

Geographic Patterns In Pollen Production In The Plant Ring Species *Euphorbia tithymaloides* In The Caribbean

N. Ivalú Cacho¹ and Daniel José-Zacatula

Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, México Distrito Federal 04510, Mexico; ivalu.cacho@gmail.com; unquited@gmail.com

¹Author for correspondence (ivalu.cacho@gmail.com)

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Abstract—We examine investment in male function in the context of geography and floral morphology in the plant ring species *Euphorbia tithymaloides*, which colonized the Caribbean from Mexico/Guatemala along two fronts that roughly correspond to the Greater and the Lesser Antilles and that meet in the vicinity of the Anegada Passage. Our results on investment in pollen relative to ovule production are consistent with *E. tithymaloides* relying on pollinators for reproduction across its range in the Caribbean. We document a geographic pattern of reduction in anther number and pollen:ovule ratios in populations of *E. tithymaloides* towards the Anegada Passage, which is consistent with a possible geographic transition towards a mixed breeding system where selfing has increased importance in this area where its two expansion fronts meet. The reduction in pollen production is correlated with inflorescence morphology, which converges to relatively shorter cyathia towards the Anegada Passage along both fronts. We discuss alternative scenarios and potential drivers of the patterns we document.

Keywords—Breeding systems, convergence, divergence, Euphorbiaceae, male investment, pollen:ovule ratios, outcrossing, reproductive isolation, selfing, speciation.

Understanding geographic variation in traits related to plant-pollinator interactions can provide important insights into the evolution and ecology of traits related to these mutualistic interactions, such as shifts in breeding system, selection on floral or pollinator traits, evolutionary or coevolutionary dynamics between taxa, or to explain biogeographic patterns of community assembly (Armbruster 1993; Inoue 1993; Barrett 1998; Temeles and Kress 2003; Johnson et al. 2010; Kalisz et al. 2012; Grossenbacher et al. 2017).

We recently reported a pattern of convergent floral evolution along the two geographic fronts of the plant ring species *Euphorbia tithymaloides* L. (Euphorbiaceae) (Cacho et al. 2019). *Euphorbia tithymaloides* colonized the Caribbean basin from Mexico/Guatemala by expanding its range along two fronts, one that travelled east towards the Greater Antilles (GA front), and a second that travelled south, through Central America, then east through northern South America, and then north through the Lesser Antilles (LA front) (Cacho and Baum 2012). Where these two fronts meet in the vicinity of the Virgin Islands and the Anegada Passage (17.9251611°N, -63.916925°W), they show convergence in floral morphology: the cyathia of these plants (modified inflorescences which resemble and function as floral units; Fig. 1) display a shorter and more compact floral shape than the elongated morphology characteristic of the area of origin in Mexico/Guatemala (Cacho et al. 2019). The shifts in morphology occurred in different fashions along both geographic expansion fronts: along the GA front, compact inflorescences are the result of a rather drastic reduction in size accompanied by a pronounced shortening of the involucre, while in the LA front, such morphology is the result of a subtle shortening of the involucre accompanied by an increase in the relative height of the cyathium (Cacho et al. 2019).

Euphorbia tithymaloides is primarily visited by hummingbirds, and exhibits classic aspects of the hummingbird pollination syndrome, such as elongated tubular floral structures with red coloration and copious nectar production (Dressler 1957; Cacho et al. 2010; Cacho and Baum 2012; Veiga Blanco et al. 2013), although formal field pollination studies across the species range are still lacking. Potential explanations for the

convergent shifts in floral form within *E. tithymaloides* include shifts away from hummingbird pollination towards increased selfing or a generalized pollinator syndrome associated with insularity, as well as more subtle changes in floral form related to the identity of potential hummingbird pollinators across the Caribbean (Lack 1973; Brown and Brown 1985; Gowda and Kress 2013). Evolutionary shifts towards predominantly selfing systems have been correlated with changes in flower traits (Wyatt 1984; Webb and Lloyd 1986; Bertin and Newman 1993; Barrett et al. 1996, 2003; Barrett 1998; Snell and Aarssen 2005; Sicard and Lenhard 2011; Kalisz et al. 2012); flowers of selfers tend to be smaller, with shorter stigmas and styles relative to closely related outcrossers (Snell and Aarssen 2005; Sicard and Lenhard 2011; Kalisz et al. 2012; Duncan and Rausher 2013). In *E. diazlunana*, an *E. tithymaloides* clademate, a transition from hummingbird to hymenopteran pollination has been accompanied by drastic morphological changes including a pronounced involucre shortening and some loss of coloration (Sahagún-Godínez and Lomelí-Senci6n 1997). Here, we extend our investigations of geographical variation in floral traits of *E. tithymaloides* to investment in male reproductive function, particularly to pollen production. To investigate whether investment in male function may provide insight into shifts in floral morphology across geography, we assess geographic patterns of pollen production and investigate how pollen production relates to floral traits that vary across the Caribbean in *E. tithymaloides*.

The relative investment by plants in male functions, particularly pollen production, has been associated with variation in plant breeding systems, in particular with the occurrence of self-fertilization (Cruden 1977; Plitmann and Levin 1990; Jürgens et al. 2002; Alarc6n et al. 2011; Duncan and Rausher 2013). Specifically, pollen to ovule (P:O) ratios have been proposed and identified as indicators of a plant's mating system (Cruden 1977) and pollination mode (Michalski and Durka 2009), and as such, have been shown to correlate, among other things, with the degree of outcrossing and floral traits (Michalski and Durka 2009; Duncan and Rausher 2013; Scalone et al. 2013). In general, P:O ratios are expected to vary with respect to pollination mode, being larger in wind pollinated species than in animal pollinated ones.

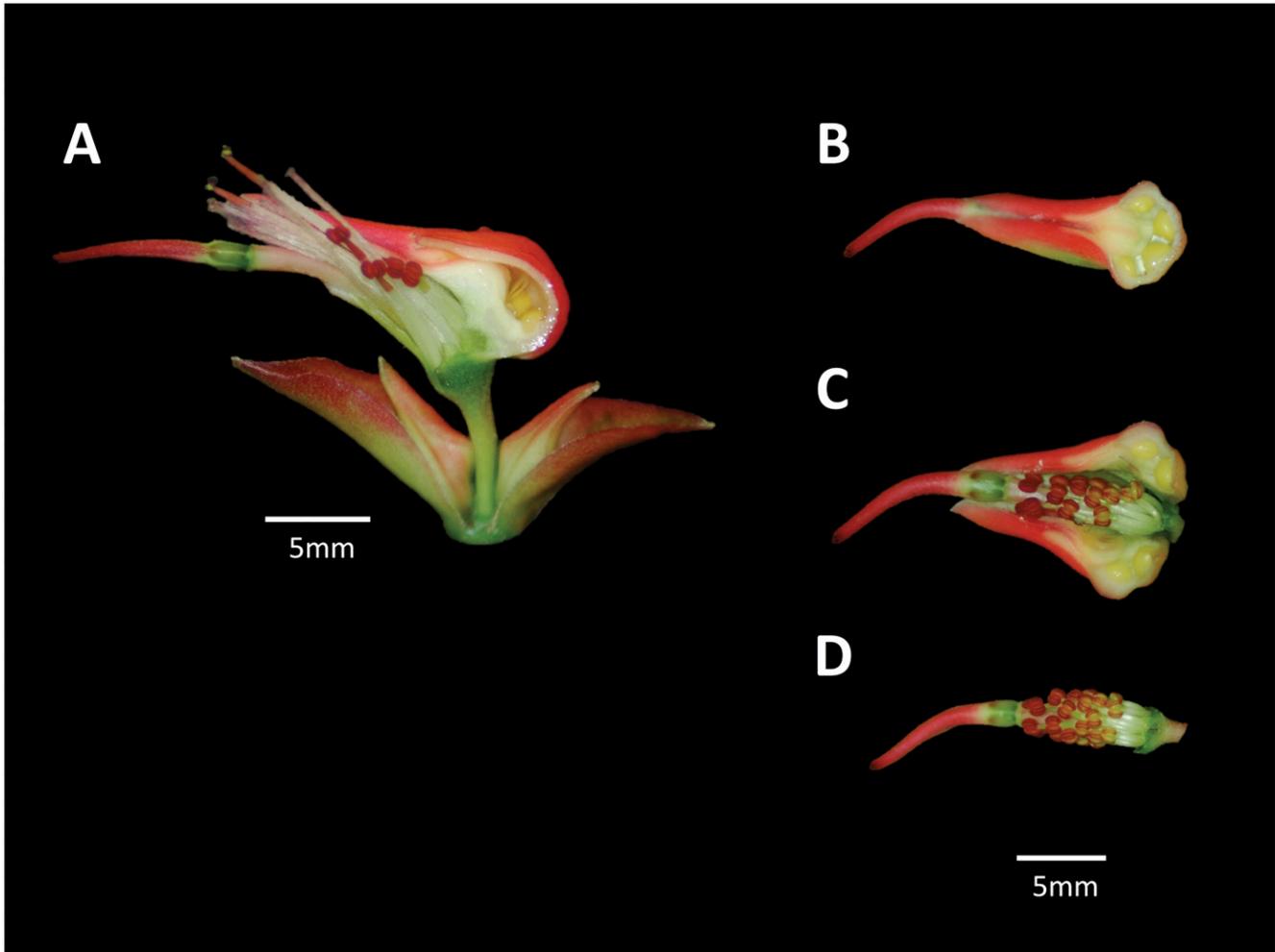


FIG. 1. The cyathia of *E. tithymaloides* is a strongly zygomorphic inflorescence that resembles and functions as a single flower. A. Longitudinal section of a cyathium (hermaphroditic phase) of *E. tithymaloides* showing the overall structure enclosing the pistillate and staminate flowers as well as the chamber where nectar glands are concealed. B. Top view of a cyathium (female phase) with the top 'flap' (involucral bract) removed, revealing its four nectar glands. C. Longitudinal section of same cyathium in B, revealing the disposition of pistillate and staminate individual flowers. D. Staminate flowers each reduced to a single anther surrounding the single central pistillate flower. Dissections and photographs by NIC.

The pattern that predominantly outcrossing plant species have higher P:O ratios than predominantly selfing ones was first documented by Cruden (1977), who inferred that P:O ratios reflect the efficiency of a pollination system and hypothesized that they should correlate with outcrossing rates across the five main breeding system classes traditionally recognized: xenogamy, facultative xenogamy, facultative autogamy, obligate autogamy, and cleistogamy (Cruden 1977; Scalone et al. 2013). While there is a general correspondence of breeding system to P:O ratios, exceptions have also been documented (Wyatt et al. 2000). The implication was that evolutionary shifts between breeding system classes would be accompanied, and reflected, by a significant change in the mean P:O. Beyond mating system, in zoophilous plants P:O ratios have been shown to vary in relation to a number of non-exclusive plant and pollinator traits, including seed mass (e.g. Shanker and Ganeshiah 1984; Götzenberger et al. 2006), pollen size (Gallardo et al. 1994; but see Götzenberger et al. 2007), stigmatic area, the pollen-bearing area of pollinators (Cruden and Miller-Ward 1981), the size of the pollen dispersal unit (Harder and Johnson 2008), the effectiveness of the pollen vector (Cruden 2000), and reward (Mione and Anderson 1992;

Cruden 2000). Thus, P:O ratios likely integrate over a multitude of factors, including both plant and pollinator traits, that act jointly on plant fitness.

Pollen:ovule ratios have been characterized across many scales. Variation in P:O ratios across angiosperms has been documented to range from four in a selfing herb (*Mimulus micranthus*) to 636,594 in a wind-pollinated tree (*Fagus sylvatica*). At a family scale, within Balsaminaceae, P:O ratios vary from 286 (*Impatiens inaperta*) to 35,184 (*I. balfourii*) (Lozada-Gobilard et al. 2018); and in Zingiberaceae, they range from 3.25 (*Caulokaempferia coenobialis*) to 616.52 (*Alpinia stachyodes*) (Wang et al. 2004). At the genus level, a study of 45 species of *Veronica* (Plantaginaceae) documented that P:O ratios range from 19 (*V. magna*) to 2,572 (*V. lycica*) (Scalone et al. 2013). In *Erodium*, P:O ratios ranged from 32 (*E. geoides*) to 1,728 (*E. recoderi*) (Alarcón et al. 2011). In *Veronica*, P:O ratios correspond well to mating system and correlate well with floral traits such as corolla size (Scalone et al. 2013). However, P:O variation among populations within one species across its range has rarely been examined systematically (Delesalle and Mazer 2009; Mazer et al. 2018, but see Scalone et al. 2013).

In this study we characterize pollen production and P:O variation across populations spanning the range of *E. tithymaloides* in the Caribbean, and relate pollen production to previously described variation in floral traits in relation to range expansion and divergence along the two fronts of this ring species across the Caribbean basin. Specifically, we address the expectation that P:O ratios (as a proxy for investment in male vs. female reproduction) in *E. tithymaloides* will decrease towards the Anegada Passage if shorter flowers are associated with a transition to selfing or a mixed breeding system where selfing plays a more significant role than it does in the center of origin in Mexico/Guatemala. To test this hypothesis, we evaluate geographical patterns of pollen production throughout the range of *E. tithymaloides*, and investigate the relative importance of geography and floral morphology as predictors of P:O ratios in *E. tithymaloides* across the Caribbean.

MATERIALS AND METHODS

Floral Morphology of *Euphorbia tithymaloides*—In *Euphorbia*, the floral unit is the cyathium, a collection of much reduced flowers organized in a structure that resembles and functions as a single flower (Prenner and Rudall 2007; Prenner et al. 2011). The cyathium has been considered one of the several “reinventions” of the flower across angiosperms, along with the capitulum of composites, inflorescences in *Cornus*, and others (Weberling 1989; Classen-Bockhoff 1990; Prenner and Rudall 2007). The general architecture of the cyathium consists of a single terminal pistillate flower surrounded by many staminate flowers each reduced to a single anther, with all flowers borne on a receptacle or involucre that also bears four or five nectar producing glands (Prenner and Rudall 2007). The pistil has a three-carpelled ovary that matures into a three-chambered capsule with a single ovule in each chamber, thus giving rise to a fixed maximum number of three seeds per cyathium.

In the Pedilanthus clade of *Euphorbia*, all flowers are enclosed by an involucre tube and the nectar glands are concealed in a chamber that can vary in shape and size (Dressler 1957). The whole structure is strongly bilateral, and resembles a slipper or shoe, hence the common name for plants in this clade: slipper spurges (Fig. 1). There is substantial variation in involucre length and cyathial height across the Pedilanthus clade (Cacho et al. 2010), which in *E. tithymaloides* can be explained by a combination of geography and history, such that the patterns reveal evolutionary convergence towards relatively shorter structures towards the Anegada Passage (Cacho et al. 2019).

Several mechanisms hypothesized to attract pollinators and avoid self-fertilization are in place in *E. tithymaloides*. Cyathia are bright red, a coloration that is presumed to be attractive to hummingbirds, and nectar sugar content and production seems to be carefully regulated to hummingbird preferences (Veiga Blanco et al. 2013). Cyathia exhibit partial temporal separation of the male and female phases (dichogamy), passing through an initial protogynous phase, followed by emergence of the anthers, at which point cyathia are functionally hermaphroditic (Dressler 1957; Prenner and Rudall 2007; Veiga Blanco et al. 2013). Beyond dichogamy, *E. tithymaloides* may also exhibit herkogamy, as anthers and style are separated spatially due to a down curving of the style that prevents contact between the stigma and the pollen (Dressler 1957).

Botanical Material—The cyathia used for this study were collected in the field (fixed in ethanol 70% or FAA) or sampled from herbarium specimens. Collections were made during multiple fieldwork trips to various localities in Mexico and the Caribbean during 2006, 2008, 2017, and 2019 by the authors (Appendix 1).

Pollen Counts—Pollen counts were performed on three anthers for one to four individuals (mean = 2.53) representing 38 populations across the range of *E. tithymaloides* in the Caribbean. Ovule production in *E. tithymaloides* is fixed to three per cyathium, so that P:O ratios are a direct reflection of pollen production per cyathium.

For cases in which specimens needed to be sampled, these were screened for presence of several cyathia in good condition so that we could destructively sample one cyathium for our study without compromising the integrity of the botanical collections. We selected specimens that represented the distribution of *E. tithymaloides* in the Caribbean as completely as possible. For each selected specimen, cyathia were rehydrated in warm

water (37°C) for 12h, and subsequently transferred to 70% ethanol, where they were preserved until further processing.

Individual cyathia were dissected to visualize the anthers. The number and state of anthers was recorded, including whether they had been attacked by enemies. Then, one undehisced anther per cyathium was selected at random and transferred to an Eppendorf tube containing 10 μ L of distilled water, where it was kept at 4°C until further processing. Anthers were macerated within the tube using a micro pestle, and the contents transferred to a Marienfeld Neubauer counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) to be examined under the microscope (Velab Prime VE-T50). The number of pollen grains in each quadrant was recorded separately and then added together to get the number of pollen grains per anther for a given sample. To ensure reproducibility, the counting exercise was repeated three times for each anther and averaged.

The number of pollen grains per cyathium was obtained by multiplying the number of pollen grains per anther by the number of anthers per cyathium. Because *E. tithymaloides* produces a single pistillate flower per cyathium with three ovules per ovary, P:O ratios were obtained dividing the amount of pollen produced per cyathium by three.

Cyathium Measurements—We contribute new cyathial measurements (length, height) for 56 cyathia representing nine populations (min = 1; mean = 6.22; max = 12 cyathia per population). Images of cyathia were taken in the field with a scale, and measured in Geogebra v. 5 (<http://www.geogebra.org>). These new data were added to data from a previous study (Cacho et al. 2019) and together are part of an ongoing morphometric data collection for this system.

Statistical Analyses—We first assessed relative male: female reproductive investment in *E. tithymaloides* in the context of P:O ratios across angiosperms using data available from previous studies. We plotted P:O ratios for *E. tithymaloides* along with available angiosperm-wide P:O ratios (Cruden 1977; Michalski and Durka 2009; Scalone et al. 2013; Lozada-Gobilard et al. 2018) in the context of five breeding system categories previously recognized (Cruden 1977; Scalone et al. 2013): cleistogamous (P:O < 10), autogamous (10 > P:O < 60), facultative autogamous (60 > P:O < 300), facultative xenogamous (300 > P:O < 2000), and xenogamous (P:O > 2000).

To take history and the geographical variation of floral morphology into account, we performed our analyses separately by colonization front (GA and LA fronts). Populations were assigned to either of the GA or LA fronts, or to the center of origin (OR) following Cacho et al. (2019). The current distribution of *E. tithymaloides* across the Caribbean basin reflects a colonization history along two geographical fronts, each representing a separate lineage. It has been documented that these two lineages are not only genetically distinct, but vary independently with respect to floral morphology (Cacho et al. 2019).

Normality and homoscedasticity were assessed with Shapiro and Levene’s tests, respectively, before analyses. Pollen count data were log transformed to meet assumptions of normality. Following transformation of pollen counts, data conformed to normality and were homoscedastic at the $p < 0.05$ significance level. Potential outliers were identified from visual examination of boxplots, and formally evaluated using a Rosner test. No significant outliers were detected on transformed data. We evaluated differences in pollen production by front (GA, LA) and populations in the center of origin (OR) using analysis of variance (ANOVA) and Tukey’s HSD for post-hoc comparisons (R function TukeyHSD).

To assess geographical patterns of pollen production along both fronts (GA, LA) of the *E. tithymaloides* species complex in relation to previously documented variation in cyathium traits, we used linear models where geography (distance to the Anegada Passage) or floral morphology (cyathium L/W) were used as independent variables. Following previous studies, we took the rather conservative approach of excluding populations from the center of origin (OR) from these regressions, which renders our analyses conservative because patterns might be less pronounced and because the exclusion of the populations from the center of origin decreases the statistical power of our correlations. All analyses were done in R version 3.5.2 (R Development Core Team).

RESULTS

Data—All data accompanying this paper, including pollen data, cyathium measurements, and images from which cyathial measurements were derived are deposited in Dryad (Cacho and José-Zacatula 2020).

Pollen:ovule ratios per cyathium in *Euphorbia tithymaloides* ranged from 254.2 to 918.4, with a mean of 507 ± 159.9 . The variation observed in P:O ratios in *E. tithymaloides* falls within the variation typically observed in facultative xenogamous plants (Fig. 2). As the number of ovules is fixed at three per cyathia, this reflects variation in pollen production rather than variation in ovule number. Pollen production per anther or cyathium (and thus P:O ratios) does not differ among fronts or center of origin (Fig. 3A, B). However, the number of anthers produced per cyathium differs significantly across groups ($F_{(2,35)} = 3.6542, p = 0.036$; Table 1A; Fig. 3C). Tukey's post-hoc

tests revealed that, in general, plants in the GA front produce more anthers per cyathium (25.9 ± 3.7) than in the LA front ($22.9 \pm 2.2; p = 0.028$); the populations from the center of origin (OR) are not different from either front (23.9 ± 2.6 ; Table 1B; Fig. 3C).

There is no significant geographic pattern in relation to distance from the Anegada Passage (AP) in the amount of pollen produced per anther along either of the two expansion fronts (Table 2; Fig. 4A, D). Pollen:ovule ratios, which are a direct reflection of the amount of pollen produced per cyathium, decrease significantly in the GA front towards the

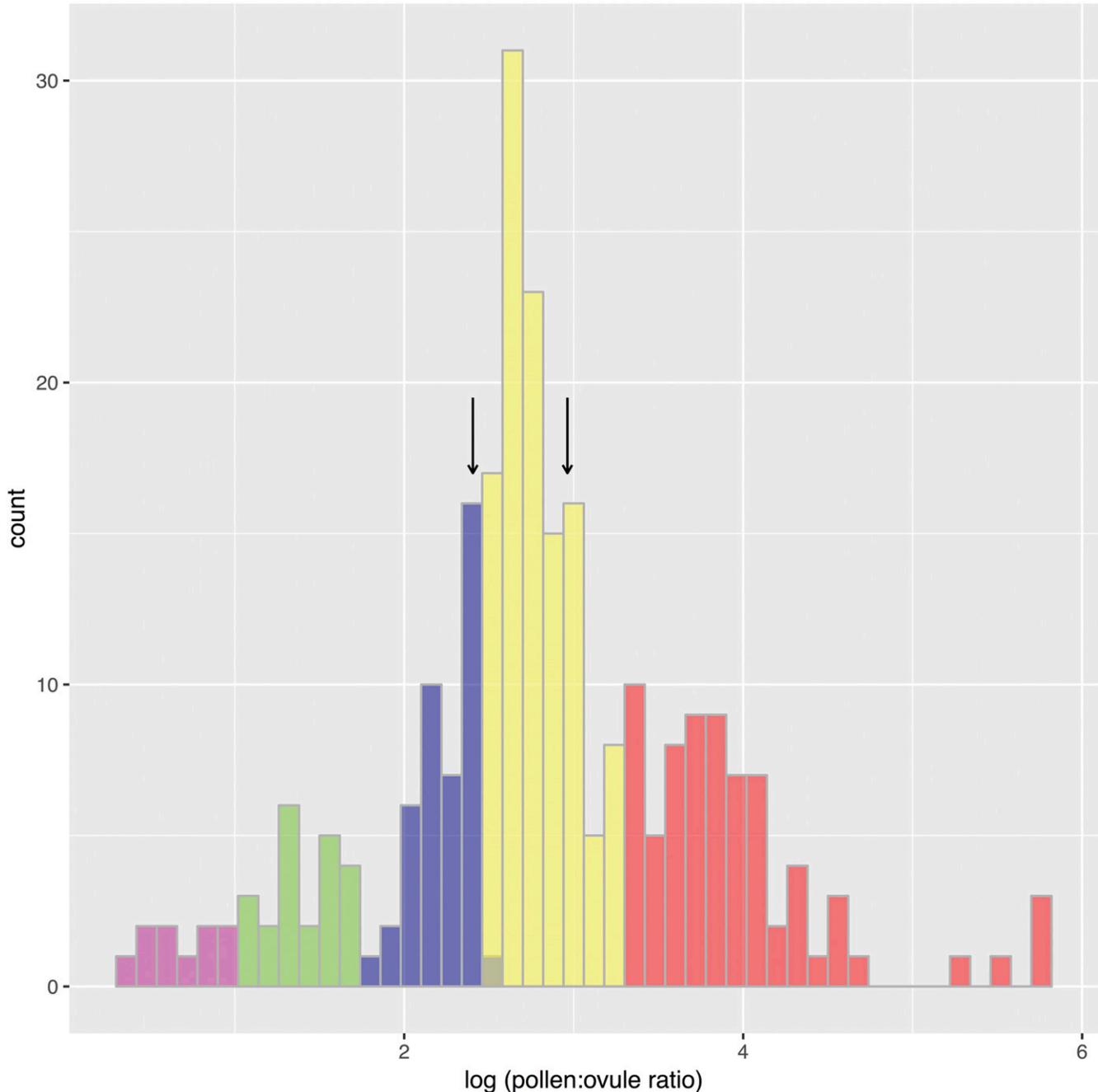


FIG. 2. The pollen:ovule (P:O) ratios of *E. tithymaloides* fall within the P:O ratios recognized as typical of facultative xenogamous flowers (data from: Cruden 1977; Michalski and Durka 2009; Scalone et al. 2013; Lozada-Gobilard et al. 2019). The two arrows show the extremes of the range in log (P:O ratios) for *E. tithymaloides*. The five categories recognized by Cruden are plotted here: cleistogamous (pink, $P:O < 10$), autogamous (green, $10 > P:O < 60$), facultative autogamous (blue, $60 > P:O < 300$), facultative xenogamous (yellow, $300 > P:O < 2000$), xenogamous (red, $P:O > 2000$).

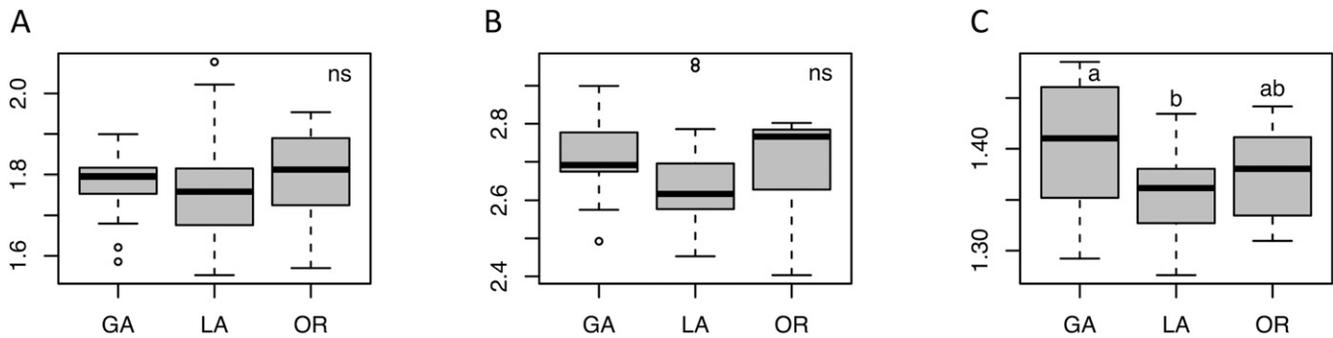


FIG. 3. Male investment in populations of *Euphorbia tithymaloides* from the Greater Antillean front (GA), the Lesser Antillean front (LA), or its center of origin (OR). A. Pollen production per anther. B. Pollen:ovule ratios. C. Number of anthers per cyathium.

AP ($p < 0.008$; Table 2) but not in the LA front (Table 2; Fig. 4E). This pattern is likely driven by a significant decrease ($p < 0.038$) in the number of anthers per cyathium in populations of the GA front of *E. tithymaloides* that are closer to the AP (Table 2; Fig. 4F).

Cyathium morphology is not a significant predictor of pollen production per anther or P:O ratios in either the GA or LA fronts (Table 3; Fig. 5A, B), but relatively shorter cyathia produce fewer anthers in both the GA and LA fronts (Table 3; $P_{GA} < 0.006$; $P_{LA} < 0.037$; Fig. 5C).

DISCUSSION

Our study contributes to an understanding of investment in male reproduction for populations of *Euphorbia tithymaloides* across its range in the Caribbean. The variation in pollen:

TABLE 1. The number of anthers produced by populations of *Euphorbia tithymaloides* differs by geographic front. Significance at the $p < 0.5$ level is highlighted by an asterisk (*).

A. Analysis of Variance					
Source of variation	Degrees of freedom	Sums of squares	Mean square	F statistic	Pr(>F)
Geographic Front	2	0.01939	0.00969	3.6542	0.0362 *
Residuals	35	0.09284	0.00265		
B. Tukey multiple comparisons of means.					
Factor comparison	Mean difference	Lower bound	Upper bound	P adjusted	
LA-GA	-0.0511	-0.0976	-0.0047	0.0283	*
OR-GA	-0.0336	-0.0903	0.0230	0.3258	
OR-LA	0.0175	-0.0365	0.0715	0.7100	

TABLE 2. Pollen and anther production in relation to distance from the Anegada Passage (AP) in *Euphorbia tithymaloides*. Distance to the AP is not a significant predictor of pollen production per anther in populations of either front. Pollen:ovule ratios decline towards the AP in populations of the Greater Antillean front, likely driven by a decrease in the number of anthers being produced towards the AP. Pollen count data were log transformed. Significance at the $p < 0.5$ level is highlighted by an asterisk (*), and at the $p < 0.01$ level by two asterisks (**).

Variable	Front	Adj. R^2	Estimate	Std. error	t value	Pr(> t)	
Pollen/anther	GA	0.118	3.54E-05	2.20E-05	1.612	0.135	ns
	LA	-0.066	-1.76E-06	2.56E-05	-0.069	0.946	ns
Pollen/ovule	GA	0.438	6.77E-05	2.10E-05	3.220	0.008	**
	LA	-0.057	9.64E-06	2.62E-05	0.368	0.718	ns
Anthers/cyathium	GA	0.276	3.23E-05	1.37E-05	2.361	0.038	*
	LA	0.098	1.14E-05	6.88E-06	1.656	0.119	ns

ovule (P:O) ratios we document in *E. tithymaloides* is contained within the lower end of the variation in P:O ratios for facultative xenogamous plants. This is consistent with what is known about pollination and breeding system of *E. tithymaloides*, which is visited by hummingbirds throughout its range. Among animal-pollinated plants, P:O ratios are expected to be lower in plants that offer nectar as a reward, like *E. tithymaloides*, compared to those where pollen is the only reward (Cruden 2000). Floral characteristics of *E. tithymaloides* are consistent with those traditionally related to hummingbird pollination: nectar-producing floral units usually with red or reddish coloration that require either force (because nectar is concealed in a somewhat sturdy structure) or an elongate structure to access the reward (Dressler 1957; Veiga Blanco et al. 2013). Nectar production and concentration as well as visitation rates in a population of *E. tithymaloides* in Brazil were consistent with hummingbird pollination (Veiga Blanco et al. 2013) but range-wide pollination studies are lacking for this species.

We find a reduction in male investment (P:O ratios) across Caribbean populations of *E. tithymaloides* towards the Anegada Passage (only significant in the Greater Antillean expansion front, GA, but same trend in the Lesser Antillean front, LA). The reduction of P:O ratios in populations closer to the AP, compared to populations at the center of origin of *E. tithymaloides*, in Mexico/Guatemala. This pattern could also be impacted by an effect of insularity, as self-compatibility is over-represented in islands (Grossenbacher et al. 2017). An increased incidence of selfing around the AP would also be consistent with observed decreasing levels of heterozygosity in *E. tithymaloides* towards this area (Cacho and Baum 2012).

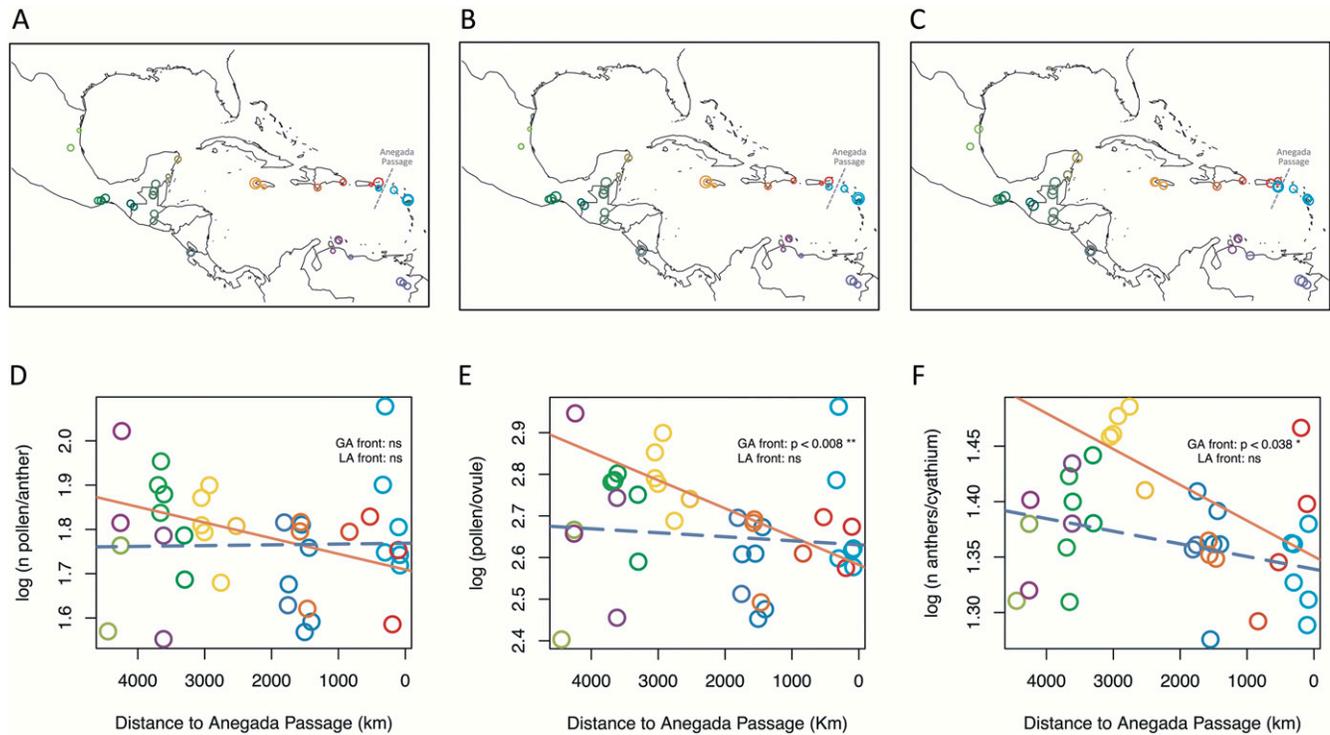


FIG. 4. Variation in pollen and anther production in the context of geography across populations of the *E. tithymaloides* species complex. Populations are color-coded by their distance to the Aneгада Passage (17.9251611°N, -63.916925°W), which has been proposed to function as a semipermeable barrier where the two geographic fronts meet (Cacho and Baum, 2012). The sizes of the circles in the maps (A–C) are proportional to the size of the relevant variable. A. Pollen per anther. B. Pollen:ovule ratio. C. Anthers per cyathium. Lower panels (D–F) show variation in relation to distance from the Aneгада Passage along the two geographic expansion fronts, Greater Antillean (GA; solid red line) and Lesser Antillean (LA; dashed blue line). D. Pollen per anther. E. Pollen:ovule ratio (equivalent to pollen per cyathium). F. Anthers per cyathium.

An alternative, non-mutually exclusive explanation for the reduced heterozygosity in populations close to the AP is their more recent origin, as such a signature of founder effects can last for generations (Matute 2013). The trend of decreasing P:O ratios towards the AP we document is likely driven by fewer anthers produced in relatively shorter cyathia, a correlation that was significant along both the GA and LA geographic fronts of *E. tithymaloides*. In cultivation, plants of *E. tithymaloides* do not self-fertilize spontaneously but anecdotal tests of selfing have revealed that hand pollinated (selfed) plants produce fruits and set seed (NIC unpubl. data), suggesting that geitonogamy or selfing from pollen transfer within flowers in an individual can be successful in this species. Range-wide studies that quantify selfing rates in populations of *E. tithymaloides* are necessary to evaluate geographical variation in selfing rates across Caribbean populations of this species.

The pattern of lower P:O ratios and anther number in shorter cyathia is also consistent with processes other than potential increases in selfing rates, and these are not necessarily mutually exclusive. A reduction in pollen production could be the result of selection for smaller floral units towards the AP and may not necessarily be related to a transition to a breeding system more reliant on selfing (changes in floral morphology can also be accompanied by the breakdown of traits that reduce selfing, such as herkogamy; Inoue 1993). In *Raphanus*, resource allocation to pollen correlates positively with corolla size (Stanton and Preston 1988); likewise, selection for smaller floral units in *E. tithymaloides* could drive a decrease in P:O ratios. Pollinators could play a key role in driving floral shifts towards relatively shorter or overall smaller structures through changes in the identity of the main pollinators, either among hummingbird species, or through an increased insect importance. Plants in insular habitats have been shown to shift towards smaller sizes compared to mainland populations as

TABLE 3. Pollen and anther production in relation to floral morphology (cyathium L/W) in *Euphorbia tithymaloides*. Cyathium morphology is not a significant predictor of pollen production per anther or pollen:ovule ratios in either front, but relatively shorter cyathia produce fewer anthers in both the GA and LA fronts. Pollen count data were log transformed. Significance at the $p < 0.5$ level is highlighted by an asterisk (*), and at the $p < 0.01$ level by two asterisks (**).

Variable	Front	Adj. R ²	Estimate	Std. error	t value	Pr(> t)	
Pollen/anther	GA	-0.089	0.010	0.082	0.124	0.904	ns
	LA	-0.066	-0.011	0.101	-0.106	0.917	ns
Pollen/ovule	GA	0.118	0.143	0.089	1.613	0.135	ns
	LA	-0.052	0.048	0.103	0.460	0.652	ns
Anthers/cyathium	GA	0.458	0.133	0.040	3.340	0.007	**
	LA	0.209	0.058	0.025	2.288	0.037	*

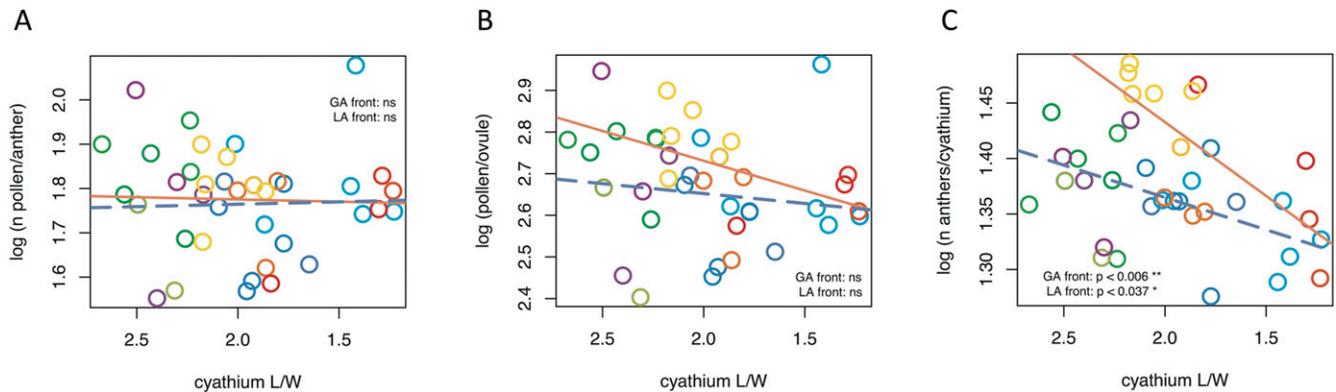


FIG. 5. Variation in pollen and anther production as a function of floral morphology (cyathium L/W) across populations of the *E. tithymaloides* species complex. Populations are color-coded by their distance to the Anegada Passage (following maps in Fig. 4), where both expansion fronts of *E. tithymaloides* meet. A. Amount of pollen produced per anther. B. Pollen:ovule ratio. C. Number of anthers produced per cyathium. Greater Antillean (GA) = solid red line; Lesser Antillean (LA) = dashed blue line.

a result of selection imposed by pollinator traits and availability (Inoue 1993; Temeles and Kress 2003; Temeles et al. 2009; Yamada and Maki 2014) or by the evolution of mixed entomophilous pollination (Feinsinger et al. 1982; Dalsgaard et al. 2009). Hummingbird-to-insect pollination shifts have been documented in the Caribbean (Feinsinger et al. 1982; Dalsgaard et al. 2009), and also in the *Pedilanthus* clade of *Euphorbia* (Sahagún-Godínez and Lomeli-Sención 1997), and trait matching between flowers and associated hummingbird species has also been documented in Caribbean insular plant systems (Temeles and Kress 2003). Evaluating the relative contribution of pollinators and their traits to the patterns in floral morphology and pollen production we report here would benefit from comparative field pollination observation in populations of *E. tithymaloides* across the Caribbean. A diversity of factors beyond pollinator related ones may influence mating systems (Karron et al. 2009, 2012) and relative investment in male vs. female functions, including variation in floral morphology and display size (Stanton and Preston 1988; Young and Stanton 1990; Karron and Mitchell 2012), natural enemies (Ivey et al. 2004), and other conditions that reduce fitness relative to other environments (Armbruster and Reed 2005).

Taken together, our results are consistent with *E. tithymaloides* being reliant on pollinators for reproduction across its range, and suggest the possibility of a geographic transition towards a mixed breeding system where selfing could be more important in populations in the vicinity of the Anegada Passage. Field observation and experimental tests of selfing rates, either in the field or a greenhouse setting, would be required to confirm what our studies on P:O ratios suggest. More broadly, an integrative approach to the study of the reproductive biology of *E. tithymaloides* encompassing pollinator observations, geographic patterns in herkogamy, dichogamy, reward, display size, and selfing rates would allow for a nuanced understanding of the factors that could be driving the geographic patterns in floral morphology and relative investment in male and female functions in this system.

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AUTHOR CONTRIBUTIONS

NIC conceived the study, carried out field collections, analyzed data, and wrote the paper; DJZ did pollen counts and participated in fieldwork. Both authors approved the final version of the manuscript.

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APPENDIX 1. Pollen count data and cyathium measurements for populations included in this study; collection numbers, herbarium and geographic data are provided.

Population, Collection number, Herbarium, Latitude, Longitude, Distance to AP (Km), Geographic front, Country, N individuals (pollen), pollen per anther, anthers per cyathium, pollen per cyathium, pollen: ovule ratio.

allighole, *NIC-084*, MEXU, 17.868, -77.392, 1464.83, GA, Jamaica, 3, 44.667, 22.333, 1010.444, 336.815, 4, 10.848, 5.809, 1.862; cahui1, *NIC-375*, MEXU, 16.998, -89.716, 3049.44, GA, Guatemala, 1, 74.333, 28.750, 2137.083, 712.361, 2, 12.110, 5.934, 2.054; cahui2, *NIC-386*, MEXU, 16.999, -89.717, 3049.36, GA, Guatemala, 2, 65.667, 28.750, 1872.500, 624.167, 2, 12.941, 6.001, 2.161; carmelas, *NIC-520*, MEXU, 18.147, -89.415, 2930.10, GA, Mexico, 1, 79.333, 30.000, 2380.000, 793.333, 4, 12.688, 5.844, 2.181; coro, *NIC-230*, MEXU, 11.130, -69.758, 1809.92, LA, Venezuela, 3, 66.000, 22.750, 1499.722, 499.907, 3, 12.028, 5.822, 2.067; dr_jaragua, *NIC-073*, MEXU, 17.792, -71.462, 835.61, GA, RepDom, 3, 62.778, 19.667, 1247.556, 415.852, 3, 4.348, 3.581, 1.233; dr_yuma, *NIC-070*, MEXU, 18.360, -68.621, 528.35, GA, RepDom, 3, 67.889, 22.333, 1538.111, 512.704, 3, 5.637, 4.474, 1.286; guacamayo, *NIC-207*, MEXU, 7.912, -62.266, 1403.45, LA, Venezuela, 3, 40.667, 23.000, 927.333, 309.111, 1, 12.657, 6.559, 1.930; guiengola1, *MEO-947*, MEXU, 16.367, -95.323, 3653.54, OR, Mexico, 3, 90.444, 20.500, 1833.556, 611.185, 6, 19.681, 8.797, 2.237; guija, *NIC-349*, MEXU, 14.272, -89.648, 4240.48, LA, Guatemala, 3, 107.000, 25.333, 2755.111, 918.370, 5, 14.361, 5.765, 2.505; hacha1, *NIC-322*, MEXU, 10.994, -85.520, 3616.12, LA, CostaRica, 1, 35.667, 24.000, 856.000, 285.333, 8, 12.067, 5.068, 2.400; hacha2, *NIC-325*, MEXU, 10.987, -85.524, 3615.24, LA, CostaRica, 2, 61.333, 27.250, 1680.667, 560.222, 12, 12.742, 5.874, 2.171; jalmarques.oax, *MEO-950*, MEXU, 16.427, -95.463, 3661.65, OR, Mexico, 3, 70.667, 26.500, 1868.778, 622.926, 6, 19.125, 8.518, 2.233; littlebay, *NIC-088*, MEXU, 18.221, -78.251, 1563.81, GA, Jamaica, 2, 66.500, 22.500, 1501.833, 500.611, 6, 11.911, 6.620, 1.804; majahual, *NIC-531*, MEXU, 18.979, -88.036, 2757.92, GA, Mexico, 2, 47.833, 31.000, 1478.667, 492.889, 2, 12.729, 6.040, 2.174; minahill, *NIC-059*, MEXU, 18.317, -64.710, 104.81, GA, St. John, 1, 56.667, 25.000, 1416.667, 472.222, 3, 7.541, 5.732, 1.304; negril, *NIC-092*, MEXU, 18.248, -78.359, 1575.61, GA, Jamaica, 3, 63.889, 23.167, 1477.778, 492.593, 5, 10.452, 5.221,

2.002; nenton, *NIC-415*, MEXU, 15.770, -91.842, 3297.48, OR, Guatemala, 3, 49.556, 24.333, 1229.556, 409.852, 12, 11.336, 5.045, 2.260; nizanda, *NIC-495*, MEXU, 16.661, -95.012, 3607.01, OR, Mexico, 3, 76.000, 25.167, 1913.444, 637.815, 1, 15.174, 6.243, 2.431; playa.daibooy, *NIC-1139*, MEXU, 12.211, -69.086, 1743.93, LA, Curacao, 3, 47.889, 25.667, 1227.000, 409.000, 8, 10.076, 5.701, 1.773; playa.stacruzA, *NIC-1137*, MEXU, 12.305, -69.148, 1755.38, LA, Curacao, 4, 42.833, 23.000, 990.333, 330.111, 1, 8.182, 4.968, 1.647; playon, *NIC-248*, MEXU, 10.491, -67.780, 1548.61, LA, Venezuela, 3, 65.444, 19.000, 1224.444, 408.148, 6, 9.765, 5.480, 1.773; portenfer1, *NIC-580*, MEXU, 16.482, -61.429, 303.42, LA, Guadeloupe, 2, 56.333, 21.250, 1192.333, 397.444, 2, 10.937, 8.912, 1.228; portenfer2, *NIC-595*, MEXU, 16.492, -61.440, 301.88, LA, Guadeloupe, 1, 119.667, 23.000, 2752.333, 917.444, 3, 9.997, 7.063, 1.417; ptchteaux, *NIC-609*, MEXU, 16.254, -61.231, 336.33, LA, Guadeloupe, 3, 86.333, 23.333, 2088.111, 696.037, 2, 10.789, 5.385, 2.014; ptevictoria, *NIC-191*, MEXU, 7.766, -61.953, 1441.51, LA, Venezuela, 3, 58.556, 24.667, 1434.111, 478.037, 5, 12.697, 6.064, 2.095; quebrada_vieques, *NIC-060*, MEXU, 18.110, -65.515, 192.95, GA, Puerto Rico, 3, 39.000, 29.333, 1154.222, 384.741, 3, 14.084, 7.709, 1.837; riohondo_guate, *NIC-361*, MEXU, 15.031, -89.587, 4259.90, LA, Guatemala, 3, 67.111, 21.000, 1377.111, 459.037, 9, 13.117, 5.773, 2.301; riohondo_mex1, *NIC-478*, MEXU, 16.426, -95.825, 3697.76, OR, Mexico, 3, 82.111, 23.000, 1839.000, 613.000, 3, 15.454, 5.730, 2.672; secreto, *NIC-538*, MEXU, 20.781, -86.950, 2526.62, GA, Mexico, 2, 64.667, 25.750, 1674.750, 558.250, 5, 11.793, 6.123, 1.922; statia, *NIC-571*, MEXU, 17.517, -62.992, 101.48, LA, Statia, 4, 64.500, 19.500, 1265.333, 421.778, 7, 10.901, 7.545, 1.442; stcroix.rd85, *NIC-076*, MEXU, 17.738, -64.688, 81.79, LA, St. Croix, 3, 53.444, 24.000, 1274.222, 424.741, 6, 13.180, 7.083, 1.868; stcroix.springut, *NIC-075*, MEXU, 17.733, -64.692, 82.17, LA, St.Croix, 2, 55.333, 20.500, 1133.000, 377.667, 3, 10.331, 7.502, 1.382; tani-nulB.slp, *NIC-443*, MEXU, 21.970, -98.850, 4256.91, OR, Mexico, 3, 58.556, 24.000, 1407.000, 469.000, 3, 15.716, 6.305, 2.494; tumeremo, *NIC-179*, MEXU, 7.430, -61.492, 1504.55, LA, Venezuela, 1, 37.000, 23.000, 851.000, 283.667, 3, 12.016, 6.126, 1.956; tzimol, *NIC-507*, MEXU, 16.113, -92.208, 3305.82, OR, Mexico, 3, 61.333, 27.833, 1692.667, 564.222, 4, 12.855, 5.134, 2.561; uaxactun, *NIC-391*, MEXU, 17.402, -89.653, 3004.19, GA, Guatemala, 3, 62.667, 29.000, 1828.000, 609.333, 2, 13.185, 7.130, 1.864; vistahermosa, *NIC-455*, MEXU, 23.799, -97.905, 4445.64, OR, Mexico, 2, 37.667, 20.500, 762.667, 254.222, 4, 15.164, 6.568, 2.312.