assessments) or long (with observed probing for reward), functional group of floral visitors, floral visitor identity and its sex (when possible), as well as relevant notes.

### Statistical analyses

### Shifts in phenology

To estimate geographic patterns in the tempo of reproductive activity (floral phenology) susceptible of promoting or maintaining reproductive isolation, we plotted number of floral units against time (day of the



**Figure 2.** A. Map depicting phenology data points, coloured by main area of occurrence (GA: red; LA: blue; ML: green) and populations where reward and visitation were studied (yellow triangles). B. Probing for nectar involved squeezing lobes and exerting force to push capillary tube to access nectar chamber. C. Cyathia bagged with micro-perforated cellophane bags experience similar light and temperature regimes to natural conditions. D. Visitation data was collected by observers positioned at a distance to minimize disturbance of potential floral visitors. All photographs by N. Ivalú Cacho.

year) and compared floral activity between our two focal areas (Greater Antilles, and Lesser Antilles; GA, LA, respectively) by estimating their overlap. We first converted calendar dates to Julian date, or ‘day of the year’ (1 to 365) using custom R scripts (R Core team 2022). Plots were smoothed using the `geom_density` function in the R package `ggplot2` v.3.5.0 (Wickham et al. 2016), and overlap in flowering activity was estimated using the function `overlap` in the R package `overlapping` v.2.1 (Pastore 2018). To assess how observed values would compare to random, non-geographically structured observations, we built a distribution of expected overlap values where geographic assignment (GA, LA) was randomized, against which we visualized our measures of overlap.

### Reward (nectar)

After visualization with histograms and evaluation of normality and equal variances with Shapiro and Levene tests respectively, data were transformed (square-root) to improve normality and homoscedasticity. Outliers were examined using Rosner’s tests. We then used linear mixed effects models to examine the effect of geography (GA, LA) on differences in reward. Nectar traits (volume, concentration, or total sugars) were the independent variables in all our models, and geographic area was the explanatory factor with fixed effects; observation sites were treated as random effects factors, nested within geographic area (model: nectar trait ~ geography + (1 | geography/site)). All models were implemented in R, using the function `lmer` in the R package `lme4` v.1.1-34 (Bates et al. 2015).

### Visitation

We tested whether visitor guilds varied in importance between focal areas (GA, LA) using the Marascuillo procedure to compare multiple proportions. Briefly, for samples from  $k$  populations, first the absolute values of the differences between proportions  $p_i - p_j$  (where  $i \neq j$ )  $\text{abs}(p[i] - p[j])$  were calculated among all  $k(k-1)/2$  pairs of proportions. Then, critical values for each proportion at the chosen level of significance ( $\alpha$ ) were calculated, as follows:  $r_{ij} = \sqrt{q\text{chisq}(\alpha, k-1)} * (\sqrt{p[i]*(1-p[i])/n_i + p[j]*(1-p[j])/n_j})$ . We used an  $\alpha$  of 0.05, and thus our statistic was  $X_{93,3}^2 = 7.8$ . Finally, each test statistic was compared against its corresponding critical value  $r_{ij}$ . Pairs with a test statistic that exceeded such critical value were considered significant at  $\alpha = 0.05$  (NIST/SEMATECH e-Handbook of Statistical Methods <https://www.itl.nist.gov/div898/handbook>, 8 Feb. 2023).

To characterize the communities of potential pollinators of *E. tithymaloides* across geography (Lesser and Greater Antilles or islands/countries), and the potential effects of changes in their composition, we built bipartite networks. Following Dáttilo et al. (2013), we calculated three indices to characterize such networks: connectance ( $C$ ), specialization index ( $H_2'$ ), and Shannon index of diversity ( $H'$ ). Connectance is the proportion of observed interactions in relation with all relationships

in the network, a proxy of complexity; high levels of connectance imply high levels of stability and resistance in the network (Jordano 1987; Beltrán and Traveset 2018). The specialization index reflects how specialized interactions are in the system, with values close to 0 reflecting low specialization and those close to 1 high levels of specialization. This index is robust to the number of interactors in the network and density of sampling (Blüthgen et al. 2006). Shannon index of diversity is widely used to characterize a system’s diversity, taking abundances into account. The more diverse a network is, the more resistant it will be to perturbations (Beltrán and Traveset 2018). Networks were built with functions `plotweb` and `networklevel` from the bipartite R library v.2.19 (Dormann et al. 2022).

All analyses and graphs were performed using base functions in R v.3.6.3 (R Core team 2022) and visualized using the R packages `ggplot2` (Pastore and Calcagni 2016), `grid` v.3.6.3 (R Core Team 2022) and `gridExtra` v.2.3 (Baptiste 2017), unless indicated otherwise.

## RESULTS

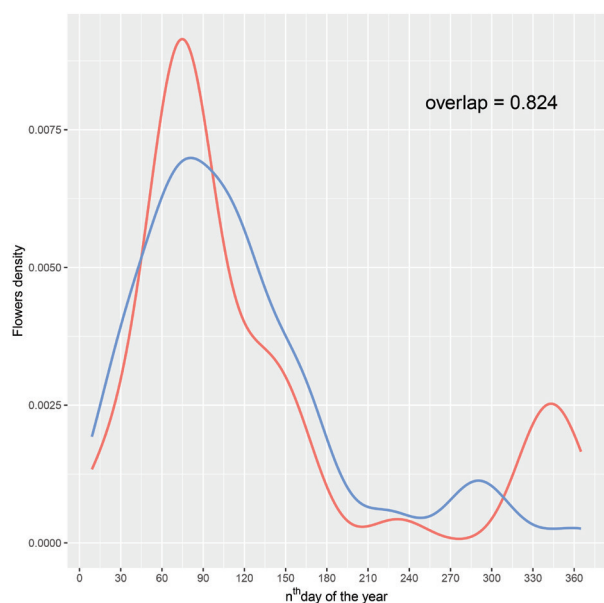
All data accompanying this paper, including phenology, reward, and visitation data are deposited in Dryad (Cacho et al. 2024).

### Phenology

The peak of floral activity in *Euphorbia tithymaloides* is in winter, when days are shorter, specifically from the ~300<sup>th</sup> day of the year (~end of October) to the 150<sup>th</sup> day of the following year (~end of May; Fig. 3). Our analyses reveal that plants from the Greater and Lesser Antilles overlap in phenological activity by 82.4% (Fig. 3), with lower values of overlap when populations of the mainland are included (~71.5–71.7%, and an overall overlap of 88%; Suppl. material 1, section 2). We see a similar pattern in our common garden plants (Suppl. material 1, section 2).

### Reward

In natural field conditions plants of *E. tithymaloides* in the Antilles produce 0–22.4  $\mu\text{L}$  of nectar (mean = 2.89  $\pm$  3.2  $\mu\text{L}$ ; median = 2.2  $\mu\text{L}$ ), with sugar concentrations of up to 391.2 °Brix (mean = 46.5  $\pm$  36.3 °Brix; median = 37.8 °Brix), which amount to up to 10.3 mg of total sugars (mean = 1.42  $\pm$  1.3 mg; median = 1.2 mg), with models detecting no significant effect of geography (Greater Antilles, Lesser Antilles; Fig. 4, Table 1, and Suppl. material 1, section 3). For all three nectar traits, measurements in common garden were slightly higher than data taken in field conditions (Suppl. material 1, section 3). In our common garden plants, those from the mainland produced significantly less nectar (~20% less on average) than those from the Lesser Antilles (Suppl. material 1, section 3).



**Figure 3.** Data from herbarium specimens, our own observations, and citizen science projects show that *Euphorbia tithymaloides* is a winter flowering plant, with an increase in its floral activity from late October to May in the Antilles, where genetic, ecological and morphological divergence has been documented (red: Greater Antilles; blue: Lesser Antilles). There is a relevant overlap (up to 82%) in flowering activity among the lineages occupying these two areas.

### Visitation

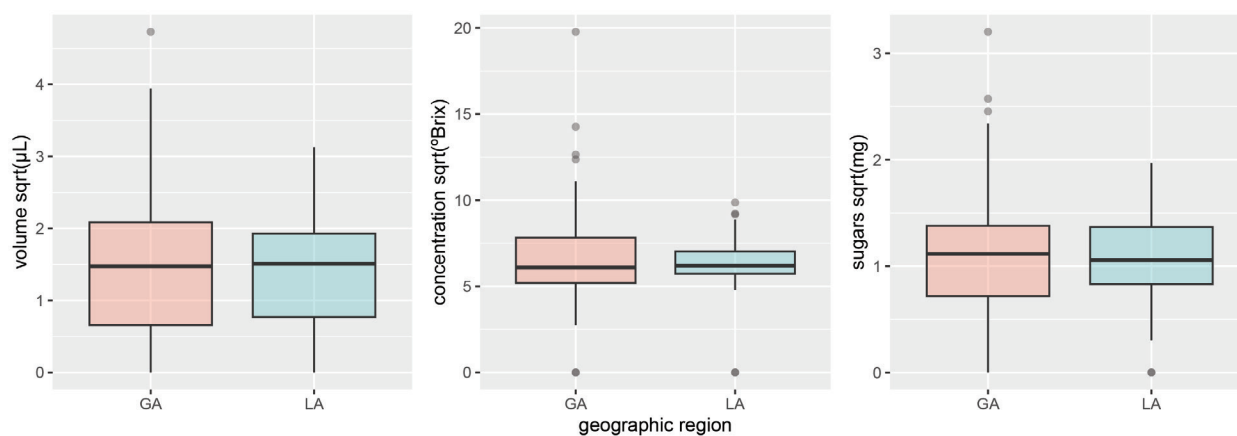
In total, we registered 4,112 visits and 170 sightings in a total of 133.23 h of observation across sites in the Antilles (mean =  $12.78 \pm 4.3$  h/site,  $n = 11$ ), and data including our mainland site is similar (Suppl. material 1, section 4). Visitors and sightings of *E. tithymaloides* included taxa in three main functional groups: hummingbirds, other birds, and insects. Our data show that hummingbirds are the main floral visitors of *E. tithymaloides* in both focal areas: Greater Antilles: 60.8%, Lesser Antilles: 84.5% (all comparisons significant at  $p < 0.05$ ; Fig. 5A and Suppl.

material 1, section 4). In the Antilles other birds visit the cyathia of *E. tithymaloides* (Greater Antilles: 36.6%, Lesser Antilles: 13.6%) but their importance as successful pollinators is still unknown. Insects are present and fly around plants of *E. tithymaloides* in both areas to the point that they account for most of the sightings (Greater Antilles: 41.8%; Lesser Antilles: 83.3%; Fig. 5B, Table 2), but since they do not stop by regularly to visit cyathia, they account for very little visitation (Greater Antilles: 2.6%, Lesser Antilles: 1.95%). Also, when they visit cyathia, they rarely contact the anthers or style of *E. tithymaloides* while searching for nectar (see Discussion and Fig. 6).

Our bipartite network by islands (fig. S4.2A in Suppl. material 1, section 4) illustrates a species turnover in hummingbirds visiting *E. tithymaloides* across the Caribbean. Given this scenario, considering communities of floral visitors at a broader scale (geographic area, GA vs LA) might not be as informative (fig. S4.2B in Suppl. material 1, section 4). Yet, the network based on geographic area exhibits a higher connectance than the one based on island/country ( $C_{\text{geography}} = 0.67$ ,  $C_{\text{island}} = 0.26$ ), but is also less specialized ( $H'_{2\text{geography}} = 0.57$ ,  $H'_{2\text{island}} = 0.76$ ), and less diverse ( $H'_{\text{geography}} = 1.93$ ,  $H'_{\text{island}} = 2.14$ ).

## DISCUSSION

We documented phenology, reward, and visitation for *Euphorbia tithymaloides* across its range in the Caribbean (with emphasis in its distribution in the Antilles) and evaluated whether divergence along two fronts (Lesser and Greater Antilles) within this species is accompanied by shifts in phenology or shifts in pollinator regimes between these two incipient lineages. Through observation and measurements in natural populations and assembling data from collections (herbaria and a common garden), databases (GBIF), and online citizen science databases (iNaturalist), we document that throughout its range, *E. tithymaloides* flowers between the end of October and the end of May and produces (per floral unit) on average  $\sim 2.9$



**Figure 4.** There are no significant differences in reward (nectar) volume (left), sugar concentration (middle) or total sugar content (right) that suggest significant shifts in pollinator regimes among the two diverging lineages of *Euphorbia tithymaloides* (red: Greater Antilles; blue: Lesser Antilles).

**Table 1.** Linear mixed models evaluating nectar production in the two focal areas in the Antilles do not support differences in nectar traits between them. Nested random models were run with function *lmer* in library ‘lme4’ in R.

Model	REML cc	N observations	N groups	Random effects			Fixed effects						
				Groups	Variance	Standard deviation	Effect	Estimate	Standard error	d.f.	t value	Pr(> t )	Significance
<b>volume sqr(μL)</b>	631.5	237	11	pop13id	0.1876	0.433	(intercept)	1.342	0.186	9.856	7.200	3.16E-05	***
				residual	0.7746	0.88	geographyLA correlation of fixed effect	0.005	0.300	9.050	0.016	0.988	
<b>sugar concentration sqr(°Brix)</b>	728.5	192	11	pop13id	0.944	0.972	(intercept)	6.559	0.424	7.652	15.460	4.79E-07	***
				residual	3.908	1.977	geographyLA correlation of fixed effect	-0.258	0.689	7.218	-0.375	0.719	
<b>total sugars sqr(°Brix)</b>	228.4	192	11	pop13id	0.086	0.294	(intercept)	0.964	0.120	9.812	8.048	1.26E-05	***
				residual	0.168	0.410	geographyLA correlation of fixed effect	0.093	0.196	9.406	0.476	0.645	

μL of nectar with a mean sugar concentration of 46.5 °Brix, and a mean net sugar content of ~1.4 mg. Hummingbirds, other birds, and insects (in that order of importance) visit the inflorescences of *E. tithymaloides*, with hummingbirds consistently being the most important floral visitor throughout the plant’s geographic range in the Caribbean.

Other authors have shown that floral phenology can be a factor of critical importance in maintaining or promoting reproductive isolation between closely related species or among diverging populations of flowering plants across a wide phylogenetic breadth, including examples in the evening primrose family (*Chamerion*, Onagraceae) (Husband and Schemske 2000), orchids (*Gymnadenia*, Orchidaceae) (Chapurlat et al. 2020), and the myrtle family (*Metrosideros*, Myrtaceae) (Osborne et al. 2020), among others. Because phenological barriers act early on in preventing reproduction, they are believed to be of relevance in instances where the process of lineage divergence leads to speciation (Coyne and Orr 2004; Martin and Willis 2007; Martin et al. 2007; Osborne et al. 2020). In *E. tithymaloides*, our results reveal a substantial overlap (above 71%) in floral phenology among plants of from the Mainland, and the Lesser Antilles or the Greater Antilles, and above 82% for plants of the Greater and Lesser Antilles (the most distantly related in this system). The relative contribution of overlap in flowering time and vegetative reproduction (Dressler 1957) to gene exchange between the diverging lineages of *E. tithymaloides* (Cacho and Baum 2012; Cacho et al. 2019; Medina-Rodríguez 2022) remains to be assessed.

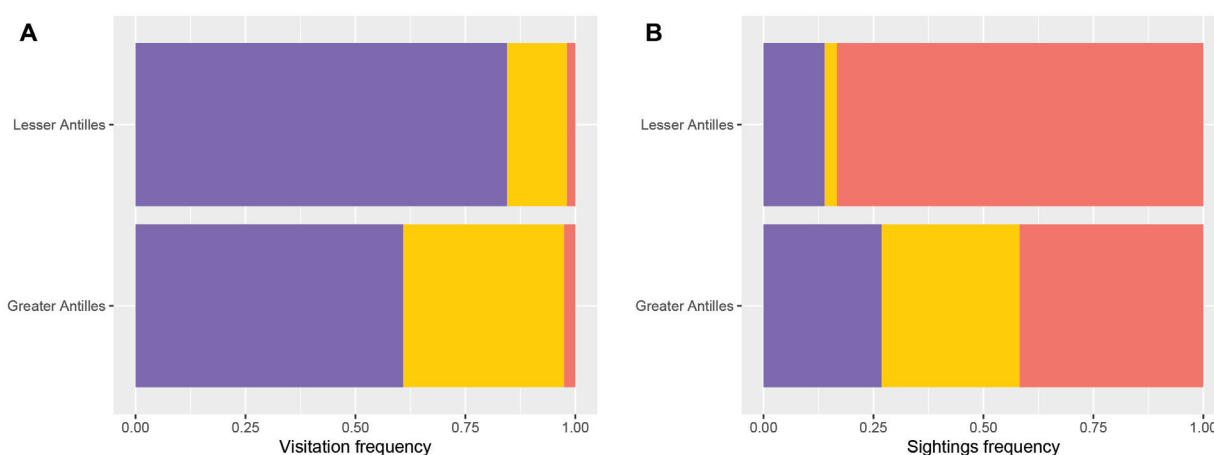
Environmental factors, including amount of light, rainfall, competition, and herbivory can contribute to variation in floral phenology (Elzinga et al. 2007; Matthews and Mazer 2016) and it has been suggested that when growing seasons contract or expand, selection could lead to changes in floral phenology ultimately due to climatic differences (Franks et al. 2007), which in turn could alter biotic interactions. To this effect, our data reveal a meaningful overlap in floral phenology between plants of *E. tithymaloides* growing in the Greater and Lesser Antilles, and this is consistent with the lack of pronounced climatic differences among these areas in the Caribbean (Cacho et al. 2019; Jacobo-Arteaga et al. 2022).

In plants, both environmental and endogenous factors (including genetic and hormonal responses) must be integrated in the control of flowering—the transition from vegetative to reproductive development (Koornneef et al. 1998; Putterill et al. 2004). While we see subtle differences in timing between plants growing in natural populations and those in our common garden, overall, the pattern is one of agreement, both in the timing of flowering activity (end of October to end of May) and in the overlap among focal areas. These results suggest that flowering in *E. tithymaloides* is genetically induced by short days, as others have observed (Dressler 1957). A strong genetic component in the control of floral phenological activity has been documented in several systems including both long-day plants like *Arabidopsis* (Johansson and Staiger



**Table 2.** Hummingbirds account for most visitation and sighting (an appearance of a potential visitor in close proximity of a focal plant that does not lead to a visit) events across 12 natural sites of *Euphorbia tithymaloides* in the Caribbean, derived from a total of 133.23 hours of observation. For data by country, see Suppl. material 1, section 4.

Geography	n visits /area	n sightings / area	Functional group	n visits	n sightings	% visits	% sightings
Greater Antilles	2160	134	hummingbird	1315	36	60.88	26.87
			other bird	790	42	36.57	31.34
			insect	55	56	2.55	41.79
Lesser Antilles	1952	36	hummingbird	1649	5	84.48	13.89
			other bird	265	1	13.58	2.78
			insect	38	30	1.95	83.33



**Figure 5.** Visitation (A) and sighting (B; appearances of a potential visitor in close proximity of a focal plant that do not lead to a visit) frequency of potential pollinators of *Euphorbia tithymaloides* in the Antilles, colour coded by functional group as follows: hummingbirds (purple), other birds (yellow), and insects (red).

2015), and short-day ones like chrysanthemums (Higuchi et al. 2013).

The study of floral reward is complex and multivariate (Abrahamczyk et al. 2017); one of the few patterns on which there is agreement is a correlation between the energetic content of nectar and the energetic requirements of potential pollinators (Smith et al. 2008b). Accordingly, high nectar volumes and ultimately high sugar contents are observed in plants that are pollinated by hummingbirds, who are mainly nectar feeders with high energetic demands (Baker 1975; Bolten and Feinsinger 1978; Lotz and Schondube 2005; Johnson and Nicolson 2008). Also, reward has been shown to be correlated with floral display. In *Iochroma* (Solanaceae), species with larger displays offer rewards higher in energy and had greater probabilities of being pollinated by hummingbirds (who are visual searchers) than those with smaller display sizes, which produced a lower reward nectar and were more likely to be pollinated by insects (Smith et al. 2008a). We did not quantify floral display in *E. tithymaloides*, and it remains a question if differences in display have evolved among populations or between diverging lineages in this system. Yet, we do not find evidence supporting

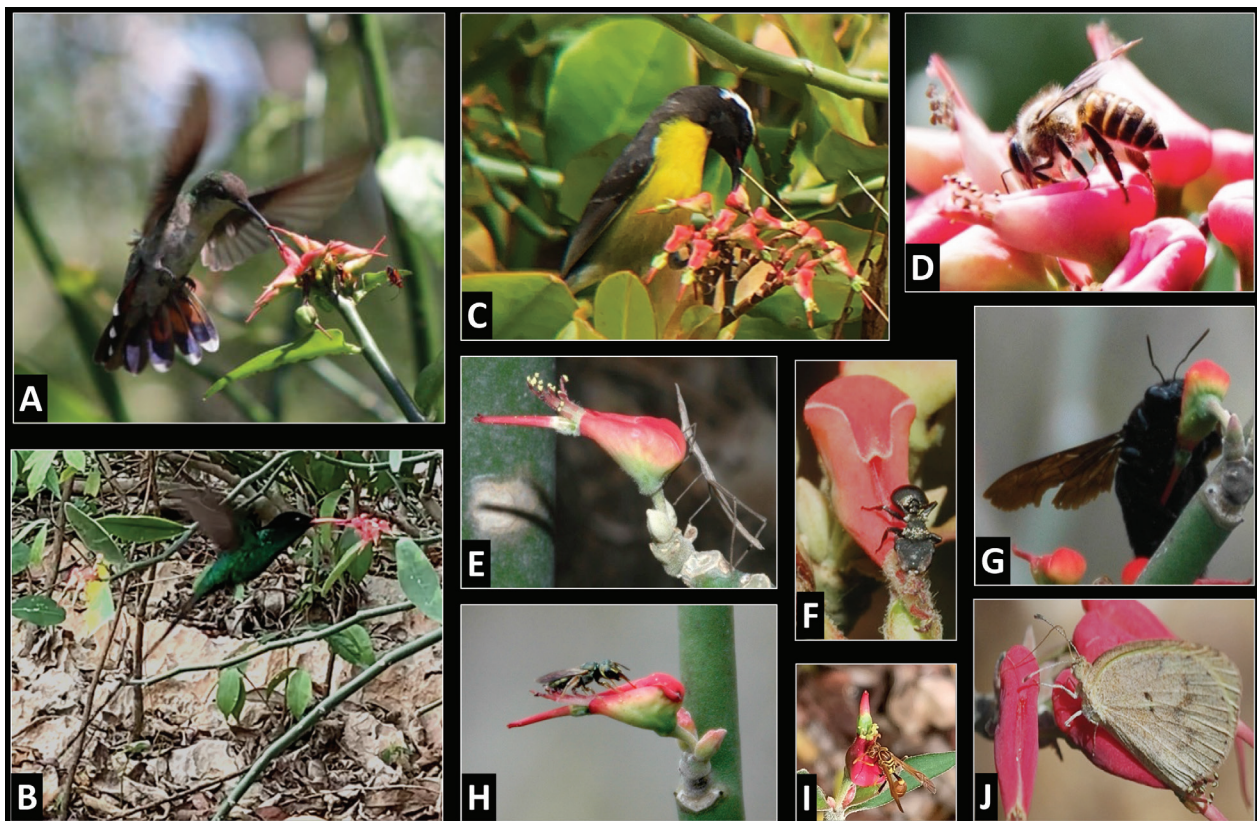
differences in amount or energetic content of nectar being offered by plants of *E. tithymaloides* from the Greater and Lesser Antilles (or those of the mainland).

Hummingbirds visit *E. tithymaloides* most frequently. They approach inflorescences in active flight and make contact with exposed anthers and stigmas with their bill when probing the inflorescence searching for nectar while hovering (Fig. 6). Other birds, mainly individuals of *Coereba flaveola* (Thraupidae), also visit and probe the cyathia of *E. tithymaloides*, but they need to perch to probe inflorescences, and thus approach them from a variety of angles several of which do not render a reliable contact of the bird's body (bill, chin, or forehead) with the reproductive parts of the plant. Insects (hymenopterans and lepidopterans to a lesser extent) account for the minority of visits in *E. tithymaloides*, and from our observations, most of these visits do not translate into contact between the insect's body and anthers and stigma of the plant: insects, while searching for nectar, land and walk on the cyathium mostly on the posterior part, where openings for the nectar chamber can be accessed for a small animal, and only rarely contact is made with its anthers or style. Altogether, these observations and data

make it unlikely that insects could be playing a role as reliable and important pollinators in this system.

Our data points to hummingbirds as the main floral visitors in *E. tithymaloides* but we cannot exclude the possibility of a mixed pollination system, which has been documented in other plants. In *Aechmea* bromeliads, the combination of effectiveness of pollen transfer and frequency of visitation translates into a bimodal pollination where bees are secondary to hummingbirds in importance as pollinators, although debate exists as to whether bimodal pollination is a stable state (Schmid et al. 2011). A limitation of our study is that we did not quantify effectiveness in pollen transfer among floral visitors of *E. tithymaloides*, but several lines of evidence point to hummingbirds as the main pollinators. Hummingbirds were overwhelmingly more important than any other functional group as floral visitors across areas for this Caribbean euphorb. Our visitation data and field observations (registered mostly in the Antilles, particularly in the Greater Antilles) related to behaviour of floral visitors revealed that in contrast to hummingbirds, bees and other birds rarely contact floral reproductive parts (stamens or styles) of *E. tithymaloides* and thus support hummingbirds as the most likely effective pollinators of this plant throughout

the Caribbean, a finding that agrees with what others have observed (Dressler 1957; Veiga-Blanco et al. 2013). Also, *E. tithymaloides* fits the pattern of plants specialized in hummingbird pollination, which tend to produce tubular-shaped flowers with red colouration (Temeles and Kress 2003; Iles et al. 2017). Moreover, hummingbirds exhibit territorial behaviour towards *E. tithymaloides*: individuals chase off other hummingbirds or other birds from plants or patches of plants that they consider their territory. Territoriality has been well documented in hummingbirds as one of their main foraging strategies (Sargent et al. 2021), and we observed this behaviour at many of our study sites: individuals of larger species of hummingbirds (or males within a species) would attack others (both smaller hummingbirds or other birds) and minimize their visitation but not fully prevent it. We did not observe a single instance of hummingbirds chasing off insects, a behaviour that has been documented (Boyden 1978), with more aggressive chasing the larger the insects (as would be predicted by energetic theory: other visitors should be chased accordingly to the threat they pose in terms of nectar depletion or territory displacement). An additional limitation of our study is that we observed floral visitation dynamics in a relatively small window of time (~13 h/study site) and therefore does not account



**Figure 6.** Floral visitors of Caribbean *Euphorbia tithymaloides*. A. Hispaniolan mango (*Anthracothorax dominicus*), Dominican Republic. Photo by Pablo Feliz. B. Doctor hummingbird (*Trochilus polytmus*), Jamaica. C. Bananaquit (*Coereba flaveola*), St. Eustatius. D. Honeybee (*Apis mellifera*), Guadeloupe. E.\* Stick insect (Phasmatoidea), Venezuela. F.\* Ant (Formicidae), Venezuela. G.\* Carpenter bee (*Xylocopa* sp.), Venezuela. H.\* *Euglossa* bee (Apidae), Venezuela. I. *Polistes* wasp, Dominican Republic. J.\* Lepidopteran (*Eurema दौरा*, Pieridae), Guatemala. An asterisk denotes anecdotal observations not included in this study. All photos by N. Ivalú Cacho unless noted otherwise.

**Table 3.** Floral visitors of *Euphorbia tithymaloides* in the Caribbean, and their relative importance (for data including the mainland, see Suppl. material 1, section 4). Sightings are appearances of potential visitors in close proximity of a focal plant that do not lead to a visit.

Country	Functional group	Visitor	Species code	n visits	n sightings	% visits	% sightings
<b>Greater Antilles</b>							
Dominican Republic	hummingbird	<i>Anthracothorax dominicus</i>	ANDO	42	6	34.71	54.55
		<i>Mellisuga minima</i>	MEMI	27	0	22.31	0.00
	other bird	<i>Coereba flaveola</i>	BAQU	52	2	42.98	18.18
		<i>Todus todus</i>	TOTO	0	2	0.00	18.18
	insect	bee	BEE	0	1	0.00	9.09
Jamaica	hummingbird	<i>Mellisuga minima</i>	MEMI	193	7	10.85	19.44
		<i>Trochilus polytmus</i>	TRPO	818	4	45.98	11.11
	other bird	<i>Coereba flaveola</i>	BAQU	719	16	40.42	44.44
		<i>Melopyrrha violacea</i>	MEVI	3	0	0.17	0.00
		<i>Todus todus</i>	TOTO	0	2	0.00	5.56
	insect	bee	BEE	46	2	2.59	5.56
		lepidopteran	LEP	0	4	0.00	11.11
		wasp	WASP	0	1	0.00	2.78
Puerto Rico	hummingbird	<i>Chlorostilbon maugaeus</i>	CHMA	25	0	100.00	0.00
		<i>Anthracothorax dominicus</i>	ANDO	0	1	0.00	1.15
St. John	hummingbird	<i>Eulampis holosericeus</i>	EUHO	5	7	2.13	8.05
		<i>Orthorhyncus cristatus</i>	ORCR	205	11	87.23	12.64
		<i>Coereba flaveola</i>	BAQU	16	9	6.81	10.34
	other bird	<i>Loxigilla noctis</i>	LONO	0	8	0.00	9.20
		<i>Setophaga striata</i>	SETR	0	3	0.00	3.45
	insect	bee	BEE	0	5	0.00	5.75
		<i>Bombus</i> sp.	BOMBUS	0	5	0.00	5.75
		lepidopteran	LEP	3	9	1.28	10.34
		odonata	DFLY	0	4	0.00	4.60
		<i>Polistes canadensis</i>	POCA	0	14	0.00	16.09
	wasp	WASP	6	11	2.55	12.64	
<b>Lesser Antilles</b>							
Curaçao	hummingbird	<i>Chlorostilbon mellisugus</i>	CHME	483	0	98.17	0.00
	other bird	<i>Coereba flaveola</i>	BAQU	9	0	1.83	0.00
	insect	bee	BEE	0	22	0.00	100.00
Guadeloupe	hummingbird	<i>Orthorhyncus cristatus</i>	ORCR	1023	3	78.15	75.00
	other bird	<i>Coereba flaveola</i>	BAQU	252	1	19.25	25.00
	insect	bee	BEE	34	0	2.60	0.00
St. Eustatius	hummingbird	<i>Orthorhyncus cristatus</i>	ORCR	143	2	94.70	20.00
	other bird	<i>Coereba flaveola</i>	BAQU	4	0	2.65	0.00
	insect	bee	BEE	0	2	0.00	20.00
		lepidopteran	LEP	4	3	2.65	30.00
		wasp	WASP	0	3	0.00	30.00

for potential resource partitioning among individuals or species of hummingbird, which could take place and quickly change (Hixon et al. 1983). Floral visitor dynamics can also vary over time, space, and with community structure and composition (of plants and their interactors), among other factors (Leimberger et al. 2022). In fact, high temporal species turnover in plant-pollinator network dynamics has been documented in different systems at the community level (Alarcon et al. 2008; Petanidou et al. 2008). Yet, both plant and animal communities in insular environments tend to be less complex than their continental counterparts (Lack 1973; Kodric-Brown et al. 1984; Brown and Browsers 1985), which could explain that we see no indication of obvious spatial or temporal variation in functional groups across sites. Thus, we conclude from our visitation data and observation that (albeit our modest window of observation), no evidence points to insects being important nectar feeders or potential pollinators of Caribbean *E. tithymaloides*. Likewise, there is no evidence of shifts in pollinator functional groups across geographic areas where *E. tithymaloides* occurs in the Caribbean. Yet, we cannot rule out the possibility of other visitors, including other birds (mainly *Coereba flaveola*) or animals in other functional groups (mainly bees) playing a role as secondary or incidental pollinators in this plant.

Other factors that could be playing an important role in contributing to divergence in *E. tithymaloides* rely not on a shift of pollinator functional group, but rather in changes in the identity of pollinators within the same functional group: different hummingbird species could be acting as main pollinators in different islands. Our network analyses of floral visitors of *E. tithymaloides* reveal relatively little overlap of hummingbird species among areas and islands/countries (as expected given their geographic ranges). Lower values of connectance and higher values of diversity and specialization in networks by islands/countries are consistent with a pattern of species replacement, especially when considering hummingbird species. Hummingbird communities in the Caribbean are complex and structure according to island size and topographic complexity (Lack 1973; Kodric-Brown et al. 1984; Brown and Browsers 1985), so that different species of hummingbirds could be imposing different selection regimes on floral morphology, limiting pollen transfer, and thus driving evolutionary divergence. This scenario would be like that observed in sister species of *Heliconia* plants in the Caribbean, where *H. bihai* and *H. caribaea* are differentially visited by males and females of the *Eulampis jugularis* hummingbird, likely promoting their reproductive isolation and preventing them from collapsing into one lineage (Temeles and Kress 2003). A similar pattern has been documented in Caribbean Gesneriaceae (Martén-Rodríguez et al. 2010) and more broadly, the pattern of trait matching between a plant's floral morphology and its hummingbird pollinators would also support this idea (Dalsgaard et al. 2021). Further

investigation would be necessary to test whether such a scenario is at play across populations of *E. tithymaloides*.

## CONCLUSIONS

Using a combination of data directly derived from field observations and measurements together with data gathered from collections and databases, including citizen science projects, we studied the timing of floral phenology, quantified and characterized reward (nectar), and evaluated floral visitation in *Euphorbia tithymaloides* across the Caribbean with emphasis in the Antilles, to evaluate if the morphological, ecological, and genetic divergence observed between incipient lineages of this plant system is accompanied by shifts in floral phenology or pollination systems. Our data are consistent with *E. tithymaloides* being a “short-day” plant, flowering mainly during the winter (between October and May). Morphology, reward, and visitation data support hummingbirds as the most likely and main pollinators of Caribbean *E. tithymaloides*, with no indication of a change of pollinator functional group in any of its main areas of occurrence. Altogether, our results do not support shifts in floral phenology or in pollinator functional groups as potential barriers to gene exchange that could be contributing to divergence in *E. tithymaloides*. Ecological factors promoting divergence in this system in incipient speciation remain to be described; possibilities include hummingbird species replacement throughout the Caribbean or geographical patterns in hummingbird morphology or behaviour, as has been shown in other systems.

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## SUPPLEMENTARY MATERIAL

### Supplementary material 1

Data on study sites (section 1), additional analyses on phenology (section 2), additional analyses on nectar (section 3), and additional analyses on floral visitation (section 4) in *Euphorbia tithymaloides* across the Caribbean, including study sites in the mainland, as well as analyses with plants in common garden. <https://doi.org/10.5091/plecevo.103095.suppl1>