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ADAPTACIÓN LOCAL EN LA INTERACCIÓN
***DATURA STRAMONIUM*-HERBÍVORO**

TESIS

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PRESENTA:

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P r e s e n t e

Me permito informar a usted, que el Subcomité de Biología Evolutiva y Sistemática, en su sesión ordinaria del día 16 de febrero de 2015, aprobó el jurado para la presentación de su examen para obtener el grado de **DOCTOR EN CIENCIAS**, del Posgrado en Ciencias Biológicas, del alumno **CASTILLO SÁNCHEZ GUILLERMO RAÚL** con número de cuenta 97201201 con la tesis titulada **"Adaptación local en la interacción Datura stramonium-herbivoro"**, bajo la dirección del **DR. JUAN SERVANDO NÚÑEZ FARFAN**:

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Sin otro particular, me es grato enviarle un cordial saludo.

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"Science is very vibrant. There are always new observations to be found. And it's all in the interest in challenging the authority that came before you. That's consistent with the punk rock ethos that suggests that you should not take what people say at face value."

Greg Graffin

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Adaptación local en la interacción *Datura stramonium*-herbívoro

Guillermo Raúl Castillo Sánchez

Resumen

Desentrañar los mecanismos que generan y mantienen la variación de atributos entre las poblaciones de una especie representa aún hoy un gran reto para la biología evolutiva. El objetivo de esta tesis fue el estudiar a nivel geográfico la variación de los atributos defensivos que median la interacción planta-herbívoro entre la planta anual *Datura stramonium* y sus herbívoros especialistas y generalistas. En este contexto, en mi tesis exploré la existencia de asociación geográfica del daño foliar por herbívoros con la defensa química y física en 28 poblaciones de *D. stramonium* y evalué si los herbívoros generalistas ejercen patrones selectivos contrastantes sobre atributos defensivos de *D. stramonium*. También evalué la existencia de adaptación local de *D. stramonium* a herbívoros generalistas y especialistas, así como a qué grado esta adaptación local es mediada por la defensa física de la planta. Finalmente exploré la evidencia de diferenciación adaptativa en atributos físicos y químicos en 13 poblaciones naturales de *D. stramonium* en el centro de México. Encontré una amplia variación geográfica en atributos defensivos (tricomas foliares, concentración de escopolamina y atropina), así como una asociación positiva significativa entre el daño foliar y la concentración de atropina. También encontré que a nivel geográfico los herbívoros especialistas seleccionan negativamente a la atropina y los generalistas favorecen a la escopolamina. Los resultados mostraron que las poblaciones de *D. stramonium* pueden estar adaptadas tanto a herbívoros generalistas y especialistas. Sin embargo, esta adaptación no está mediada por los tricomas foliares. Finalmente detecté evidencia de diferenciación adaptativa para la concentración de atropina y de escopolamina. Esta tesis resalta la necesidad de combinar experimentos de campo con evidencia obtenida de poblaciones naturales para responder preguntas contemporáneas acerca de la evolución de la defensa en plantas.

Abstract

Unveiling the mechanisms that generate and maintain variation of traits between species populations remains as a major challenge for evolutionary biology. The objective of this thesis was to study the geographic variation of defensive traits mediating the plant-herbivore interaction between *Datura stramonium* and its specialist and generalist herbivores. To do so, I explored the geographic association between leaf damage and chemical and physical defense of 28 natural populations of *D. stramonium* and evaluated whether specialist and generalist herbivores exert contrasting selective patterns on plant defense. I also assessed the local adaptation of *D. stramonium* to specialist and generalist herbivores. Finally I aimed to detect evidence of adaptive differentiation of defensive traits of *D. stramonium* in 13 natural populations in central Mexico. I found ample geographic variation in defensive traits (leaf trichome density, atropine and scopolamine concentration) and a positive geographic association between leaf damage and atropine. Results showed that, at a geographic level, specialist herbivores select negatively atropine concentrations, whereas generalist herbivores favor scopolamine concentration. I also found that *D. stramonium* populations can be locally adapted to specialist and generalist herbivores, but this adaptation is apparently not mediated by trichome density. Finally, I found evidence of adaptive differentiation of atropine and scopolamine concentration. This thesis highlights the need to combine field experiments with evidence obtained form natural populations to address further questions about the evolution of plant defense.

Introducción general

La mayoría de las especies están compuestas de una serie de poblaciones que frecuentemente están diferenciadas fenotípica y genéticamente (Rice & Jain, 1985; Thompson, 2005). Esta diferenciación puede originarse por mecanismos, como la deriva génica, cuellos de botella o efectos fundadores (Gomulkiewicz *et al.*, 2007). Sin embargo, también puede originarse debido a la existencia de variación espacial en las presiones selectivas ejercidas por las condiciones bióticas y abióticas del ambiente (Lande & Arnold, 1983; Holsinger & Weir, 2009). La variación espacial en los patrones selectivos puede producir la diferenciación adaptativa entre poblaciones en atributos involucrados en una interacción (Thompson, 1994; Thompson, 2005; Züst *et al.*, 2012). Como consecuencia, se espera que a lo largo de grandes áreas geográficas, se promuevan diferentes trayectorias evolutivas en diferentes poblaciones (Mayr, 1947; Thompson, 2005). Debido a esto, conocer que tan común es la diferenciación adaptativa de atributos en la naturaleza es relevante para entender cómo se genera y se mantiene la variación dentro de las especies así como entre poblaciones (Muola, 2010).

La Teoría del Mosaico Geográfico de la Coevolución (TMGC) nos provee de un marco teórico para estudiar la evolución de las interacciones a una escala geográfica amplia (Thompson, 2005). De acuerdo a esta teoría, el resultado del proceso coevolutivo (cambio evolutivo recíproco entre especies interactuantes) está determinado por la variación geográfica en las interacciones entre especies. En particular, la TMGC postula que son tres los procesos que están moldeando el resultado coevolutivo a escala geográfica: *(1)* La existencia de un mosaico selectivo, *(2)* la presencia de "Hot-spots" y "Cold-spots" coevolutivos y *(3)* la remezcla de caracteres (mutaciones, flujo génico entre poblaciones, deriva génica o extinción de poblaciones). De acuerdo a la TMGC la intensidad y sentido de las presiones selectivas ejercidas entre atributos de especies interactuantes varía entre poblaciones (debido a interacciones genotipo × genotipo × ambiente sobre la adecuación de especies interactuantes). Por ello, a lo largo de una región geográfica existirán poblaciones en las que exista selección recíproca entre especies (*i.e.* "Hot-spots" coevolutivos), que estarán embebidas en una matriz de localidades en dónde sólo una

especie ejerce presiones selectivas o donde simplemente no existirán presiones selectivas (*i.e.* "Cold-spots" coevolutivos). Este proceso de diferenciación en las poblaciones estará también mediado por nuevas mutaciones, deriva génica y por el flujo génico entre poblaciones (remezcla de caracteres). Esto altera continuamente la distribución espacial de genes y atributos potencialmente relevantes para la evolución de la interacción. Como resultado, la TMGC predice tres patrones observables: *(i)* variación espacial en atributos que median una interacción, *(ii)* desajuste de atributos entre especies interactuantes en algunas localidades y *(iii)* pocos atributos favorecidos por coevolución a nivel de especie (Thompson, 2005; Gomulkiewicz *et al.*, 2007).

La TMGC sugiere así la existencia de un patrón geográfico de adaptaciones y maladaptaciones locales (Gómez *et al.*, 2009). La hipótesis de adaptación local propone que los individuos originarios de una población mostrarán mayor adecuación en su hábitat original en comparación con individuos originarios de otro hábitat (Kawecki & Ebert, 2004). Sin embargo, las especies interactuantes pueden estar adaptadas recíprocamente en unas poblaciones y no en otras, e incluso estar maladaptadas localmente (individuos de una población presentar menor adecuación en su hábitat original en comparación con individuos originarios de otro hábitat) (Thompson & Cunningham, 2002). Esto, como resultado de las dinámicas coevolutivas locales de las especies interactuantes (Laine, 2009). Por ello estudiar la ocurrencia de adaptación local es fundamental para entender la manera en la que se genera y se mantiene la variación geográfica de este tipo de interacciones (Kawecki & Ebert, 2004; Thompson, 2005; Laine, 2009).

La interacción planta-herbívoro ha sido ampliamente estudiada en el contexto de la coevolución y de la adaptación local (Ehrlich & Raven, 1964; Garrido *et al.*, 2012). En este tipo de interacción (y desde el punto de vista de las plantas) es posible detectar adaptación local cuando las plantas de una población presentan mayor adecuación cuando están expuestas a herbívoros simpátridos en comparación a cuando son expuestas a herbívoros alopátridos (Kawecki & Ebert, 2004). Dado que, en principio, el daño foliar provocado por herbívoros reduce el éxito reproductivo de las plantas (Crawley,

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1989), esperaríamos que esta adaptación local esté mediada en buena medida por atributos defensivos exhibidos por las plantas (Laukkanen *et al.*, 2012).

Las plantas poseen diferentes atributos defensivos que les permiten evitar el daño provocado por los herbívoros, por un lado están las defensas físicas (*e. g.* tricomas, espinas) y por el otro están las defensas químicas (e. g. terpenos, alcaloides) (Rausher, 2001; Schaller, 2008; Anderson & Mitchell-Olds, 2011). La expresión de estos atributos defensivos puede ser inducida (cuando es promovida por el daño provocado por herbívoros) o constitutiva (cuando está presente de manera previa al daño por herbívoros) (Agrawal & Karban, 2000). Se ha propuesto que la evolución y mantenimiento de estos atributos ha sido resultado de presiones selectivas ejercidas por los herbívoros, por ello se esperaría que se seleccionen a favor aquellos atributos que reduzcan el daño por herbívoros y se favorezcan "niveles defensivos altos" (Simms & Rausher, 1987; Rausher, 2001). Sin embargo, a lo largo del área de distribución geográfica de una especie, las poblaciones naturales de plantas suelen estar expuestas una gran variedad de herbívoros, que en los extremos pueden ir de los generalistas (se alimentan de una gran variedad de plantas que no están emparentadas) hasta los especialistas (consumen exclusivamente un grupo de especies relacionadas filogenéticamente) (Thompson, 2005; Lankau, 2007). Se ha hipotetizado que los atributos defensivos de las plantas son una barrera efectiva en contra de herbívoros generalistas, debido a que pueden alimentarse de diferentes especies de plantas, tendiendo diferentes alternativas en su dieta (Agrawal & Heil, 2012). Por el contrario, diferentes estudios han sugerido que los herbívoros especialistas han evolucionado mecanismos que les permiten sobreponerse a la defensa de las plantas (Shonle & Bergelson, 2000; Rausher, 2001; Kliebenstein *et al.*, 2002; Lankau, 2007); incluso se ha propuesto que podrían estar utilizando estos atributos para elegir entre plantas (Nieminen *et al.*, 2003; Bidart-Bouzat & Kliebenstein, 2008; Ali & Agrawal, 2012). Por ello, a lo largo de una región geográfica los atributos defensivos de una especie vegetal podrían estar sujetos a presiones selectivas contrastantes (Lankau, 2007). Esta selección variable espacialmente sería la "materia prima" necesaria para que ocurra la diferenciación poblacional en la interacción planta-herbívoro, promoviendo diferenciación fenotípica y variación espacial en los patrones de adaptación local entre especies

interactuantes (Thompson, 1994; Thompson, 2005). Así, investigar la variación geográfica de la interacción planta-herbívoro es de suma importancia para entender la evolución de las especies interactuantes (Muola, 2010) y para ayudarnos a explicar la gran variación en atributos defensivos que se observa en la naturaleza (Ali & Agrawal, 2012).

En este contexto esta tesis tuvo como objetivo estudiar a una escala geográfica (múltiples poblaciones), los atributos defensivos que median la interacción planta-herbívoro entre la hierba anual *Datura stramonium* y sus herbívoros en el centro de México. Para ello en el capítulo I estudié la asociación geográfica del daño foliar por herbívoros con la defensa química y física en 28 poblaciones de *D. stramonium*. En el capítulo II comparé si las presiones selectivas ejercidas por herbívoros generalistas y especialistas sobre atributos defensivos difieren significativamente en sentido y magnitud. El capítulo III evalué la existencia de adaptación local de *D. stramonium* a herbívoros generalistas y especialistas, así como a qué grado esta adaptación local es mediada por la defensa física de la planta. Finalmente en el capítulo IV exploré la evidencia de diferenciación adaptativa en atributos físicos y químicos en 13 poblaciones de *D. stramonium* en el centro de México.

Capítulo I

Asociación geográfica y variación temporal de la defensa química y física y el daño foliar en Datura stramonium

ORIGINAL ARTICLE

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Geographic association and temporal variation of chemical and physical defense and leaf damage in *Datura stramonium*

Abstract The evolution of plant defense traits has traditionally been explained trough the "coevolutionary arms race" between plants and herbivores. According to this, specialist herbivores have evolved to cope effectively with the defensive traits of their host plants and may even use them as a cue for host location. We analyzed the geographic association between leaf trichomes, two tropane alkaloids (putative resistance traits), and leaf damage by herbivores in 28 populations of *Datura stramonium* in central Mexico. Since the specialist leaf beetles *Epitrix parvula* and *Lema trilineata* are the main herbivores of *D. stramonium* in central Mexico, we predicted a positive association between plant defense and leaf damage across populations. Also, if physical environmental conditions (temperature or precipitation) constrain the expression of plant defense, then the geographic variation in leaf damage should be explained partially by the interaction between defensive traits and environmental factors. Furthermore, we studied the temporal and spatial variation in leaf trichome density and leaf damage in five selected populations of *D. stramonium* sampled in two periods (1997 vs. 2007). We found a positive association between leaf trichomes density and atropine concentration with leaf damage across populations. The interaction between defensive traits and water availability in each locality had a significant effect on the geographic variation in leaf damage. Differences among populations in leaf trichome density are maintained over time. Our results indicate that local plant–herbivore interaction plays an important role in shaping the geographic and temporal variation in plant defense in *D. stramonium*.

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Introduction

Plants have evolved a vast array of defensive traits that prevent/reduce damage by herbivores (Rausher 2001; Núñez-Farfán et al. 2007). These defensive traits include thorns, spines, trichomes, and the so-called secondary chemical compounds like cyanogenic glycosides, cardenolides, or tropane alkaloids, among others (Ehrlich and Raven 1964; Berenbaum 1981; Mauricio and Rausher 1997; Ballhorn et al. 2009). The evolution of plant defenses and counter-defense traits by herbivores has traditionally been explained trough the "coevolutionary arms race" between plants and herbivores (Ehrlich and Raven 1964; Dawkins and Krebs 1979; Janzen 1980). Therefore, damage to plants exerted by herbivores is expected to reduce plant fitness components like growth rate, survival, and reproductive success (Strauss 1991).

In turn, plant resistance traits that prevent or limit damage negatively affect herbivores' performance (Duffey and Isman 1981; Van Dam et al. 2000).

However, plant populations are commonly distributed across wide geographic areas facing a diverse array of herbivores, ranging from specialists (i.e., that feed upon a restricted group of plants) to generalists (i.e., those that feed upon several unrelated plant species). It has been proposed that generalist herbivores are effectively deterred by plant defenses whereas specialist herbivores are adapted and have evolved to cope effectively with the resistance traits of their preferred host plants (Rausher 2001; Cornell and Hawkins 2003). Furthermore, in many instances specialist herbivores have even evolved the ability to use these defensive traits as a cue to find their host plants (Dobler et al. 2011). Thus, at the geographic scale, the outcome of plant–herbivore interactions is likely to vary across populations as a function of the expression of defensive traits by local plants (e.g., related to genetic variance in defense, the environment, and their interaction; see Fornoni et al. 2004), and the degree of specialization and adaptation of herbivores (Van der Meijden 1996; Lankau 2007; Garrido et al. 2012). Moreover, constant selective pressure of herbivores on defensive traits along time within populations may lead to a stable geographic structure in defense traits (Nuismer et al. 2000).

Simultaneous analysis of multiple populations and temporal dynamics is needed in order to fully understand the variability in the ecological outcome of antagonistic interactions (Thompson and Fernandez 2006). Although numerous studies have demonstrated adaptive processes occurring within populations, much less evidence exists regarding how geographic variation in herbivory and plant defense traits is affected by ecological interactions within populations.

We studied the spatial variation in herbivory and defensive traits in 28 natural populations of the annual plant *Datura stramonium* across central Mexico (Brummitt & Powell): L., where it is consumed mainly by two specialist herbivores, the chrysomelid beetles *Epitrix parvula* and *Lema trilineata*, and on occasion by the generalist grasshopper *Sphenarium purpurascens* (Núñez-Farfán and Dirzo 1994). *D. stramonium* is a ruderal plant widely distributed in Mexico. Because of its broad geographic distribution, *D. stramonium* faces diverse biotic and abiotic conditions, constituting an ideal system for the study of the evolution of plant defensive in a geographical context (Thompson 1999).

Previous studies of *Datura stramonium* indicate that leaf trichomes reduce damage by herbivores. For instance, Valverde et al. (2001) found that leaf trichome density is a component of resistance against herbivory in *D. stramonium*, but the role of trichomes as a defensive character differs among populations in Central Mexico (i.e., spatial variation of selection on plant resistance). Recently, Kariñho-Betancourt (2009) found genetic variation and positive directional selection on leaf trichome density in *Datura stramonium* indicating its potential evolutionary response to variation in this character. Likewise, species in the genus *Datura* are known for the production of tropane alkaloids

(Andersson et al. 2008), that affect the activity of acetylcholine (Brown and Taylor 2006) impairing insects' performance (Wink and Latz-Brüning 1994; Shonle 1999; Alves et al. 2007). Hyoscyamine and scopolamine are the two main alkaloids of *D. stramonium* (Shonle and Bergelson 2000), and atropine is formed by racemization. Atropine has the same pharmacological properties of hyoscyamine, but requires twice the dosage to achieve the same effect (Alexander et al. 2008). In a population of *D. stramonium* from Indiana USA, genetic variation of and natural selection on alkaloid concentration (scopolamine and hyoscyamine) has been detected (Shonle and Bergelson 2000). Directional phenotypic selection to reduce scopolamine and stabilizing selection for hyoscyamine concentration were detected despite low damage imposed by herbivores (ca. 1 % of total leaf area loss on average) (Shonle and Bergelson 2000). Furthermore, no genetic variance for resistance (1-relative damage) to *Epitrix parvula* in *D. stramonium* has been detected in a natural population from Mexico (Núñez-Farfán and Dirzo 1994). Variation in resistance can be partially attributed to the genetic load imposed by inbreeding in *D. stramonium* (Bello-Bedoy and Núñez-Farfán 2010, 2011).

Since tropane alkaloids and leaf trichomes are components of resistance to herbivores in *D. stramonium*, we hypothesized that the among-population variation in these resistance traits would be related to the level of leaf damage exerted by herbivores. Considering that *D. stramonium* is commonly consumed by its main specialist herbivores, *Epitrix parvula* and *Lema trilineata* (Coleoptera: Chrysomelidae), we predict a positive association between plant defense expression and leaf damage across populations. Further, if physical environmental conditions (temperature or precipitation) act as a selection agent or constrain the expression of defensive attributes, then the geographic variation in leaf damage should be partially explained by the interaction between defensive traits and environmental factors.

In order to assess the temporal and among-population variation in the relationship between trichome density and leaf damage, we studied five selected populations of *D. stramonium* that were previously analyzed for the same traits (see Valverde et al. 2001).

Our goals were to (1) analyze geographic variation in defensive characters (leaf trichome density, and tropane alkaloid concentration, atropine and scopolamine) and leaf damage imposed by herbivores; (2) assess the relationship between leaf damage, defensive traits, and environmental variables, and (3) analyze the temporal variation in leaf trichome density and leaf damage for a set of populations sampled 10 years apart.

Method

Study system

Datura stramonium L. (Solanaceae), known as jimsonweed, is a widely distributed annual herb that grows

along roadsides and disturbed environments in Mexico, USA, Canada, and Europe (Weaver et al. 1985; Núñez-Farfán and Dirzo 1994; Shonle and Bergelson 2000; Valverde et al. 2001). In central Mexico, *D. stramonium* is consumed mainly by the specialist leaf beetles *Lema trilineata* and *Epitrix parvula*. In some populations of *D. stramonium*, the generalist grasshopper *Sphenarium purpurascens* (Orthoptera: Pyrgomorphidae) can also occasionally consume jimsonweed leaves (Núñez-Farfán and Dirzo 1994). A full description of the plant, insects, and leaf damage type produced by folivores can be found elsewhere (Núñez-Farfán and Dirzo 1994). Previous studies in *D. stramonium* have shown that leaf damage caused by these insects reduces plant fitness, and that alkaloids and leaf trichomes are can serve both as defensive traits and feeding stimulants to herbivores (Shonle and Bergelson 2000; Valverde et al. 2001; Kariñho-Betancourt 2009; Bello-Bedoy and Núñez-Farfán 2011).

Sampling

In September–October 2007, we sampled 28 populations of *D. stramonium* in central and eastern Mexico (Fig. 1) over a wide range of habitat types occupied by this species (Hernández-Cumplido 2009). The geographic location and climatic characteristics of each locality are summarized in Table S1. In order to measure the extent of damage by herbivores in each population, ten randomly chosen plants of *D. stramonium* were sampled to estimate the population mean of leaf trichome density, concentration of two tropane alkaloids (atropine and scopolamine), and the proportion of leaf damage by herbivores. From each individual, 20 randomly selected leaves (including the petiole) were harvested, extended horizontally on paper sheets, labeled, and kept flat using a botanical press. Pressed leaves were dried at ambient temperature in the glasshouse for further analysis in the laboratory (leaf area, trichome density, and tropane alkaloids). All individuals were sampled during the reproductive stage to avoid bias due to plant age (Avery et al. 1959; Núñez-Farfán 1991). Additionally, we counted the number of branches per individual in order to obtain mean plant size per population as an estimator of each population's productivity (Bello-Bedoy and Núñez-Farfán 2010).

Leaf damage and trichome density

The proportion of damage by herbivores per plant (p_i) was estimated by dividing the damaged leaf area (DLAi) by estimated total leaf area (TLAi) in a sample of ten randomly chosen leaves. Total leaf area was estimated using a regression analysis of leaf area as a function of leaf length following (Núñez-Farfán and Dirzo 1994; Valverde et al. 2001), using a sample of undamaged leaves. Since leaf shape and area differed slightly among populations, we used a different equation for each population (R^2 ranging from 0.887 to 0.954, $p < 0.001$, $n \geq 30$). The remaining leaf area was measured using a Win-Dias image analyzer (Delta-T Devices Ltd., Cambridge, UK).

Leaf trichome density per plant was obtained from the same sample of leaves used to estimate proportion of leaf damage by herbivores. Average leaf trichome density was estimated by counting the trichomes in five observation fields of 2.5 mm^2 using a dissecting microscope. Observation fields were located at different

Fig. 1 Central and eastern Mexico map showing *Datura stramonium* populations studied during September–October 2007
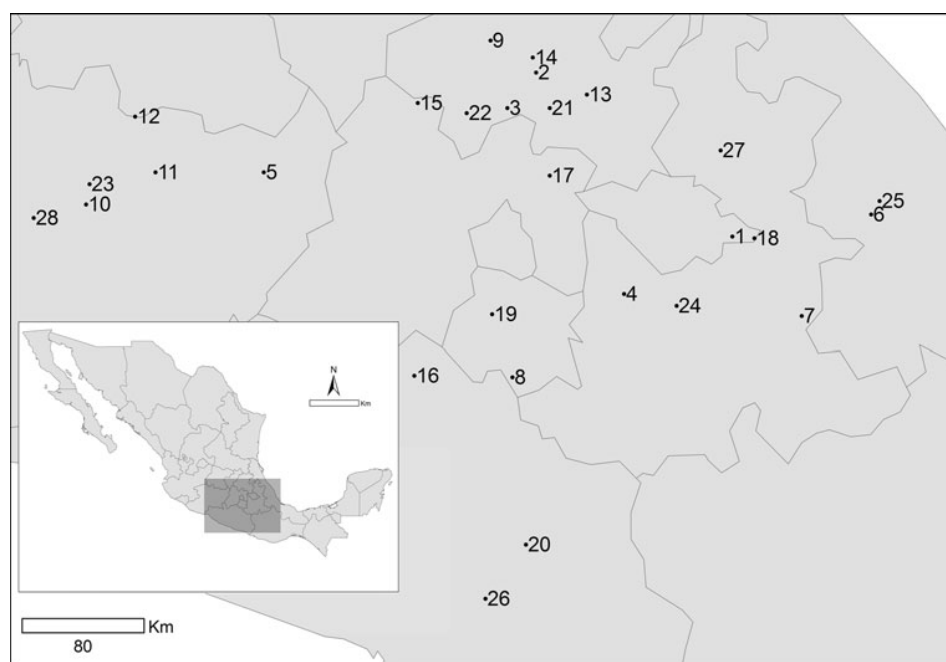
regions in the adaxial side of the leaf in order to account for spatial variation within the leaf: (1) Basal central area, (2) bottom right edge, (3) lower left edge, (4) top right edge, and (5) upper left edge.

Alkaloid concentration

For each plant, we quantified the two most abundant alkaloids of *D. stramonium* (atropine and scopolamine) by high-performance liquid chromatography (HPLC). Dried leaves were macerated and maintained for 12 h in 20 ml of methanol (MeOH). The supernatant was filtered and MeOH was evaporated completely at 60 °C. Subsequently, 10 ml of hydrochloric acid (HCl) 0.1 N was added and rinsed twice with 10 ml of chloroform ($CHCl_3$), recovering the aqueous phase. HCl was neutralized with 0.8166 g of sodium bicarbonate ($NaHCO_3$) and rinsed twice with 10 ml of $CHCl_3$, and this time the organic phase was recovered. Finally, chloroform was evaporated to dryness at 65 °C. The dried samples were re-suspended in 1 ml of methanol before being injected into the HPLC. The samples were injected into HP/Agilent 1100 HPLC equipment with a reverse phase column Discovery C-18 (Supelco Analytical) at 23 °C. The injection volume was 30 μl and the flow rate was 1 ml/min. Following Kursinszki et al. (2005), the mobile phase was a solution of acetonitrile, methanol, and a 30 mM phosphate buffer, pH 6.00 (12:7.9:80.1 v/v/v). DAD detector was used at a wavelength of 210 nm. The curves obtained in each run were compared with a standard solution of atropine and scopolamine (Sigma-Aldrich, St. Louis, MO, USA) 1 mg/ml. Mean population alkaloid concentration was estimated averaging ten plants per population. Concentrations are expressed in mg/g.

Environmental variables

Mean annual temperature and precipitation of each locality of *D. stramonium* were obtained from the Worldclim database (Hijmans and Graham 2006). For each population, Lang's aridity index (Rehman 2010) was calculated by dividing population mean annual rainfall by mean annual temperature, obtaining values close to zero in arid locations and high values in humid locations (Oliver 2005).

Data analysis

To detect differences in leaf trichome density, alkaloid concentration, and leaf damage among populations, we performed a one-way MANOVA. Subsequently, univariate one-way ANOVAs were performed for each response variable independently. Prior to statistical analyses, trichome density was square root transformed, the proportion of leaf damage was arcsine transformed, and the alkaloid concentration was log-transformed (Sokal and Rohlf 1995). Statistical analyses were performed with JMP statistical package v9.0 (SAS, 2010).

To assess whether the amount of plant damage exerted by herbivores in each population was associated with geographic variation in mean trichome density, mean alkaloid concentration, population plant vigor (mean plant size), and physical environmental conditions (Lang's index), a multiple regression analysis was performed. Due to sample-size limitations, the model included only second-order interactions. In addition, we evaluated the correlation between predictor variables with Pearson correlations.

Spatial and temporal variation in trichome density and foliar damage

To evaluate the spatial and temporal variation in leaf trichome density and leaf damage by herbivores, we compared the data collected in 1997 by Valverde et al. (2001) and 2007 for the same populations (see Valverde et al. 2001 for further details on data collection and sample size in 1997). The included populations were Actopan, Patria Nueva, Teotihuacán, Ticumán, and Zirahuén. We assessed spatial and temporal variation in trichome density and leaf damage by means of ANOVA that included the term year of collection (1997 vs. 2007), population, and their interaction as predictor variables. We also assessed whether the relationship between trichome density and leaf damage changed spatially and temporally (1997 vs. 2007 collections) by ANCOVA. This model included year, population, and trichome density as a covariate; leaf damage was the response variable. Whenever population or year was significant, a Tukey–Kramer LSD post hoc test was conducted to determine which means differed. For the analyses, we estimated leaf trichome density and leaf damage following the same methods described in Valverde et al. (2001). Trichome density was estimated as the total number of trichomes within an area of 2.5 mm^2 on the basal central area of the adaxial side of the leaf using a dissecting microscope. The proportion of leaf damage was estimated as explained above. Sample sizes ranged from 10 to 20 plants per population.

Results

Among-population variation in leaf damage and defense traits

One-way MANOVA revealed a significant multivariate effect for the term population (Wilks' $\lambda = 0.033$, $F = 10.4$, $df = 108, 828.22$, $p < 0.0001$). Given the significance of the overall test, univariate ANOVAs for each variable were performed. The average proportion of leaf area damaged by herbivores was 22 ± 12 % (SD) with a high coefficient of variation (CV) of 54.96 %. ANOVA showed significant differences among populations in leaf damage ($F = 10.44$, $df = 27$, $p < 0.0001$)

(Fig. 2a). The average leaf trichome density ($\pm$SD) was 7.28 $\pm$ 1.95 trichomes (in 2.5 mm^2) with a coefficient of variation of 26.87 %. ANOVA detected significant differences in the leaf trichome density among populations ($F = 6.56$, $df = 27$, $p < 0.0001$), (Fig. 2b). Furthermore, mean atropine concentration ($\pm$SD) was 0.78 $\pm$ 0.67 mg/g with a coefficient of variation of 86.31 %, and mean scopolamine concentration was 0.89 $\pm$ 0.75 mg/g with a coefficient of variation of 84.59 %. ANOVA showed significant differences among populations for both atropine ($F = 6.91$, $df = 27$, $p < 0.0001$) and scopolamine ($F = 16.14$, $df = 27$, $p < 0.0001$) (Fig. 2c). Finally, we found significant differences among populations on average population plant size (number of branches) ($F = 6.71$, $df = 27$, $p < 0.0001$). Average population plant size was 14.49 $\pm$ 6.9.

Geographic patterns in defense

The multiple regression model was significant ($F_{21} = 5.69$, $p = 0.0065$); the included factors explained 60 % of variance in the mean proportion of leaf damage among populations. Significant effects of leaf trichome density and atropine concentration were detected. The interactions trichome density $\times$ atropine (Fig. 3a), atropine $\times$ Lang's index (Figure S2a), and trichome density $\times$ Lang's index (Fig. 3b) were also significant. Finally, interactions between atropine $\times$ plant size (Fig. 3c), scopolamine $\times$ plant size (Figure S2b), and trichome density $\times$ plant size (Fig. 3d) were also significant (Table 1). We found significant correlations between factors, however these were generally low (Table S3).

Spatial and temporal variation in foliar damage and trichome density

We found significant differences among populations and years (1997 vs. 2007) in the proportion of leaf damage on plants of *D. stramonium*. A significant effect of the population $\times$ year interaction was also detected (Table 2a). Average leaf damage by herbivores was significantly higher in 2007 than in 1997 for all five populations (Fig. 4a). Similarly, significant differences among populations and between years in leaf trichome density were detected. Moreover, population $\times$ year interaction was significant (Table 2b). In the population of Actopan, leaf trichome density was significantly lower in 2007 than 1997 (Fig. 4b).
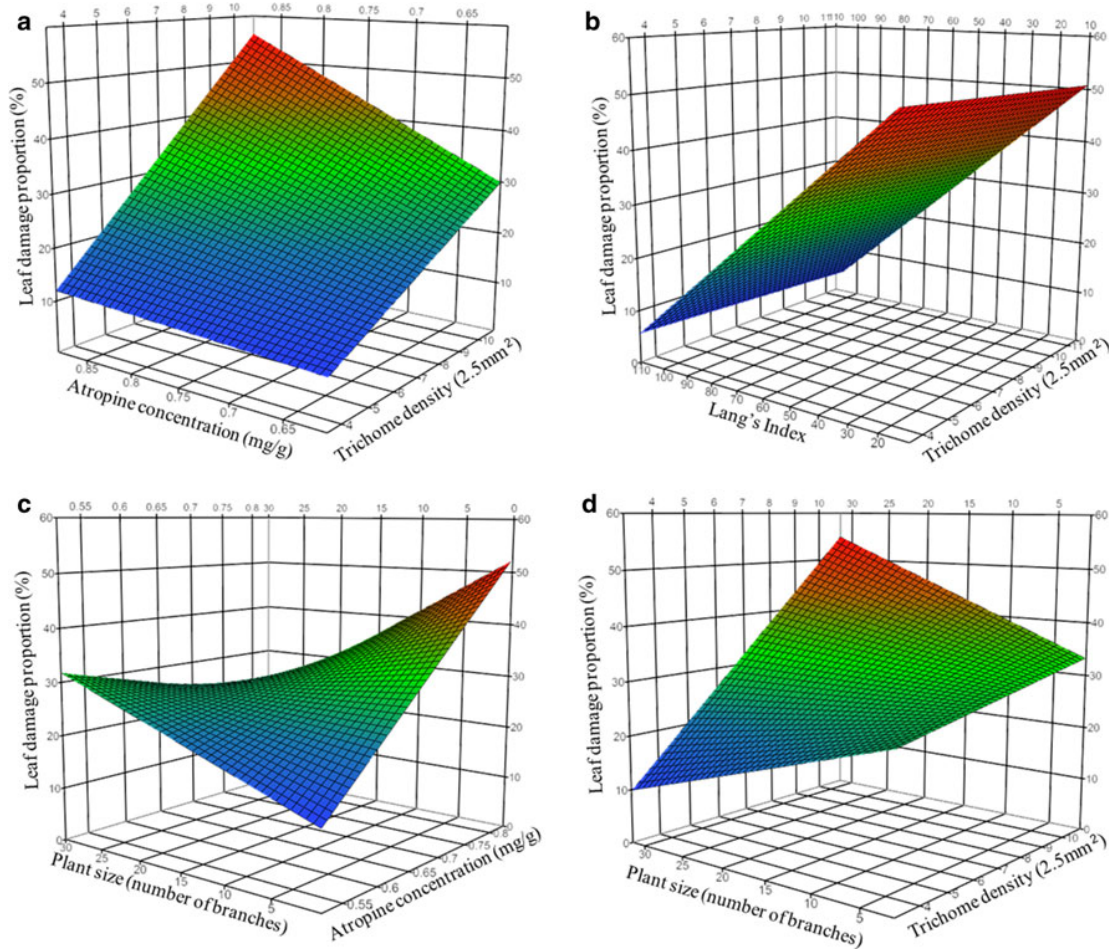
Fig. 3 Interaction surfaces between predictor variables resulting from the multiple regression model. **a** Leaf trichome density (in 2.5 mm^2) × leaf scopolamine concentration (mg/g), **b** leaf trichome density × mean population plant size, **c** leaf atropine concentra-tion × mean population plant size, and **d** leaf trichome density × Lang's Index. Response variable in the four panels was the proportion leaf damage

The ANCOVA analysis, again, detected differences among populations and years, and an effect of trichome density on leaf damage only in interaction with population. The population × trichome density and population × year × trichome density interactions were also statistically significant (Table 3).

Discussion

Datura stramonium displays high among-population variation in leaf damage by herbivores and defensive traits. We detected a geographic association between defensive characters, environmental factors, and leaf damage. According to our expectation, multiple regression analysis detected a positive significant effect of trichome density and atropine concentration on leaf damage, indicating that populations that showed high leaf trichome density also received high levels of leaf damage. Since *D. stramonium* is primarily consumed by

the two specialists, *Epitrix parvula* and *Lema trilineata*, our results suggest that, at least for these two herbivores, defensive characters do not prevent or reduce herbivory, but rather, that these specialist herbivores are adapted to the defenses of *D. stramonium*. Thus, higher investment in defensive characters would not reduce damage by herbivores since herbivores are able to overcome the barriers that probably functioned to prevent or diminish their attack in the past (Janzen 1980; Berenbaum et al. 1986; Bowers and Puttick 1988; Zangerl and Berenbaum 1993; Lively et al. 2004). Herbivores of *D. stramonium* may have temporarily "escaped" from the "arms race" in some populations (Hanifin et al. 2008).

Furthermore, specialist herbivores may use tropane alkaloids as cues for plant location and perhaps as a defense against parasitoids (Agrawal and Heil 2012). In *D. stramonium*, Shonle and Bergelson (2000) have previously shown that scopolamine can act as a phago-stimulant for the specialist flea beetle *Epitrix parvula*. Recently, Castillo et al. (in preparation) found a positive

Table 1 Multiple regression analysis of the average proportion of leaf damage by herbivores in different populations of *Datura stramonium* from central Mexico

Source of variation	Estimate	SE	t	p
Intercept	−0.236621	0.347096	−0.68	0.5084
Trichome density	0.076559	0.023581	3.25	**0.007**
Lang's index	−0.003599	0.003108	−1.16	0.2694
Scopolamine	−0.770713	0.963115	−0.8	0.4391
Atropine	5.638847	1.159169	4.86	**0.0004**
Plant size	−0.002734	0.012347	−0.22	0.8285
Trichome density × Lang's index	0.002437	0.000782	3.12	**0.0089**
Trichome density × scopolamine	0.646523	0.41855	1.54	0.1484
Trichome density × atropine	1.172646	0.43811	2.68	**0.0202**
Trichome density × plant size	0.012043	0.004463	2.7	**0.0194**
Scopolamine × Lang's index	0.031263	0.037723	0.83	0.4234
Atropine × Lang's index	−0.210931	0.062216	−3.39	**0.0054**
Plant size × Lang's index	0.000552	0.000539	1.03	0.3255
Scopolamine × atropine	18.2762	13.38017	1.37	0.197
Scopolamine × plant size	−0.394301	0.147689	−2.67	**0.0204**
Atropine × plant size	−0.472894	0.176812	−2.67	**0.0202**

p-values equal to or lower than 0.05 are shown in bold type
Besides population, the model included alkaloids concentration, trichome density, and Lang's index as predictor variables

Table 2 ANOVA of (a) the proportion of leaf damage and (b) leaf trichome density among 28 populations of *Datura stramonium* in 1997 and 2007

Source	df	S. S.	M.S.	F	p	R^2
(a) Proportion of leaf damage by herbivores						
Population	4	1.2687299	0.317182	44.5745	**< 0.0001**	0.89
Year	1	6.0783616	6.078362	854.2088	**< 0.0001**	
Population × year	4	0.8523307	0.213083	29.9451	**< 0.0001**	
Error	138	0.9819776	0.007116			
Total	147	9.6089484				
(b) Leaf trichome density						
Population	4	40.746443	10.18661	54.5497	**< 0.0001**	0.69
Year	1	0.936728	0.93673	5.0162	**0.0267**	
Population × year	4	9.539837	2.38496	12.7715	**< 0.0001**	
Error	138	25.770142				
Total	147	91.302568				

p-values equal to or lower than 0.05 are shown in bold type

relationship between atropine concentration and herbivory by the specialists *Lema trilineata* and *Epitrix parvula* in four and two, respectively, *D. stramonium* populations. However, this specialist herbivore is absent in some populations of *D. stramonium*, which may give rise to differences in leaf damage (Andraca-Gómez 2009). In these populations, generalist herbivores like *Sphenarium purpurascens* are likely to be the main consumers of jimsonweed (G. Castillo and J. Núñez-Farfán, pers. obs.). If specialist and generalist herbivores respond differently to the plants' defensive traits they may impose differential/contrasting selection on plant defense (Lankau 2007).

Previous studies in *D. stramonium* point out that the specificity and abundance of herbivores is a key aspect determining the outcome of the plant–herbivore interaction. In some populations, tolerance, rather than resistance to herbivores may be favored by selection, and standing levels of damage may be uncoupled from defensive attributes (Fornoni et al. 2004; Carmona and Fornoni 2013). Unfortunately, we were not able to separate leaf damage according feeding habit of the herbivores. Future studies must analyze explicitly the relationship between plant defense and specialization of herbivores at the local population level.

Multiple regression analysis also found that populations with higher atropine concentration and increased trichome density received more leaf damage. This supports the idea that multiple-trait defense is more common compared to single-trait defense (Paul and Hay 1986; Hartmann and Dierich 1998; Agrawal and Fishbein 2006), pointing that defense in *D. stramonium* is composed of multiple traits. Moreover, trichome density interacts with plant vigor, an indirect estimator of habitat's productivity. We found high levels of herbivory in populations of *D. stramonium* with high trichome density and high productivity (in terms of average plant size). These results are consistent with the hypothesis of Hochberg and Van Baalen (1998), which predicts that both plant defense and the impact of herbivores will be maximized in highly productive populations (due to the positive effect of plant productivity on herbivores). However, the interaction of atropine and scopolamine with plant vigor indicates that populations with higher
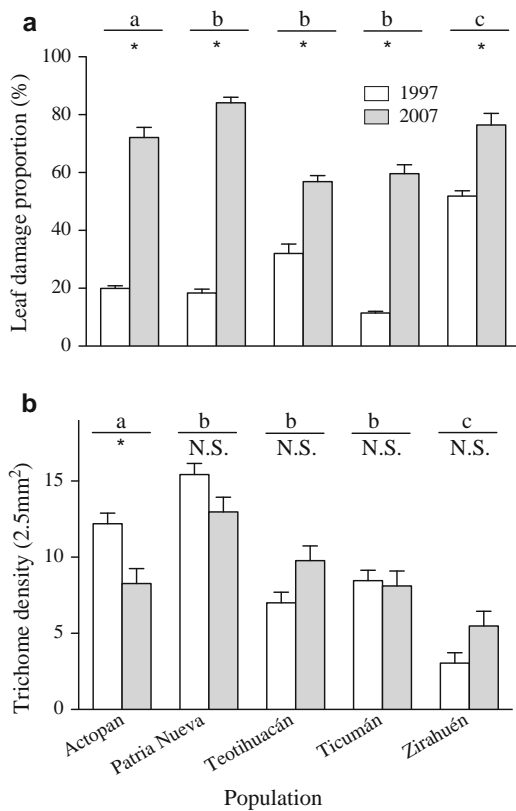
Fig. 4 Spatial and temporal among population variation in **a** mean leaf trichome density and **b** mean leaf damage in five populations of *Datura stramonium* collected in 1997 and 2007. *Different letters* indicate significant differences among populations and *asterisks* indicate significant differences between years of collection at $p > 0.05$, following Tukey–Kramer LSD post hoc test. *Error bars* represent 1 SE

leaf damage were those with low productivity and high concentrations of both alkaloids. This contrast with predictions of Hochberg and Van Baalen (1998) but is consistent with the geographic pattern predicted when herbivores overcome plant defenses. Furthermore, these patterns may reflect the costs associated with alkaloid production (Shonle and Bergelson 2000).

Finally, we detected a significant interaction of Lang's index with both trichome density and atropine concentration. Plant populations in drier habitats with high atropine concentration and trichome density showed higher levels of leaf damage. These patterns are consistent with the positive geographic association between plant defense traits and herbivory, but suggest that the interaction of water availability with alkaloids is different than its interaction with trichomes. Both leaf trichomes and alkaloid concentration are likely to be influenced by water availability (Wilkens et al. 1996; Baricevic et al. 1999; Gonzáles et al. 2008), but in opposite directions. High leaf trichome density has been found to reduce water loss indirectly by increasing light reflectance in dry environments (Ehleringer 1984; Rotondi et al. 2003), favoring higher densities in more arid environments, whereas alkaloid production may water-limited, potentially decreasing alkaloid concentration in dry habitats (Baricevic et al. 1999).

Overall, differentiation among populations of *D. stramonium* in trichome density is maintained over time (in an interval of 10 years). Leaf trichome density differed between years only in the Actopan population, suggesting that despite environmental variation over time, local adaptive processes can promote and maintain population differences in trichome density (Kawecki and Ebert 2004; Siepielski et al. 2009). Leaf damage experienced in all five populations was higher in 2007 than in 1997, whereas trichome density remained stable in most populations. This may reflect that 10 years is not enough time to plant populations to adapt, and suggest the existence of environmental and/or genetic correlations with others traits, which might limit the evolutionary response of trichome density (Lande and Arnold 1983; Merilä et al. 2001; Wilson et al. 2006).

Furthermore, the relationship between trichome density and leaf damage changed among populations and time. Among-population differences in the relationship between trichomes and leaf damage may promote geographic structuring of the plant–herbivore interactions. Nevertheless, since the relationship between defensive traits and damage by herbivores changes over time, it is unlikely that such a structure remains stable, and thus populations may not attain an optimal leaf trichome density at each locality (Grant and Grant 2002). Our results suggest that the defensive role of tri-

Table 3 ANCOVA leaf damage proportion by herbivores among populations of *Datura stramonium* between 1997 and 2007

Source	df	S. S.	M.S.	F	p	R^2
Population	4	6.949429	1.73736	11.2851	**< 0.0001**	0.86
Year	1	15.845978	15.84598	102.9283	**< 0.0001**	
Trichome density	1	0.41272	0.41272	2.6808	0.104	
Population × year	4	3.678313	0.91958	5.9732	**0.0002**	
Population × trichome density	4	2.55901	0.63975	4.1555	**0.0034**	
Year × trichome density	1	0.060727	0.06073	0.3945	0.5311	
Population × year × trichome density	4	3.681963	0.92049	5.9791	**0.0002**	
Error	128	19.70581	0.15395			
Total	147	148				

p-values equal to or lower than 0.05 are shown in bold type

Leaf trichome density was used as a covariate

chome density changes over time as reported for other traits that mediate interactions (Siepielski et al. 2009). However, it is necessary to explicitly assess the consistency of the role of leaf trichome density as a defense in *D. stramonium*, in the face of spatio-temporal variation in the herbivore community.

Altogether, our results suggest that plant–herbivore interactions occurring at a local scale may have an important role in the shaping of the geographic and temporal variation of leaf damage by and plant defense to herbivores in *Datura stramonium*. However, further studies are needed to explicitly evaluate the role of natural selection imposed by herbivores in producing among-population variation in chemical and physical defensive traits, as well as the role of specialist vs. generalist herbivores as agents that give rise to the selection mosaic in *D. stramonium*. Accomplishing this will increase our knowledge of the evolution of plant–herbivore interactions at a large geographic scale.

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Capítulo II

Mosaico selectivo ejercido por herbívoros especialistas y generalistas en la defensa química y física de *Datura stramonium*

Selection Mosaic Exerted by Specialist and Generalist Herbivores on Chemical and Physical Defense of *Datura stramonium*

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Abstract

Selection exerted by herbivores is a major force driving the evolution of plant defensive characters such as leaf trichomes or secondary metabolites. However, plant defense expression is highly variable among populations and identifying the sources of this variation remains a major challenge. Plant populations are often distributed across broad geographic ranges and are exposed to different herbivore communities, ranging from generalists (that feed on diverse plant species) to specialists (that feed on a restricted group of plants). We studied eight populations of the plant *Datura stramonium* usually eaten by specialist or generalist herbivores, in order to examine whether the pattern of phenotypic selection on secondary compounds (atropine and scopolamine) and a physical defense (trichome density) can explain geographic variation in these traits. Following co-evolutionary theory, we evaluated whether a more derived alkaloid (scopolamine) confers higher fitness benefits than its precursor (atropine), and whether this effect differs between specialist and generalist herbivores. Our results showed consistent directional selection in almost all populations and herbivores to reduce the concentration of atropine. The most derived alkaloid (scopolamine) was favored in only one of the populations, which is dominated by a generalist herbivore. In general, the patterns of selection support the existence of a selection mosaic and accounts for the positive correlation observed between atropine concentration and plant damage by herbivores recorded in previous studies.

Introduction

Coevolution, the reciprocal evolutionary change between interacting species, has been considered a key process in the evolution of both plants and their natural enemies [1–4]. In particular, it has been used to explain the evolution of the great diversity of defensive traits in plants, such as trichomes, spines, resins, or secondary metabolites [5,6]. This theory assumes that herbivores exert selective pressures on traits that reduce herbivore damage [4,7]. Given that herbivory generally decreases plant fitness, natural selection is expected to favor high levels of these defensive traits [8–10].

Nonetheless, plant populations are often distributed along wide geographic areas, and are thus exposed to different herbivore communities, ranging from generalists (which feed upon a wide diversity of hosts), to specialists (which feed on a related group of species) [11–13]. It has been hypothesized that defensive traits are an effective barrier against generalist herbivores, because these herbivores can feed on alternative plants, (but see [14]). On the other hand, several studies have suggested that specialist herbi-

vores have evolved mechanisms to overcome host defenses [6,12,15,16]. Moreover, specialist herbivores may be able to identify their hosts based on defensive traits such as secondary metabolites, imposing negative selection on these traits [11,17,18]. Thus, along the distribution of a plant species, defensive traits such as trichomes and secondary metabolites may be under contrasting selective pressures arising from multiple interacting species [19]. Such spatially variable selection is expected to change the population mean of traits, promoting population differentiation in defensive traits [20–22]. Although there is much evidence of selection on plant defenses, there is less evidence regarding spatially variable selection by herbivores on defensive traits (see [23,24]). Furthermore, few studies have explicitly evaluated whether specialist and generalist herbivores exert different selection pressures on defensive traits [12].

Datura stramonium (Solanaceae) is an ideal system for studying variable selection patterns acting on defensive traits at a geographic scale. It typically grows in disturbed and agricultural habitats in Mexico, Canada, and the United States [25–27]. Due to its wide distribution, *D. stramonium* is exposed to a wide variety

of herbivores and diverse environmental conditions. Most Mexican populations of *D. stramonium* are attacked by the specialist herbivore *Lema daturaphila* [28]. However, there are populations where *L. daturaphila* is absent, and where the main herbivores are *Epitrix parvula*, (specialist herbivore of the Solanaceae family) [29], and the generalist *Sphenarium purpurascens* (Núñez-Farfán and Guillermo Castillo, pers. obs.). Specifically, *D. stramonium* features leaf trichomes and tropane alkaloids as defensive traits that prevent herbivory [16,26]. Previous studies have documented that these traits can evolve by selection by herbivores [16,30]. Atropine is the substrate alkaloid used to produce the derived and more toxic scopolamine [31]. Recently, Castillo et al. [32] found a positive geographic association between atropine concentration and leaf damage across 28 *D. stramonium* populations in central Mexico, suggesting that atropine may not be an effective deterrent against herbivory. However, it is unclear whether selection exerted by generalist and/or specialist herbivores of *D. stramonium* drive this pattern. Specialized herbivores are expected to promote a more intense coevolutionary dynamic with the host plant, as they are more likely to adapt to the host chemical and physical barriers, imposing strong selection, and promoting counter-resistance host response [33–35]. Thus, while atropine may be less effective against herbivores than scopolamine [36], the benefits of tropane alkaloids should be higher against generalist rather than specialist herbivores.

Here, we evaluated whether selection imposed by specialized and/or generalized herbivores on plant defenses matches the among-population variation in defensive traits of *D. stramonium* recorded in a previous study [32]. To do so, we performed phenotypic selection analyses to explore the mode and intensity of selection acting on chemical (atropine, scopolamine) and physical (leaf trichomes) defensive traits in eight populations of *D. stramonium* attacked mainly by generalist or specialist herbivores. Next, we explored whether higher concentration of the derived alkaloid (scopolamine) is associated with higher plant fitness benefits, and whether selection for this secondary compound is more intense against generalist than specialist herbivores.

Methods

Ethics Statement

No specific permissions were required to make observations and to collect plant material of *D. stramonium* in the locations sampled in this study, nor is this species endangered and protected by the Mexican Government.

Study system

Datura stramonium L. (Solanaceae) is an annual herb commonly distributed in cultivated areas, roadsides and disturbed environments in Mexico, the United States, Canada, and Europe [16,26,28,37,38]. This species reproduces mainly by self-fertilization, and has limited pollen and seed dispersal [39]. Leaves of *D. stramonium* are consumed primarily by the specialist folivorous beetle *Lema daturaphila* [28], the oligophagous flea beetle *Epitrix parvula* (which consumes other members of the Solanaceae family) [29], and the generalist grasshopper *Sphenarium purpurascens* [28]. *Lema daturaphila* damage is characteristic, as both adults and larvae consume the leaf blade while avoiding the main vascular bundles (pers. obs.). *Epitrix parvula* damage consists of small holes on the leaves. Although damage can be severe, whole leaves are rarely totally consumed [28]. Damage exerted by *Sphenarium purpurascens* consists of round-to-ragged holes in the leaves, typically originating from the leaf margins. Although leaf damage can be complete, grasshoppers usually leave partially

defoliated leaves (G. Castillo personal observation). Previous studies have found that leaf damage significantly reduces plant fitness [28,40] and that leaf trichomes and tropane alkaloids are defensive traits against herbivores [16,26,30,32,41].

Sampled Population

From August-September 2011 we sampled eight natural populations of *D. stramonium* in central Mexico (Fig. 1). The sampled populations occurred within different plant communities (see Table S1). The linear distances between populations ranged from 20 to 300 km. In each population we sampled ±30 randomly selected individual plants. From each plant we collected a random sample of 20 leaves, and all the fruits produced. In addition, we recorded the predominant damage type caused by each herbivore feeding on *D. stramonium* in each population. The most frequent herbivore species at each population is listed in Table 1. Our field observations and leaf damage records during 3 years indicate that the predominant herbivores at each population remained stable throughout the 2010–2012 period.

Trichome density and plant fitness

Trichome density was estimated as the total number of trichomes in an observation field of 2.5 mm^2 located in the central basal region of the adaxial side of the leaf [26]. Average trichome density per plant was obtained from a sample of 20 randomly chosen, fully expanded leaves. The mean trichome density for each population was calculated for a sample of approximately 30 individuals. We used total fruit number as a proxy of plant fitness. Since *D. stramonium* is an annual selfing plant, fruit number is a good estimator of lifetime maternal fitness [8].

Tropane alkaloid concentration

We used HPLC to quantify the concentration of atropine and scopolamine (two major alkaloids in *D. stramonium*) from a sample of 20 leaves per plant. The extraction method consisted of a series of acid-base reactions (see [32]). The samples were injected into a Waters Alliance 2695 HPLC device. We used a reverse-phase column (Discovery C-18 Supelco Analytical) at 30°C. The injection volume was 30 μL with a flow rate of 1 mL/min. The mobile phase was a solution of acetonitrile, methanol and a 30 mM phosphate buffer at a pH of 6.00 (9:6.9:84.1, v/v/v). The DAD detector used a wavelength of 210 nm. The curves obtained from each sample were compared against a standard solution of atropine and scopolamine (Sigma-Aldrich Laboratories, 1 mg/mL). The mean alkaloid

Figure 1. *Datura stramonium* populations sampled in Central Mexico (See Table S1).
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Table 1. Selection differentials (S) of trichome density, atropine, and scopolamine concentration in eight populations of *Datura stramonium*.

Population	Main herbivore	Defensive trait	S	SE	t	P
Acolman (N = 31)	*Lema daturaphila*	Trichome density	0.116	0.192	0.604	0.55
		Atropine	**0.416**	**0.182**	**−2.284**	**0.029**
		Scopolamine	−0.089	0.197	−0.456	0.652
Patria Nueva (N = 30)	*Lema daturaphila*	Trichome density	−0.197	0.103	−1.905	0.067
		Atropine	−0.183	0.104	−1.753	0.09
		Scopolamine	**−0.269**	**0.097**	**−2.756**	**0.01**
Joquicingo (N = 31)	*Lema daturaphila*	Trichome density	−0.234	0.184	−1.277	0.211
		Atropine	−0.303	0.187	−1.62	0.116
		Scopolamine	−0.036	0.195	−0.186	0.854
San Martín (N = 29)	*Lema daturaphila*	Trichome density	0.205	0.151	1.36	0.186
		Atropine	**−0.331**	**0.142**	**−2.332**	**0.027**
		Scopolamine	−0.106	0.154	−0.685	0.499
Tzin Tzun Tzan (N = 30)	*Epitrix parvula*	Trichome density	**−0.347**	**0.099**	**−3.488**	**0.001**
		Atropine	−0.151	0.122	−1.233	0.228
		Scopolamine	0.107	0.128	0.837	0.41
Valsequillo (N = 33)	*Epitrix parvula*	Trichome density	−0.051	0.099	−0.552	0.605
		Atropine	**−0.255**	**0.088**	**−2.89**	**0.006**
		Scopolamine	−0.052	0.099	−0.533	0.598
Sanabria (N = 34)	*Sphenarium purpurascens*	Trichome density	−0.188	0.187	−1.004	0.324
		Atropine	−0.228	0.178	−1.281	0.209
		Scopolamine	−0.071	0.182	−0.39	0.699
Santo Domingo (N = 30)	*Sphenarium purpurascens*	Trichome density	−0.083	0.204	−0.409	0.686
		Atropine	**−0.392**	**0.191**	**−2.055**	**0.049**
		Scopolamine	**0.65**	**0.164**	**3.965**	**0.001**

Significant values appear in bold-type fonts. Standard error (SE) of estimates, *t*-test value, and probability (P) are provided.
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concentration (mg/g) per population was estimated from a sample of ±30 plants.

Data analysis

Among-population variation in plant defenses. Before conducting statistical analyses, we assessed among-population differences in defensive traits of the studied eight populations. A multivariate analysis of variance (MANOVA) was performed on leaf damage, trichome density, and concentrations of atropine and scopolamine. These analyses were equivalent to those published elsewhere (see [32]), but applied in this case to our eight selected populations. Our analyses detected significant differences in defensive traits among the eight populations of *D. stramonium* (Wilks' $\lambda = 0.1477$, $F_{28, 848.73} = 30.697$, $P < 0.0001$). Univariate ANOVAs showed significant differences in trichome density ($F_{7, 238} = 7.55$, $P < 0.0001$), atropine ($F_{7, 239} = 2.96$, $P = 0.0053$) and scopolamine concentration ($F_{7, 239} = 4.10$, $P = 0.0003$) (Fig. S1).

Phenotypic selection on defensive traits. Following the Lande and Arnold approach [42], we used multivariate selection analyses to estimate the magnitude and direction of linear and non-linear selection acting on defensive traits for each population. Standardized partial linear selection gradients (β) were obtained by fitting a linear regression that considered relative plant fitness as the response variable and all three defensive traits as predictor variables. Because our sample size precluded the estimation of reliable non-linear selection gradients, only directional selection

gradients are presented here. Defensive traits were standardized ($\mu = 0$, $\sigma^2 = 1$) and fitness was relativized for each population prior to the analyses. Regression analyses were performed using the function *lm* in R 3.0.2 [43].

Before conducting selection analyses, correlations between predictor variables were examined within each population to avoid strong multicollinearity in subsequent analyses. These analyses revealed that concentrations of atropine and scopolamine were positively correlated in six out of eight populations (Table S2). Only in two populations scopolamine was positively correlated with trichome density (Table S2). All other correlations between defensive traits were non-significant (Table S2). Therefore, we estimated selection differentials, as the slope of the univariate regression of population relative fitness on standardized traits [44]. These estimates measure changes in the distribution of a trait due to direct and indirect selection, when traits are correlated.

Differential selection among herbivore species. To assess whether patterns of selection on defensive traits are consistent among populations and herbivores, we estimated effect sizes for each differential and selection gradient. Effect sizes were used to compare estimates of phenotypic selection corresponding to populations consumed by different herbivore species [45]. Because the differentials and selection gradients were obtained from regression models with the same covariance structure, slopes are reliable metrics to estimate effect sizes [46]. Effect sizes were estimated using the slopes and their corresponding variances

(estimated as: $V_\beta = SE_\beta{}^2$) [47] to weight each of them by level of certainty. In order to account for between-population variation, we applied a random-effect model following an Omnibus Test (Q_m) [45]. We concluded that, when confidence intervals around mean effect size did not overlap with zero, a particular species of herbivore exerted a significant effect on the pattern of selection of a focal defensive trait. We used *metafor* [48] from R package to perform the analyses.

Results

Phenotypic selection on defensive traits

Multiple regression analyses revealed significant directional selection acting on defensive traits in five out of the eight studied populations (see Table 1). Trichome density was negatively selected in the Tzin Tzun Tzan population, and positively selected in the San Martín and Santo Domingo populations. Atropine concentration was negatively selected in the Acolman, San Martín and Valsequillo populations, whereas scopolamine concentration was selected positively in the Acolman population (Table 1).

Univariate association between traits and fitness (selection differentials) indicated strong geographic variation in the pattern of selection acting on defensive traits. Secondary metabolites were more responsive than physical defenses in the presence of natural herbivores. Tropane alkaloids had positive, negative or neutral effects on fitness. Atropine concentration was selected against in three populations (San Martín, Santo Domingo and Valsequillo), and positively selected in one population (Acolman) (Table 2). Scopolamine concentration was positively selected in one population (Santo Domingo) and negatively selected in another population (Patria Nueva). Trichome density was negatively selected in one population only (Tzin Tzun Tzan) (Table 2). In two populations (Joquicingo and Sanabria) no evidence was detected of selection on plant defensive traits (Table 2).

Differential selection among herbivore species

Trichome density was selected against in those populations eaten by *E. parvula*, while no consistent pattern was detected for the other herbivore species (Table S3). A comparison of selection differentials among herbivore species indicated a consistent trend in all species to select against the production of atropine, since mean effect sizes did not overlap zero and were negative (Figure 2). Mean effect sizes for scopolamine also showed a trend indicating that this alkaloid was selected against by the more specialized herbivore (*L. daturaphila*) followed by the less specialized beetle (*E. parvula*), and it was favored by the generalist grasshopper (Figure 2, Table S3). Mean effect sizes for scopolamine also showed a trend indicating that this alkaloid was selected against by the more specialized herbivore (*L. daturaphila*) followed by the less specialized beetle (*E. parvula*), while it was favored by the generalist grasshopper (Figure 2, Table S3). Differences between the mean effect sizes of selection differentials and those estimated for gradients of selection indicate that indirect selection is also consistent at this level of analysis. However, the contrast between the mean effect sizes (from differentials and gradients) indicates that direct selection is acting in a dominant fashion against scopolamine concentration (Table S3).

Discussion

Our findings revealed significant geographic variation in selection patterns on defensive traits of *D. stramonium* in central Mexico. Despite this spatial variation, we were able to detect herbivore-specific effects on selection in plant defenses. All

herbivore species selected for a reduction in the concentration of the "older" tropane alkaloid (atropine) suggesting that this secondary compound is no longer beneficial as a deterrent against herbivory, and that it entails a fitness cost to the host plant. In addition, the more toxic derived alkaloid (scopolamine) was more effective against the generalist rather than the specialist herbivores, which supports our initial expectation. Although previous studies in natural populations of *D. stramonium* showed significant selection favoring higher levels of trichome density [26], the present analyses detected a marginal fitness effect of trichome density in almost all populations examined. Overall the strong pattern of selection against the production of atropine is consistent with the previous finding of a positive geographic association between atropine and leaf damage within the same region [32].

Empirical evidence suggests that the spatial variation of traits that mediate the plant-herbivore interaction is a common phenomenon in nature. Yet understanding the origin and maintenance of such variation has proven to be challenging. Selection by herbivores is a major force shaping the evolution of plant defensive traits such as trichomes or secondary metabolites [5,6]. However, along their distribution, plant species are exposed to specialized and/or generalist herbivores [11,12]. Depending of their level of specialization, herbivores are expected to exert contrasting selective pressures on plant defense [11]. This is likely to produce spatially variable selection on defensive traits along the distribution of a species [49]. According the Geographic Mosaic Theory of Coevolution (GMCT, Thompson, [13]), selection mosaics constitute the raw material that promotes and maintains variation in those traits that are involved in species interactions. Here, we found evidence of spatially variable selection exerted by herbivores in both the chemical and physical defense of *D. stramonium*. Furthermore, in line with GMCT predictions, we found ample geographic variation in the defensive traits, similarly to what has been previously reported [26,32] for trichome density, atropine and scopolamine concentrations of *D. stramonium*.

Estimation and interpretation of selection patterns is fundamental to form predictions about the evolution of defensive traits [50]. Differences in selective patterns among populations can lead to among-population differences in defensive traits [49]. In this study we found evidence of spatially variable phenotypic selection on defensive traits in populations facing different herbivore species (putative selective agents). Selection differentials indicate that both atropine and scopolamine were selected against in populations consumed by *L. daturaphila* and/or *E. parvula*. Thus, in the presence of genetic variation underlying the expression of tropane alkaloids, we suggest that atropine and scopolamine concentrations should be reduced in these populations. The contrast between differentials and gradients of selection for the studied alkaloids indicates that, while direct selection reduces atropine concentration, indirect selection reduces scopolamine. Since atropine is the precursor of scopolamine [51] and their concentrations are positively correlated (as detected in this study; see also Shonle & Bergelson, [16]), direct selection acting on atropine is likely conditioning the adaptive value of scopolamine as well. Nevertheless, in one of the studied populations (Santo Domingo), consumed by the generalist grasshopper *S. purpurascens*, selection favored an increase in scopolamine and a reduction in atropine concentrations. Although in this population no evidence of a positive correlation between alkaloids was detected, this contrasting selection could still explain why these costly chemical defenses are maintained. In addition, differences between selection differentials and gradients for trichome density and scopolamine concentration suggest that indirect selection represents an important force driving the evolution of plant chemical defense.

Table 2. Multiple regression analyses to estimate linear selection gradients (β) for trichome density, atropine, and scopolamine concentration in eight populations of *Datura stramonium*.

Population	Main herbivore	Defensive trait	β	SE	t	P
Acolman (N = 31)	*Lema daturaphila*	Trichome density	−0.014	0.174	−0.08	0.937
		Atropine	**−0.985**	**0.293**	**−3.359**	**0.002**
		Scopolamine	**0.703**	**0.29**	**2.422**	**0.022**
Patria Nueva (N = 30)	*Lema daturaphila*	Trichome density	−0.096	0.124	−0.778	0.444
		Atropine	−0.068	0.13	−0.526	0.603
		Scopolamine	−0.178	0.153	−1.163	0.255
Joquicingo (N = 31)	*Lema daturaphila*	Trichome density	−0.194	0.196	−0.992	0.33
		Atropine	−0.258	0.196	−1.318	0.199
		Scopolamine	0.038	0.197	0.19	0.851
San Martín (N = 29)	*Lema daturaphila*	Trichome density	**0.328**	**0.146**	**2.244**	**0.034**
		Atropine	**−0.522**	**0.171**	**−3.054**	**0.005**
		Scopolamine	0.291	0.178	1.631	0.115
Tzin Tzun Tzan (N = 30)	*Epitrix parvula*	Trichome density	**−0.316**	**0.115**	**−2.759**	**0.01**
		Atropine	−0.208	0.14	−1.49	0.149
		Scopolamine	0.253	0.132	1.908	0.068
	Epitrix parvula					
Valsequillo (N = 33)		Trichome density	−0.009	0.089	−0.104	0.917
		Atropine	**−0.357**	**0.113**	**−3.154**	**0.003**
		Scopolamine	0.166	0.113	1.47	0.152
Sanabria (N = 34)	*Sphenarium purpurascens*	Trichome density	−0.189	0.2	−0.945	0.353
		Atropine	−0.335	0.222	−1.507	0.143
		Scopolamine	0.17	0.231	0.739	0.466
Santo Domingo (N = 30)	*Sphenarium purpurascens*	Trichome density	**0.04**	**0.014**	**2.769**	**0.01**
		Atropine	−0.012	0.014	−0.82	0.419
		Scopolamine	−0.018	0.013	−1.374	0.181

Significant gradients appear in bold-type fonts. Standard error (*SE*) of estimates, *t*-test value, and probability (*P*) are provided.
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The specialist-generalist paradigm of host plant use by herbivores predicts that specialized herbivores should be less affected by plant defenses than generalists [52]. This expectation is based on the existence of a trade-off, such that being able to consume a diverse diet constrains the opportunities to specialize on a given host [53–55]. In turn, if plants are involved in a coevolutionary arms-race with herbivores through chemical defenses [56], recently evolved secondary plant compounds should be more effective against consumers than their ancestors [36]. Our results provide correlative evidence in support of theoretical expectations, since the precursor tropane alkaloid atropine had no positive effect on plant fitness, while the more derived alkaloid (scopolamine) was still effective against the generalist but not to the specialist herbivore (*L. daturaphila*). Accordingly, specialized herbivores may be even using atropine in order to select *D. stramonium* plants. Nonetheless, a pattern of directional selection for an increase in scopolamine was detected in one of the populations consumed by the generalist grasshopper, so it not possible to draw conclusions about the potential of this herbivore to affect the evolution of this tropane alkaloid. In addition, in one of the populations dominated by the specialized beetle *L. daturaphila*, atropine was favored by selection suggesting that the current coevolutionary state of the interaction at each population, together with gene flow among populations, could

also account for the maintenance of these secondary compounds. Overall, the strong directional selection found for all herbivore species against atropine may explain the positive correlation recorded between herbivory damage and atropine concentration for a set of 28 plant populations within the same studied region in central Mexico [32].

Conclusion

Empirical evidence suggests that spatial variation of traits that mediate interactions is a common phenomenon in nature. However, understanding the origin and maintenance of such variation has proven to be challenging. In this study, we provide evidence of spatially variable selection exerted by herbivores on physical and chemical defensive traits of *D. stramonium*. Local selective pressures are likely to produce the observed divergence in defensive traits at a geographic scale. However, further studies are still needed that explicitly evaluate the role of selection by herbivores in shaping trait divergence. In addition, future research should evaluate whether local adaptation to specialist and generalist herbivores occurs in nature, and to what extent it is mediated by defensive traits. Such research would increase our understanding of the great variation in defensive trait diversity in the wild.
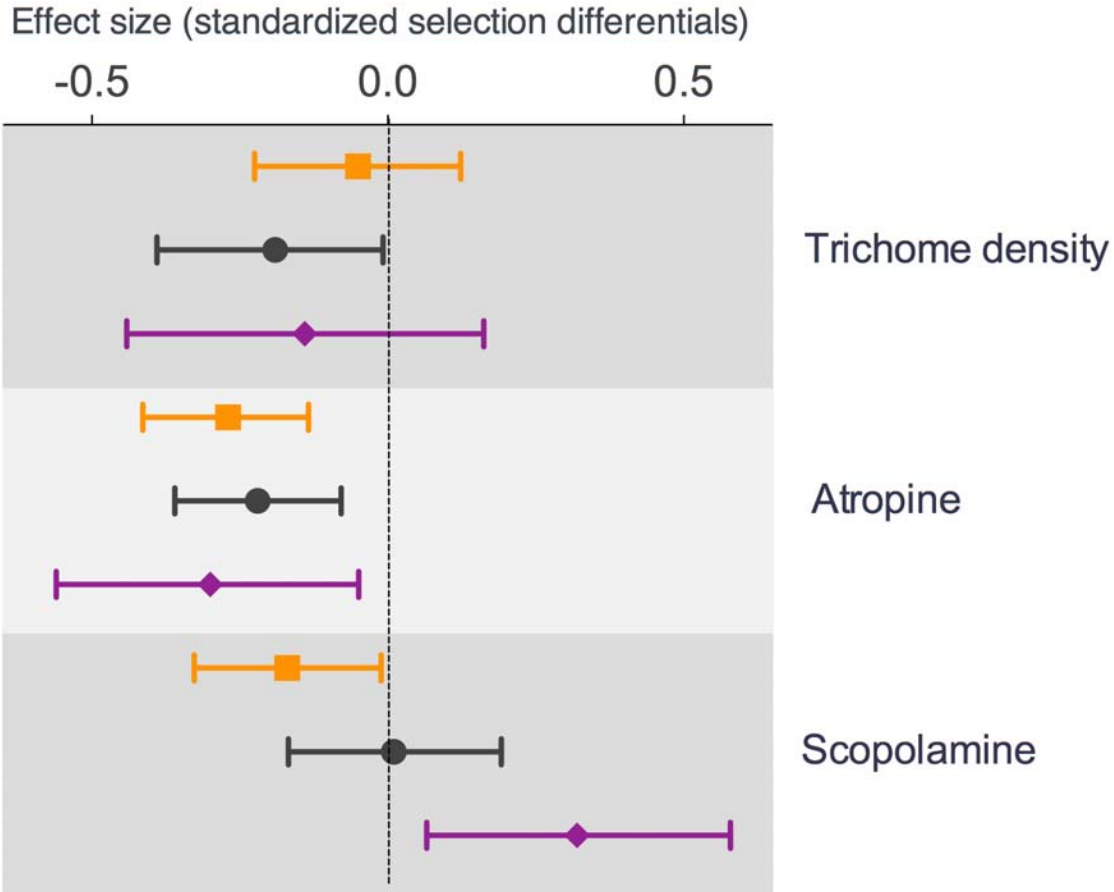
Figure 2. Forest plot showing the mean effect size for standardized selection differentials (S) of each defensive trait and corresponding confidence interval at 95%. Different colors and forms denote different species of herbivores: Tangerine squares: *Lema daturaphila*; black circles: *Epitrix parvula*, and purple diamonds: *Sphenarium purpurascens*. Corresponding values are reported in Table S3.
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Supporting Information

Figure S1 Among-population variation in a) leaf trichome density, b) atropine concentration, and c) scopolamine concentration in eight populations of *Datura stramonium* in central Mexico. Bars represent average value +1 standard error.
(DOC)

Table S1 *Datura stramonium* populations sampled in August-September 2011. DS = Desert shrub, POF = Pine-Oak forest TDF = Tropical deciduous forest.
(DOC)

Table S2 Correlations (r) between trichome density, scopolamine, and atropine concentration in eight populations of *Datura stramonium* in central Mexico. Significant correlations appear in bold-type fonts.
(DOC)

Table S3 Effect sizes for selection differential (S) and gradients (β) of selection with their corresponding confidence intervals at 95% (in parentheses). An omnibus test (Q_m) evaluates whether parameters are equal among groups (*i.e.*, $H_0 = \beta_1 = \ldots = \beta_p = 0$). *, $P<0.05$; ***, $P<0.001$; n. s., not significant. Confidence intervals at 95% in bold-type font do not overlap with zero value.
(DOC)

Author Contributions

Conceived and designed the experiments: GC JNF. Performed the experiments: GC LLC EOV RTL GAG. Analyzed the data: GC DC EOV GAG RTL AAL. Contributed reagents/materials/analysis tools: GC LLC JNF JF AAL. Wrote the paper: GC DC PLV JNF.

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Capítulo III

Adaptación local a herbívoros múltiples en *Datura stramonium*

1   Local adaptation to multiple herbivores in *Datura stramonium*

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19   Abstract

20   Local adaptation is a central process for creating and maintaining variation of traits

21   mediating antagonistic interactions. Despite this, few studies have evaluated the local

22   adaptation of plants to their biological counterparts such as herbivores. Most studies

23   evaluating local adaptation to herbivores have focused on specialist systems, where local

24   adaptation is likely to occur. However, there is less evidence regarding the existence of

25   local adaptation on generalist systems, where local adaptation is not expected. Since leaf

26   damage has in general a negative impact on plant fitness it is expected that local

27   adaptation is mediated by the herbivores feeding preferences and thus by the defensive

28   traits exhibited by plants. We conducted a reciprocal transplant experiment that aimed to

29   detect the existence of local adaptation of the annual herb *Datura stramonium* to specialist

30   and generalist herbivores. We also explored if leaf trichome density (a defensive trait of *D.*

31   *stramonium*) was mediating local adaptation to herbivores. Results showed that *D.*

32   *stramonium* populations can be locally adapted to both generalist and specialist herbivores,

33   but also that this is not a generalized feature of all populations. Although trichome density

34   showed a significant effect on individual fruit production, we did not find a consistent

35   pattern indicating that this trait was mediating the observed local adaptation patterns.

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40   Introduction

41   Most species consist of a group of populations genetically and phenotypically differentiated

42   (Rice & Jain, 1985; Thompson, 2005). Such differentiation may arise through neutral

43   processes such as gene flow, genetic drift or founder effects (Gomulkiewicz *et al.*, 2007).

44   However, it may also be originated by spatially variable selection exerted by biotic and/or

45   abiotic factors (Lande & Arnold, 1983; Holsinger & Weir, 2009). Uncovering the relative role

46   of these forces in generating and maintaining phenotypic variation remains as a current

47   challenge for evolutionary ecology (Mazer & Damuth, 2001; Leinonen *et al.*, 2006;

48   Leinonen *et al.*, 2013).

49   Local adaptation studies have highlighted the role of selection in generating divergence

50   through adaptations to the local conditions (Vesakoski & Jormalainen, 2013). According

51   Kawecki and Ebert (2004) local adaptation is a pattern such that individuals from a

52   population have higher relative fitness in their original habitat compared with individuals

53   originated in other habitat (local vs. foreign criterion). Local adaptation can be also

54   detected when individuals of a population show higher fitness in their own habitat than in

55   other habitats (home vs. away criterion). Though, it has been suggested that the "home vs.

56   away" criterion alone does not provide enough evidence of local adaptation, because it

57   may be reflecting the differences in habitat quality, rather than the effects of divergent

58   selection (Kawecki & Ebert, 2004; DeFaveri & Merilä, 2014). Recently, Blanquart *et al.*

59   (2013) introduced a third criterion, namely the sympatric vs. allopatric criterion. These

60   authors propose that local adaptation should be considered a property of a metapopulation

61   (rather than a property of a single population) and should be defined as the differences in

62   fitness between populations in their home sites (simpatry) and fitness of populations when

63   transplanted to other sites (allopatry).

64   Several studies have explored the local adaptation of plants to local abiotic conditions (see

65   Leimu & Fischer, 2008). However, few studies have evaluated plant's local adaptation to

66   their biological counterparts (Ortegón-Campos *et al.*, 2009; Garrido *et al.*, 2012; Laukkanen

67   *et al.*, 2012). In the case of the plant-herbivore interaction (and from the standing point of

68   the plants), local adaptation is exhibited when plants originated from a population show

69   higher fitness when are exposed to sympatric herbivores in comparison to when are

70   exposed to allopatric herbivores (Kawecki & Ebert, 2004; Laukkanen *et al.*, 2012). Most of

71   the evidence supporting this hypothesis comes from specialist systems, where interacting

72   species are engaged in a strong coevolutionary arm-race, and local adaptation is likely to

73   occur (Gandon & Michalakis, 2002). In contrast, less evidence arises from generalist

74   systems, where multi-specific selection is thought to predominate and where local

75   adaptation is not predicted (Gómez *et al.*, 2009; Ortegón-Campos *et al.*, 2009). Since leaf

76   damage by herbivores has generally a negative impact on plant fitness (Crawley, 1989), it

77   is expected that local adaptation to herbivores be mediated by herbivores feeding

78   preferences and the defensive traits exhibited by plants (Laukkanen *et al.*, 2012).

79   The annual herb *Datura stramonium* is a good system for testing local adaptation to

80   generalist and specialist herbivores. In Mexico, most of the *D. stramonium* populations are

81   attacked mainly by the specialist folivore *Lema daturaphila* (Chrysomelidae) (Nuñez-Farfan

82   & Dirzo, 1994; Valverde *et al.*, 2001). However, there are populations where *L. daturaphila*

83   is absent and where the oligophagous *Epitrix parvula* (Chrysomelidae) and the generalist

84   *Sphenarium purpurascens* (Orthoptera) are the main plant consumers (Castillo *et al.*,

36

85   2014). Previous studies have documented that *D. stramonium* features leaf trichomes as

86   defensive traits against herbivory (Valverde *et al.*, 2001), and can evolve by selection

87   exerted by herbivores (Kariñho-Betancourt, 2009). Recently, (Garrido *et al.*, 2012) found

88   local adaptation of *D. stramonium* to its specialist herbivore *Lema daturaphila*

89   (Parachnowitsch & Lajeunesse, 2012); however it is still unknown if *D. stramonium* can be

90   locally adapted to generalist herbivores and if defensive traits, such as trichome density,

91   are mediating the local adaptation of *D. stramonium* to specialist and generalist herbivores.

92   In this context, we conducted a reciprocal transplant experiment that aimed to *(i)* detect

93   local adaptation of *D. stramonium* to specialist and generalist herbivores and *(ii)* to

94   evaluate to what extent local adaptation is mediated by leaf trichome density. We

95   hypothesized that there will be local adaptation exclusively in specialist populations and

96   leaf trichome density should be mediating the local adaptation of *D. stramonium* to both

97   generalist and specialist herbivores.

98   Methods

99   *Study system - Datura stramonium* L. (Solanaceae) is an annual herb commonly

100   distributed in cultivated areas and disturbed environments in Mexico, the United States,

101   Canada, and Europe (Weaver & Warwick, 1984; Shonle & Bergelson, 2000; Valverde *et*

102   *al.*, 2001). The species reproduces mainly by self-fertilization, and has limited pollen and

103   seed dispersal (Motten & Antonovics, 1992). Previous studies have shown that *D.*

104   *stramonium* has a moderate genetic structure in Central Mexico, $R_{ST} = 0.265$ (Andraca-

105   Gómez, 2009).

106   Most populations of *D. stramonium* in Mexico are consumed by the specialist folivorous

107   beetle *Lema daturaphila* (Nuñez-Farfan & Dirzo, 1994). However, there are populations

108   where *L. daturaphila* is absent, and where the oligophagous flea beetle *Epitrix parvula*

109   (which consumes other members of the Solanaceae family), or the generalist grasshopper

110   *Sphenarium purpurascens* are the main consumers of the plant (Castillo *et al.*, 2014). Leaf

111   damage exerted by each of these herbivores is characteristic and recognizable (a more

112   detailed description of leaf damage type by each herbivore can be found elsewhere,

113   Nuñez-Farfan & Dirzo, 1994; Carmona & Fornoni, 2013; Castillo *et al.*, 2014).

114

115   *Reciprocal garden experiment* – During September-November 2011 we conducted a

116   reciprocal garden experiment with plants originated from four different natural populations

117   of *D. stramonium.* For this experiment, we used plants originated from four *D. stramonium*

118   populations in central Mexico (Fig. 1). Populations were selected based on the main

119   herbivore present at each locality. Teotihuacán and Joquicingo populations are consumed

120   primarily by the specialist herbivore *L. daturaphila,* whereas Morelia and Santo Domingo

121   are populations consumed mainly by the generalist *Sphenarium purpurascens.* Field

122   observations and leaf damage records carried out for 3 years performed by our laboratory

123   indicate that the main herbivores at each population remained stable throughout the 2010-

124   2012 period. Distance between populations ranged from 70 to 290 km. Geographic

125   coordinates and habitat characteristics of each population are shown in Table S1.

126   Plants were obtained germinating seeds from 30 fruits collected randomly at each

127   population of origin. Seeds where germinated individually in plastic pots using a

128   commercial soil mix in a glasshouse at the Instituto de Ecología of the Universidad

129   Nacional Autónoma de México. This allowed us to generate four groups of 30 plants by

130   each of the four sites of origin. When plants showed their first true leaves were

131   transplanted to four experimental plots at each site of origin in a full-randomized block

132   arrange. In order to separate the biotic and abiotic effect on plant fitness in the experiment,

133   we included an herbivore exclusion treatment. For this, we applied the commercial

134   systemic insecticide Furadan ® (1g/50ml). Thus, the experimental design was 4 *sites of*

135   *origin* × 4 *experimental sites* × 2 herbivory levels.

136   *Leaf damage -* The proportion of leaf damage per plant was calculated as the ratio of

137   removed leaf tissue area in ±20 leaves divided by the total area of those same 20 leaves.

138   Following Valverde *et al.* (2001), total leaf area was calculated as a function of leaf length

139   using a sample of undamaged leaves. Leaf shape varied among populations, so we used a

140   different equation for each population (R^2 ranged from 0.934 to 0.958, $p < 0.001$, n ± 30).

141   Remaining leaf area was measured using Image Pro-Plus image analysis software [version

142   7.0, 46].

143   *Trichome density and plant fitness -* We estimated leaf trichome density as the total

144   number of trichomes in an observation field of 2.5 mm^2 located in the central basal region

145   of the adaxial side of the leaf, following Valverde et al. (2001). We obtained the trichome

146   density per plant averaging trichome number of a random sample of 20 fully expanded

147   leaves. We used fruit number as a proxy of individual maternal fitness. Because *D.*

148   *stramonium* is a annual selfing plant, fruit number is a reliable proxy of lifetime fitness

149   (Mauricio & Rausher, 1997).

150   Statistical analyses

151   *Herbivore exclusion* – To evaluate if the herbivore exclusion treatment was efficient, we

152   used a multifactorial ANOVA that used the terms *insecticide*, *experimental site* as well as

153   the *insecticide × experimental site* interaction as predictor variables, and *leaf damage*

154   *proportion* as response variable. Leaf damage proportion was log-transformed prior to

155   analyses. Similarly, we evaluated the effect of the insecticide treatment on fruit production

156   using a quasi-poisson GLM with a log link function. Predictor variables used in this latter

157   model were the same as described above but using fruit production as response variable.

158   *Local adaptation* – We tested for local adaptation of *D. stramonium* to generalist and

159   specialist herbivores using a negative binomial GLM that used a logit link function. This

160   analysis was performed on subset of the data that excluded plants under the herbivore

161   exclusion treatment. The model included the terms *origin, experimental site* and *trichome*

162   *density* as a covariate as predictor factors, and *fruit number* as the response variable.

163   Whenever a significant interaction *origin × experimental site* was detected (first requisite for

164   detecting local adaptation), we carried out directed contrasts for testing the *(i)* "local vs.

165   foreign", *(ii)* "home vs. away" and *(iii)* "sympatric vs. allopatric" criterions of local adaptation,

166   as described in Blanquart *et al.* (2013).

167   *Leaf trichome density contribution to local adaptation* – To evaluate if trichome density

168   account for the local adaptation of *D. stramonium* to generalist and specialist herbivores,

169   we tested the correlation between leaf trichome density and fruit number using simple

170   Spearman's *p* for each population at ach experimental site. All analyses were performed in

171   R statistical software version 3.0.2 (R Development Core Team, 2011).

172

173　Results

174　*Herbivore exclusion* – We found a significant effect of the insecticide treatment on leaf

175　damage proportion ($F_{1, 65.53}$ = 292.42, P < 0.0001). Overall efficiency of the insecticide

176　treatment was 58.82% (in terms of leaf damage reduction). We also found a significant

177　effect of the term *experimental site* ($F_{3, 48.02}$ = 71.42, P < 0.0001). Mean leaf damage

178　reduction at each experimental site was: Joquicingo 61.11%, Teotihuacán 55.11%, Santo

179　Domingo 48.81% and Morelia 46.81%. A significant effect of the *experimental site* ×

180　*insecticide* interaction was also detected ($F_{3, 2.9}$ = 4.37, P = 0.0046) (see Fig. S2). In the

181　other hand, herbivore removal treatment increased in 7.5% the overall fruit production

182　(GLM, Wald X^2 = 4.39, P = 0.036). Significant differences in fruit production among

183　experimental sites were also detected (GLM, Wald X^2 = 4.39, P < 0.0001). Finally, a

184　significant *experimental site* × *insecticide* interaction was detected (GLM, Wald X^2 = 44.95,

185　P < 0.0001) (see Fig. S3).

186　*Local adaptation* – We found a significant effect of the term *experimental site* on fruit

187　production (Table 1). Mean fruit production was 3.78 in Joquicingo, 7.8 in Teotihuacán, 2.1

188　in Santo Domingo and 9.38 in Morelia. A significant effect of trichome density was also

189　detected (Table 1). Moreover, the *experimental site* × *trichome density* and *origin* ×

190　*trichome density* interactions resulted significant (Table 1). Finally, we detected a

191　significant effect of the interaction *experimental site* × *origin* (Table 1). Direct contrasts that

192　aimed to detect local adaptation according the *local vs. foreign* criterion (*i. e.,* higher mean

193　fitness of a focal population at home, in comparison of the average mean fitness of all the

194　other populations when transplanted into the focal population) evidenced the existence of

195   local adaptation for plants originated in Morelia population (Fig. 2). Accordingly, the *home*

196   *vs. away* criterion (higher mean fitness of the population at home compared to the average

197   mean fitness of the same population when transplanted in all other habitats) also showed

198   evidence for local adaptation of the Morelia population (Fig. 2). Finally, the sympatric *vs.*

199   allopatric criterion (higher mean fitness in sympatric combinations of populations and sites

200   compared to average fitness in allopatric combinations) showed evidence of local

201   adaptation in Teotihuacán and Morelia populations (Fig. 3).

202   *Leaf trichome density contribution to local adaptation* – We found significant correlations

203   between trichome density and fruit production in the Santo Domingo ($r = -0.55$, $P = 0.0024$)

204   and Morelia ($r = -0.6$, $P = 0.0002$) populations in the Joquicingo experiment. We also found

205   significant correlations in the Santo Domingo population in the Santo Domingo

206   experimental site ($r = -0.41$, $P = 0.0336$) and in the Santo Domingo population in the

207   Teotihuacan experimental site ($r = -0.52$, $P = 0.0002$).

208   Discussion

209   Local adaptation is a fundamental process for creating and maintaining trait variation of

210   antagonistic interactions (Laukkanen *et al.*, 2012). However, few studies have evaluated

211   local adaptation of plants to their biological counterparts (Leimu & Fischer, 2008). Most of

212   the empirical evidence of local adaptation to herbivores come from specialist systems,

213   where local adaptation is likely to occur (Gómez *et al.*, 2009; Ortegón-Campos *et al.*,

214   2009). Yet, there is scarce evidence regarding the existence of local adaptation on

215   generalist systems, were local adaptation is in principle not expected (Gómez *et al.*, 2009).

216   Here we evaluated local adaptation of *D. stramonium* to its generalist and specialist

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217 herbivores. To sum up, our results indicate that *D. stramonium* can be locally adapted to

218 both generalist and specialist herbivores, but also indicate that it is not a generalized

219 pattern across populations. Since local adaptation to herbivores is likely to be mediated by

220 the feeding capacities of the herbivores, we also explored if leaf trichome density (a

221 defensive trait of *D. stramonium*) was mediating local adaptation to herbivores. In this line,

222 we found that trichome density have indeed a significant effect on individual fruit

223 production, however we did not find a consistent pattern indicating that this trait is related

224 to the observed local adaptation patterns.

225 In order to know if our local adaptation estimates were reflecting actual adaptations to

226 herbivores and not merely to abiotic conditions, we included an herbivore exclusion

227 treatment in our reciprocal garden experiment. Although the application of insecticide did

228 not prevent herbivores from feeding on *D. stramonium* completely, we found a significant

229 reduction on leaf damage of plants under this treatment. Moreover, variation in the

230 efficiency of the insecticide across experimental sites and populations was similar.

231 Likewise, we found a significant effect of the herbivore reduction treatment on plant fruit

232 production. In general, fruit production under the herbivore reduction treatment was higher

233 compared to plants fully exposed to herbivores, probably as a result reduced herbivory.

234 Despite the observed variation of herbivore exclusion across experimental sites and

235 populations, we consider that our results represent good estimates of local adaptation to

236 herbivores.

237 Results indicate the existence of local adaptation of *D. stramonium* for the Morelia

238 population to the generalist herbivore *Sphenarium purpurascens*. This population fulfilled

239 the requirements for the three local adaptation criterions contemplated in our study.

240   Although this is not expected in theory (Gómez *et al.*, 2009), recent studies of *D.*

241   *stramonium* have detected selection by generalist herbivores on defensive traits (Castillo *et*

242   *al.*, 2014). We suggest that if there is moderate or low genetic flow between plant and

243   herbivore populations, local adaptive processes between *D. stramonium* and *S.*

244   *purpurascens* could arise. We also found evidence of local adaptation in the Teotihuacán

245   population to the specialist herbivore *L. daturaphila* based in the sympatric *vs.* allopatric

246   contrast. This result is in line with previous studies that have found mutual local adaptation

247   of *D. stramonium* to *L. daturaphila* (Garrido et al., 2012; Parachnowitsch & Lajeunesse,

248   2012). Interestingly, local adaptation patterns for generalist and specialist herbivores don't

249   appear to be a generalized feature of *D. stramonium* populations, and resembles a

250   geographic mosaic of local adaptations as predicted by GMTC (Thompson, 2001;

251   Thompson, 2005), reflecting a system where local adaptation to herbivores is fluctuating as

252   the result of structured coevolutionary dynamics at each population.

253   We also explored to what extent trichome density accounts for local adaptation of *D.*

254   *stramonium*. Our results showed that trichome density alone does not account for local

255   adaptation of the populations. Despite that other empirical evidence have found strong

256   selective pressures by herbivores acting on leaf trichome density (Valverde *et al.*, 2001;

257   Kariñho-Betancourt, 2009) and that we detected a significant effect of trichome density on

258   plant fitness, we did not find a consistent pattern indicating that leaf trichome density is

259   mediating with local adaptation. A recent study detected correlational selection acting on

260   trichome density and tropane alkaloids in central Mexico *D. stramonium populations*

261   (Castillo et al., 2014), suggesting that defense syndromes are locally mediating the plant

262   herbivore interaction as has been proposed in the literature (Agrawal & Fishbein, 2006;

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263   Leimu & Koricheva, 2006). Thus, exploring leaf trichome density together with other

264   defensive traits such as tropane alkaloids is fundamental to evaluate the role of plant

265   defense to local adaptation processes.

266   Unveiling the forces that maintain and generate phenotypic variation is a current challenge

267   for evolutionary biologists (Mazer & Damuth, 2001; Leinonen *et al.*, 2006; Leinonen *et al.*,

268   2013). Here we have provided evidence of a plant species that can be locally adapted to

269   both specialist and generalist herbivores. Our results also support the notion that adaptive

270   processes involved in the plant-herbivore interaction occur in a structured fashion across

271   populations. It is also worth to mention that, in this study we were not able to deal with

272   other sources of variation such as, transgenerational induction and epigenetic inheritance

273   of plant defensive traits. Several studies have found that herbivore and pathogen attack of

274   plants can generate particular defense phenotypes across generations (Holeski *et al.*,

275   2012; Kim & Felton, 2013). Generating this kind of evidence was beyond the scope of this

276   research, but including explicitly these factors in the context of the local adaptation could

277   help us to explain those patterns that do not fit into the traditional theory predictions.

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413   Table 1. Results of the GLM model on the effects of population of origin, experimental sites

414   (i.e., "Site") and leaf trichome density on fruit production of *D. stramonium.* Significant

415   values appear in bold-type fonts.

Variable	d.f.	*F*	*P*
Site	3	62.0781	**< 0.001**
Origin	3	1.4951	0.2135942
Trichome density	1	14.892	**< 0.001**
Site × Origin	9	7.3266	**< 0.001**
Site × Trichome density	3	3.8664	**0.0088906**
Origin × Trichomes	3	3.0473	**0.0274643**
Site × Origin × Trichome density	9	1.3427	0.2086155

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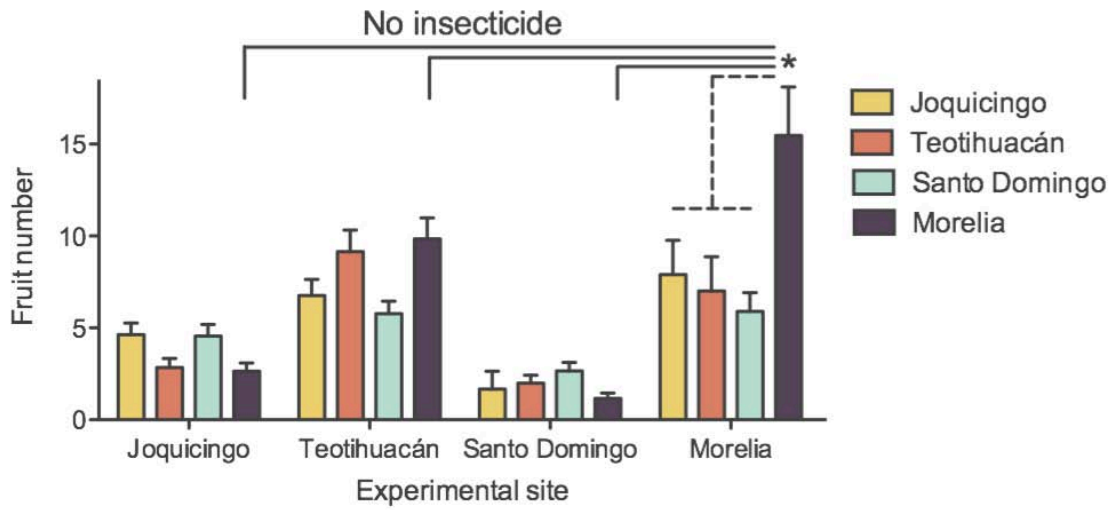
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428   Figure 1.
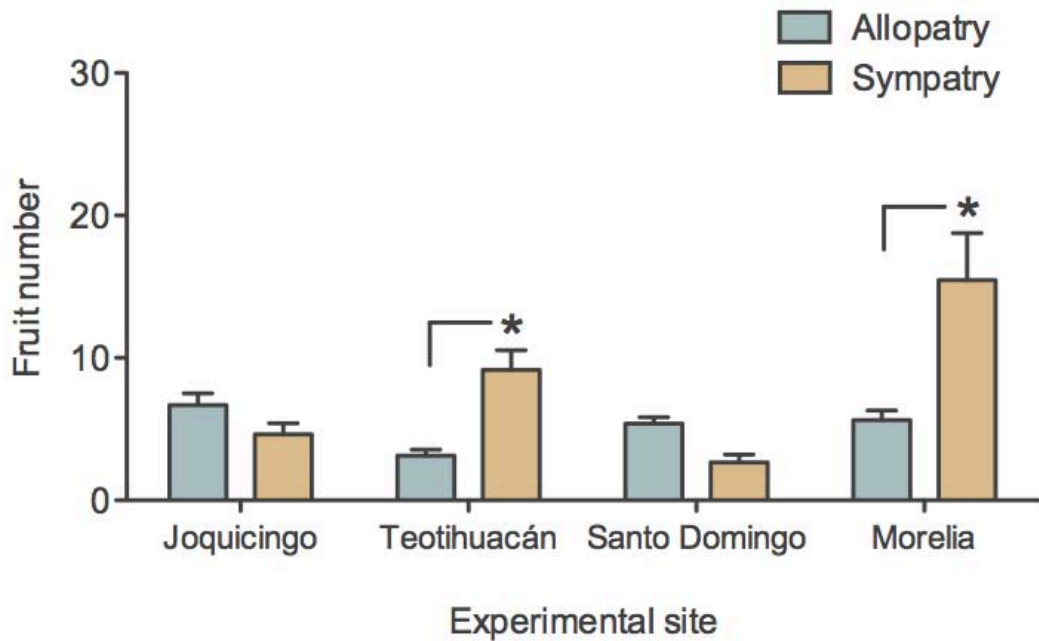

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430   Figure 2.

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434   Figure 3.

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436

437   Figure legends

438   Figure 1. Geographic location of source populations and experimental sites used in the

439   reciprocal garden experiment. Letters between parentheses indicate the main herbivore at

440   each locality. SP = *Sphenarium purpurascens*, LD = *Lema daturaphila.*

441   Figure 2. Mean fruit production of four populations of *D. stramonium* in a reciprocal garden

442   experiment. The asterisk indicates significant differences ($P < 0.05$) of contrasts comparing

443   fruit production according to the "local *vs.* foreign" (dashed line) and "home *vs.* away"

444   (continuing lines) criteria for testing local adaptation. Error bars represent ±1SE.

445   Figure 3. Mean fruit production in allopatric and sympatric combinations of experimental

446   sites and populations of origin. The asterisk indicates significant differences ($P < 0.05$) of

447   contrasts comparing fruit production according the "sympatric *vs.* allopatric" criterion for

448   testing local adaptation. Error bars represent ±1SE.
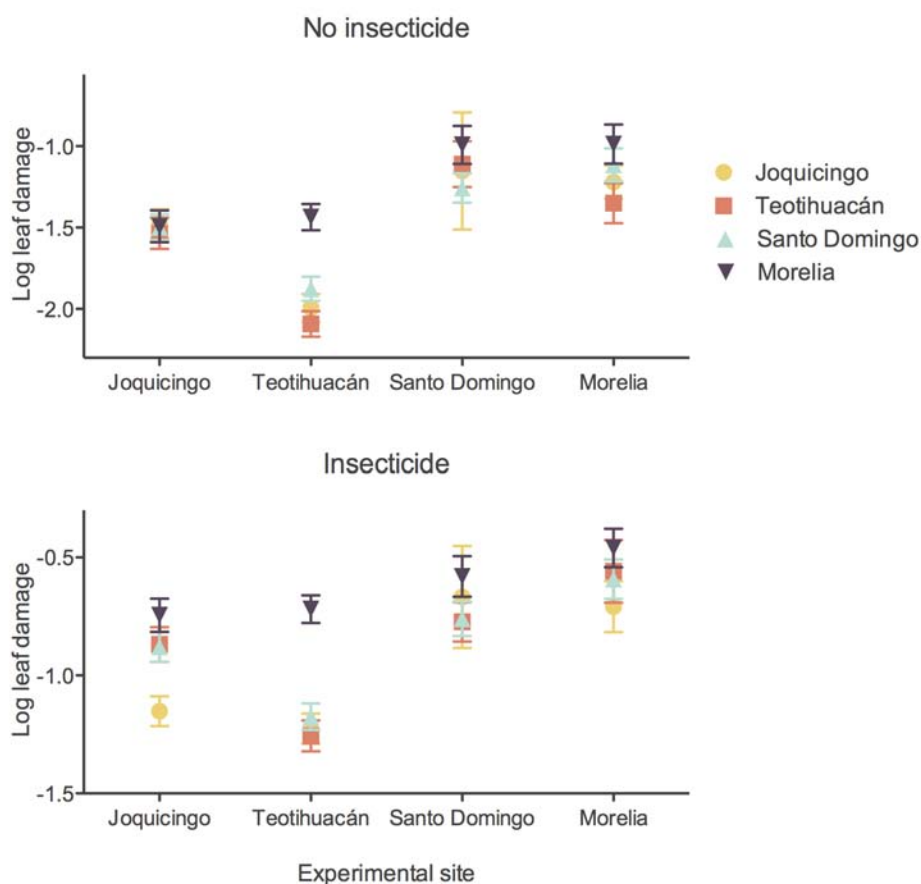
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450   Figure S2. Logarithm of leaf damage by herbivores in the reciprocal garden experiment

451   with four populations of *D. stramonium* and two herbivore reduction levels, a) no insecticide

452   applied, b) insecticide applied. Error bars represent ±1SE.

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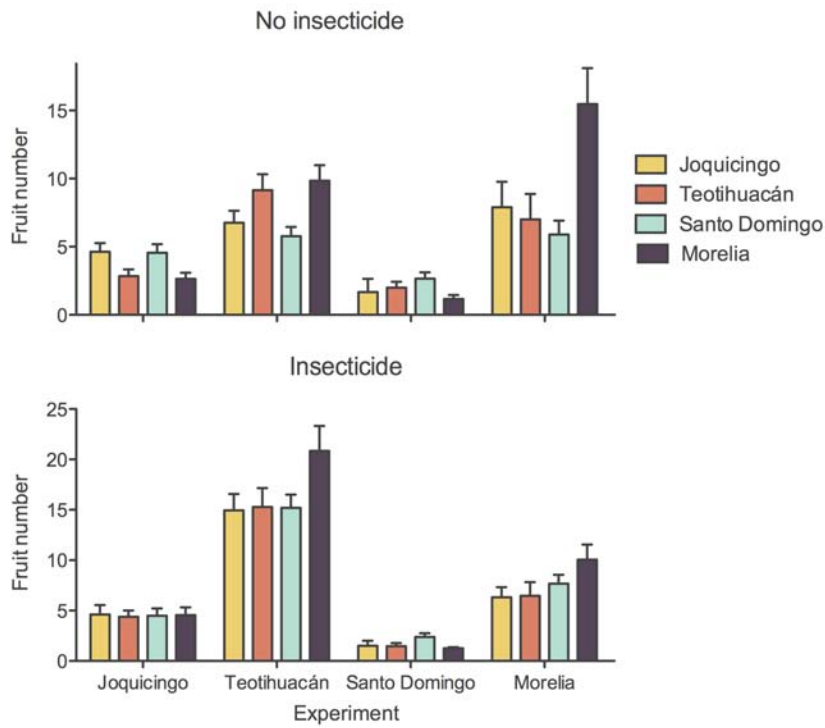
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456   Figure S3. Fruit production in the reciprocal garden experiment with four populations of *D.*

457   *stramonium* and two herbivore reduction levels, a) no insecticide applied, b) insecticide

458   applied. Error bars represent ±1SE.

459

Capítulo IV.

Diferenciación adaptativa de atributos defensivos en *Datura stramonium*

1 Adaptive differentiation in defensive traits of *Datura stramonium*

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24   **Introduction**

25   Most species consist of a series of populations that are often phenotypically differentiated

26   (Rice & Jain, 1985; Thompson, 2005). Such phenotypic differentiation in multiple traits can

27   be effectively produced by processes like genetic drift, mutation, founder effects or

28   population isolation (Gomulkiewicz *et al.*, 2007). However, phenotypic differentiation in

29   traits that contribute to individuals' fitness may also have a spatial structure caused by

30   varying selective regimes exerted by biotic and/or abiotic factors (Holsinger & Weir, 2009).

31   Elucidating to what extent these factors promote character differentiation among

32   populations is crucial, if we are to fully understand the prevalence of among-population

33   variation in the wild (Lynch, 1990; Althoff & Thompson, 1999; Criscione *et al.*, 2006; Kelly,

34   2006; Gomulkiewicz *et al.*, 2007).

35

36   Defensive traits exhibited by plants (*i.e.,* traits that prevent/reduce damage by

37   natural enemies) vary widely across populations (Núñez-Farfán *et al.*, 2007; Züst *et al.*,

38   2012). Selection exerted by herbivores is a major force driving the evolution of plants

39   defensive traits (Rausher, 2001; Anderson & Mitchell-Olds, 2011). Thus, among-population

40   differentiation in defensive traits is likely to be produced by spatial variation in the local

41   selective regimes exerted by herbivores. Such spatially variable selection can be

42   generated by among-population variation in the abundance, species composition, feeding

43   styles, and degree of specialization of herbivores to their host plants (Falconer & Mackay,

44   1996; Charlesworth *et al.*, 1997; Parchman & Benkman, 2002; Arany *et al.*, 2008; Hare,

45   2012). However, the among-population variation in defensive traits may also result from

58

46   other causes rather than selection exerted by herbivores (Gomulkiewicz *et al.*, 2007).

47   Adaptive responses to spatial variation in habitat quality or neutral processes as genetic

48   drift or limited gene flow can also promote spatial variation in traits mediating plant-

49   herbivore interactions (Nuismer *et al.*, 1999; Gandon & Nuismer, 2009). Thus, it is relevant

50   to determine if selection by herbivores or neutral processes can account for the observed

51   among-population differentiation of defensive traits in plants.

52   *Datura stramonium* L. (Solanaceae) provides an optimal system for studying among-

53   population differentiation in defensive traits. Because of its wide distribution (Mexico,

54   Canada, United States, and Europe), *D. stramonium* is exposed to different environmental

55   conditions and to a wide diversity of herbivore species (Weaver & Warwick, 1984; Valverde

56   *et al.*, 2001; Cuevas-Arias *et al.*, 2008). Recent studies have found ample geographic

57   variation in leaf trichome density and atropine and scopolamine concentration in central

58   Mexico (Castillo *et al.*, 2013; Castillo *et al.*, 2014). However, it is unclear if selection by

59   herbivores or neutral processes can account for the observed among-population

60   differentiation in these defensive traits.

61   Here, we assessed to what extent gene flow and natural selection account for

62   population differentiation observed in defensive leaf traits (trichome density, atropine and

63   scopolamine concentrations) in 13 populations of *D. stramonium* in central Mexico. To do

64   so, we compared the degree of phenotypic differentiation of defensive traits by means of

65   P_{ST} index (analogue of Q_{ST} but influenced by environmental effect) (Spitze, 1993) against

66   the neutral expectation set by allelic divergence at microsatellite loci (F_{ST}).

67

68   **Methods**

69   *Study system*

70   *Datura stramonium* is an annual herb commonly found in roadsides, cultivated areas and

71   disturbed environments in Mexico, the United States, Canada, and Europe (Weaver *et al.*,

72   1985; Valverde *et al.*, 2001; van Kleunen *et al.*, 2007). In Mexico, leaves of *D. stramonium*

73   are consumed by a specialist herbivore, the chrysomelid *Lema trilineata* (Nuñez-Farfan &

74   Dirzo, 1994), the oligophagous *Epitrix parvula* (Chrysomelidae), which also feed from other

75   members of the Solanaceae family (Glass, 1940), and by the generalist grasshopper

76   *Sphenarium purpurascens* (Nuñez-Farfan & Dirzo, 1994). *D. stramonium* features leaf

77   trichomes and tropane alkaloids (atropine and scopolamine) as defensive traits against

78   herbivory. These traits have shown heritable basis (Shonle & Bergelson, 2000; Valverde *et*

79   *al.*, 2001; Kariñho-Betancourt & Núñez-Farfán, 2015), and are under selection by specialist

80   and generalist herbivores (Castillo *et al.*, 2014). Furthermore, among-population

81   differentiation in leaf trichome density and atropine and scopolamine concentration was

82   recently found in a common garden experiment (Castillo et. al., in prep.).

83

84   *Fieldwork*

85   During August-September 2007 we sampled 13 natural populations of *D. stramonium* in

86   central Mexico (Fig. 1). Selected populations inhabit a wide range of habitat types. The

87   geographic location and climatic characteristics are shown in Table 1. From each

88   population we sampled 30 randomly chosen individual plants.

89   *Defensive traits quantification*

90   Following Valverde et al., (2001) we estimated leaf trichome density as the total number of

91   trichomes in an observation field of 2.5 mm^2 located in the central basal region of the

92   adaxial side of the leaf, using a stereoscopic microscope. Then we averaged the trichome

93   density per plant from a random sample of 20 fully expanded leaves. We also quantified

94   the concentration of atropine and scopolamine (two major alkaloids in *D. stramonium*) from

95   a sample of 20 leaves per plant by means High Precision Liquid Chromatography (HPLC).

96   Details of the extraction method and HPLC conditions can be found elsewhere (see

97   Castillo *et al.*, 2013).

98

99   *Data analysis*

100 We estimated the neutral genetic differentiation among populations of *D. stramonium* using

101 F_{ST} values obtained from five nuclear microsatellite markers designed specifically for *D.*

102 *stramonium* as reported by Andraca-Gómez (2009). F_{ST} values were calculated using

103 FSTAT 2.9.3.1 (Goudet, 2001) employing approximately 30 individuals per population.

104

105 *Phenotypic divergence in defensive traits* – We used the degree of among-population

106 phenotypic divergence (P_{ST}) to explore to what extent natural selection and gene flow

107 account for phenotypic differentiation of defensive traits. P_{ST} is an analogous index to Q_{ST},

108 but is affected by environmental and non-additive genetic effects (Merilä & Crnokrak,

109 2001). However, the use of P_{ST} instead of Q_{ST} is justified when estimates of the latter are

110 not available (see Leinonen *et al.*, 2006; Lehtonen *et al.*, 2009). Thus:

111
$$P_{ST} = \frac{\sigma^2_{GB}}{\sigma^2_{GB} + 2(h^2 \bullet \sigma^2_{GW})} ,$$

112 where σ^2_{GB} is the variance among populations σ^2_{GW} is the variance within population and h^2

113 is the trait heritability. Because environmental conditions influence P_{ST} values, we

114 calculated P_{ST} for two contrasting scenarios. The first scenario assumed that

115 environmental and non-additive genetic effects account for half of the observed variation

116 ($h^2 = 0.5$), while the second scenario assumed that all of the observed phenotypic variance

117 is due to genetic and additive variation ($h^2 = 1$). To estimate P_{ST} values we fitted a linear

118 model for each defensive trait, under the assumption that the distribution of defensive traits

119 was normally distributed. The *population* term was considered as a random effect. To test

120 the hypothesis that P_{ST} is higher than F_{ST}, a Monte Carlo test was carried out, approaching

121 a sample of 10,000 deviates from both P_{ST} and F_{ST} by means of their estimated error. P_{ST}

122 error was estimated from the likelihood errors of its components (variances among- and

123 within-populations), while F_{ST} error was obtained by bootstrapping (Goudet, 2001). The

124 10,000 random deviates of F_{ST} and P_{ST} were compared and the *p*-value was obtained as

125 the proportion of comparisons in which the F_{ST} was equal or higher than the P_{ST} (null

126 hypothesis).

127   We further evaluated the correlation between the F_{ST} and P_{ST} pair-wise

128 differentiation matrices of each trait using Mantel tests (Mantel, 1967). Neutral marker

129 variation can be used as a neutral expectation against which the phenotypic divergence of

130   traits can be compared (Gomulkiewicz *et al.*, 2007). If defensive phenotypic differentiation

131   between populations (P_{ST}) is the result of neutral processes rather than selection,

132   differentiation among populations in these traits should correlate with differentiation in

133   selectively neutral markers (F_{ST}) (Gomulkiewicz *et al.*, 2007; Leinonen *et al.*, 2013). All

134   data analyses were performed using R statistical software (R Development Core Team,

135   2011).

136

137   **Results**

138   *Among-population variation in defensive traits and leaf damage* - A multivariate analysis of

139   variance (MANOVA) detected significant multivariate differences in the studied defensive

140   traits of 13 populations of *D. stramonium* (Wilks' $\lambda = 0.091$, $F_{36,\,331.64} = 11.51$, $P < 0.0001$).

141   After the subsequent univariate ANOVAs were applied, we found significant differences in

142   trichome density ($F_{12,126} = 5.10$, $P < 0.0001$), atropine ($F_{12,126} = 7.85$, $P < 0.0001$) and

143   scopolamine concentration ($F_{12,\,126} = 23.33$, $P < 0.0001$). Mean leaf trichome density and

144   mean atropine and scopolamine concentration per population are shown in Figure S1. Post

145   hoc comparisons between populations for defensive traits are provided in Table S2.

146   *Phenotypic divergence in defensive traits* – - Comparison of phenotypic (P_{ST}) and neutral

147   genetic marker divergence (F_{ST}) showed that P_{ST} for atropine concentration was

148   significantly higher than the F_{ST} only in the scenario where $h^2 = 0.5$ (Table 2a, Figure 2).

149   Moreover, P_{ST} for scopolamine concentration was significantly higher than the F_{ST} in the

150   both heritability scenarios ($h^2 = 0.5$ and $h^2 = 1$), (Table 2b, Figure 2). In contrast, P_{ST} of leaf

151 trichome density did not differ significantly from the F_{ST} in neither scenario (Table 2c,

152 Figure 2).

153   Finally, we found no significant correlation between F_{ST} and P_{ST} pair-wise

154 differentiation matrices of any of the studied variables (see Table 3).

155

156 **Discussion**

157 Overall, our results showed that the overall phenotypic differentiation (P_{ST}) of tropane

158 alkaloids, scopolamine and atropine, was higher than differentiation in neutral marker

159 genes (F_{ST}). In addition, we did not find a correlation between pair-wise neutral genetic

160 distances and phenotypic distances of any of the three defensive traits. This suggests a

161 leading role of selection over neutral processes in driving the divergence of chemical

162 defense of *D. stramonium*.

163   Previous studies have recorded ample geographic variation in trichome density and

164 tropane alkaloids in populations of *D. stramonium* in Central México (Castillo *et al.*, 2013).

165 Accordingly, we detected significant geographic variation in the studied defensive traits

166 (see Table 1). Moreover, we found that phenotypic differentiation values of both atropine

167 and scopolamine concentration exceeded F_{ST}, pointing that contrasting natural selection

168 exerted by herbivores play a relevant role in the among-population differentiation of

169 chemical defense of *D. stramonium*. The lack of correlation between pair-wise neutral

170 genetic distances and phenotypic distances from the studied defensive traits also suggests

171   that neutral processes alone do not account for among-population variation of plant

172   defense.

173   Selection by herbivores is considered a major force that maintains the diversity of

174   defensive traits within and among species (Rausher, 2001; Anderson & Mitchell-Olds,

175   2011). However, few studies have explored whether natural enemies constitute a

176   predominant force promoting geographic variation on plant defense. Recently, studies by

177   Fine et al., (2013) and Misiewicz & Fine (2014) shown evidence of natural selection (trough

178   change in herbivores composition in different soils types) promoting phenotypic divergence

179   of leaf thickness, leaf pubescence and chemical investment among populations of the

180   Amazonian tree *Protium subserratum.* Similarly, Züst et al. (2012) elegantly inferred that

181   the abundance and selection by two specialist herbivores have promoted the geographic

182   variation of chemical defense of *Arabidopsis thaliana*. Here, we found evidence of adaptive

183   differentiation on atropine and scopolamine concentration in Mexican populations of *D.*

184   *stramonium*. In support of this, Castillo et al. (2014) recently found evidence of spatially

185   variable selection by specialists and generalists herbivores, reducing atropine and favoring

186   scopolamine concentration. This pattern of selection is likely to account for the observed

187   among-population differentiation of chemical defense of *D. stramonium*.

188   Although P_{ST} is a suitable option for estimating phenotypic divergence when Q_{ST}

189   estimates are unavailable, it can be biased due to environmental and non-additive genetic

190   effects (Merilä & Crnokrak, 2001; Leinonen *et al.*, 2006). However by testing two plausible

191   scenarios of heritability ($h^2 = 0.5$ and $h^2 = 1$), we found higher differentiation in putative

192   adaptive traits (tropane alkaloids) than expected by random differentiation. Although these

193   estimates are likely to be influenced by environmental and non-additive genetic effects,

194 previous studies have found genetic variation and among population differences for

195 resistance to herbivory (Fornoni *et al.*, 2003). Furthermore, a common garden experiment

196 (that included four of the populations used here) showed traces of genetic among-

197 population differentiation for scopolamine and atropine concentration (G. Guillermo

198 Castillo, unpublished. data). This indicates that our estimates of population differentiation in

199 defensive traits in *D. stramonium* are realistic.

200

201 **Conclusion**

202 Uncovering the mechanisms behind patterns of phenotypic differentiation of natural

203 populations remains as a major challenge for evolutionary ecology (Mazer & Damuth,

204 2001; Leinonen *et al.*, 2006; Leinonen *et al.*, 2013). Here, we showed evidence congruent

205 with adaptive divergence in chemical defensive traits of *D. stramonium*. Further studies

206 must assess explicitly the role of selection by multi-specific herbivores communities and

207 local adaptation to herbivores in generating and maintaining the ample among-population

208 divergence of defensive traits observed in natural populations.

209

210 **Acknowledgements**

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378 Table 1. Vegetation type, latitude, longitude, altitude and population means of leaf trichome density, and atropine and

379 scopolamine concentrations of 13 populations of *Datura stramonium* in Central and Southern Mexico. DS = desert shrub,

380 POF = Pine-Oak Forest, TDF = tropical deciduous forest.

381

	Vegetation Type	Latitude	Longitude	Altitude (m)	Trichome density (2.5×nmm²)	Atropine (mg/g)	Scopolamine (mg/g)
1. - Acatzingo	DS	-97.78	19.32	2160	9	0.296	0.159
2. - Atlixco	DS	-98.42	18.98	1840	9.48	0.665	0.611
3. - Esperanza	DS	-97.37	18.85	2278	7.29	0.477	0.331
4. - Patria Nueva	DS	-98.96	20.38	2040	9.9	0.255	0.39
5. - Taxco	TDF	-99.66	18.5	1582	8.32	0.826	0.146
6. - Teotihuacán	DS	-98.86	19.68	2294	8.73	0.437	0.354
7. - Ticumán	TDF	-99.2	18.86	1210	7.12	0.843	2.06
8. - Tlaxiaca	DS	-98.86	20.08	2340	9.37	0.289	0.458
9. - Tula	DS	-99.35	20.05	2020	6.3	2.373	2.134
10. - Tzin Tzun Tzan	POF	-101.58	19.63	2050	4.28	0.935	1.532
11. - Valsequillo	DS	-98.11	18.91	2209	6.1	1.768	0.045
12. - Xalmimilulco	POF	-98.38	19.20	1200	4.77	2.045	2.024
13. - Zirahuén	POF	-101.91	19.43	2174	4.91	0.619	1.968

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386   Table 2. Significance values after a Monte Carlo procedure testing the hypothesis that P_{ST}

387   values of defensive traits (in two heritability scenarios) are larger than F_{ST} in 13 populations

388   of *Datura stramonium.*

| Trait	$h_2 = 1$	$h_2 = 0.5$
a) Atropine	0.4074	**0.0426**
b) Scopolamine	**0.0382**	**0.0109**
c) Trichomes density	0.6461	0.0826

389

390

391   Table 3. Correlation between neutral differentiation matrix (F_{ST}) and phenotypic

392   differentiation matrix (P_{ST}) of defensive traits of *Datura stramonium* under two scenarios of

393   heritability ($h^2 = 1$ and $h^2 = 0.5$).

Variable	by	*r*	*P*	*r*	*P*
		$h^2 = 1$		$h^2 = 0.5$	
F_{ST}	Atropine	-0.0637	0.5795	-0.0655	0.5689
F_{ST}	Scopolamine	0.0278	0.8091	0.0348	0.7623
F_{ST}	Trichomes density	-0.0855	0.4568	-0.1053	0.3589

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404   Figure 1.

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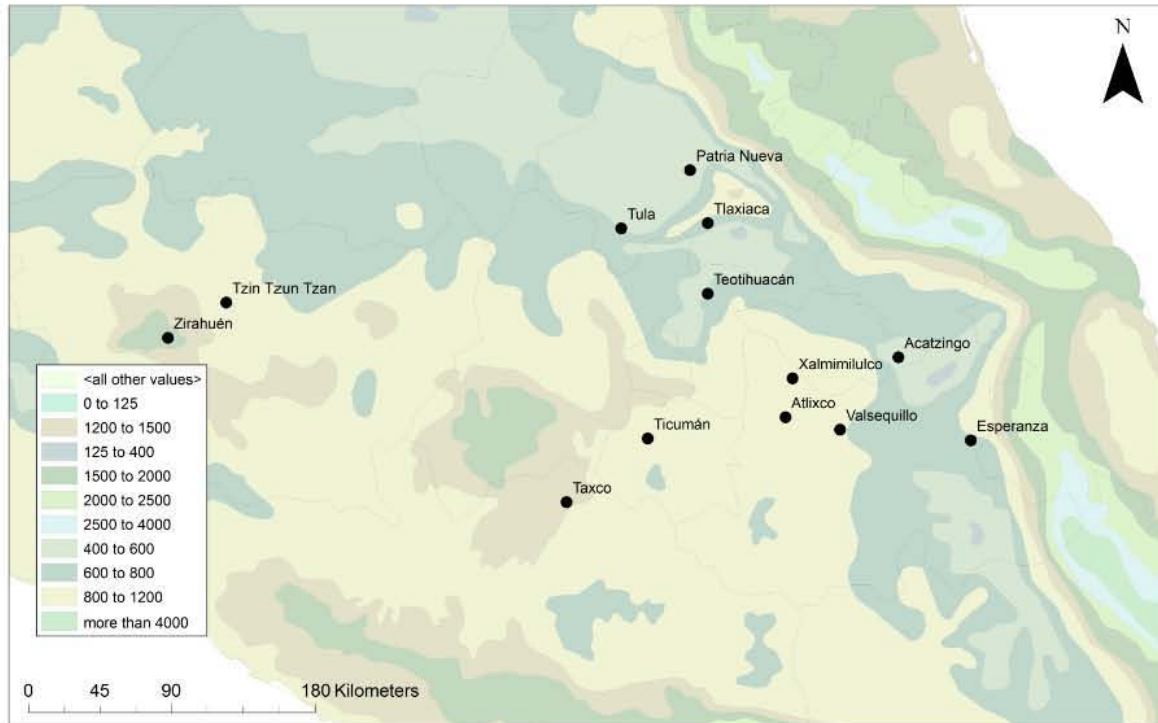
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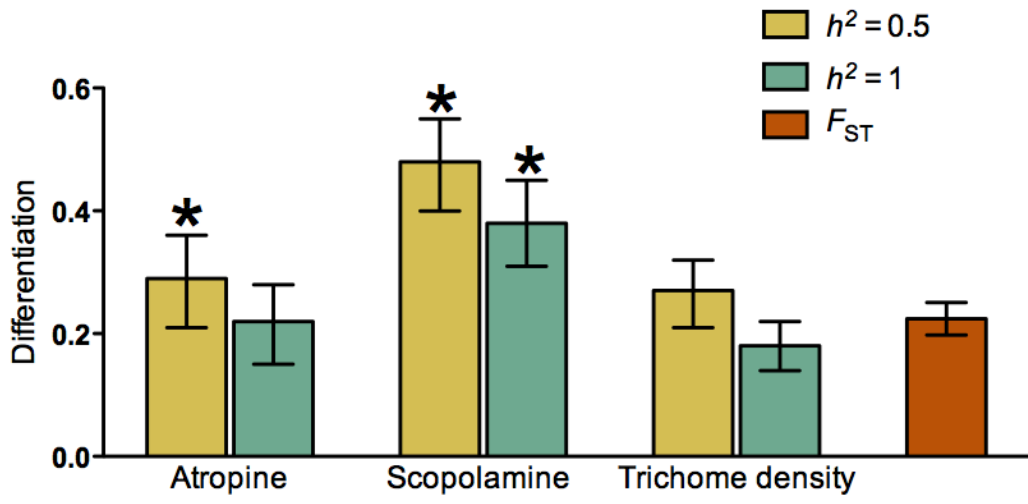
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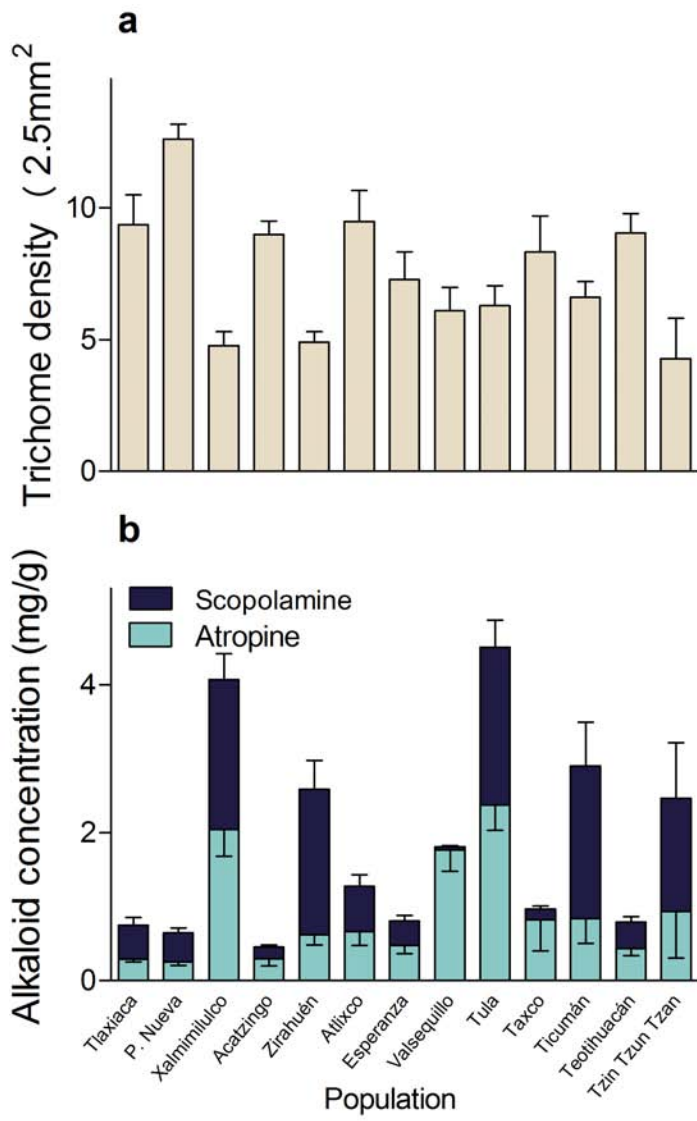417   Figure 2

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421   Figure S1

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427   Figure legends

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429   Figure 1. *Datura stramonium* populations sampled in Central México (see Table 1).

430

431   Figure 2. P_{ST} values of defensive traits of *Datura stramonium* in two scenarios of heritability

432   (h^2 = 0.5 and h^2 = 1). Error bars represent 95% confidence intervals. * Represents overall

433   P_{ST} values that differ significantly from F_{ST} after a Monte Carlo test (10,000 deviates from

434   both P_{ST} and F_{ST}).

435

436   Figure S1. Among-population variation in *a)* leaf trichome density and *b)* atropine

437   concentration, and scopolamine concentration in 13 populations of *Datura stramonium* in

438   central Mexico. Bars represent average value +1 SE.

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Discusión

Desentrañar los mecanismos que generan y mantienen la variación de atributos entre las poblaciones de una especie ha representado desde los tiempos de Darwin y hasta ahora gran reto para los biólogos evolutivos (Darwin, 1859; Mazer & Damuth, 2001; Leinonen *et al.*, 2013). El objetivo de esta tesis fue el estudiar a nivel geográfico la variación de los atributos defensivos que median la interacción planta-herbívoro entre la planta anual *D. stramonium* y sus herbívoros.

Los resultados del capítulo I revelaron una gran variación geográfica en el daño foliar ejercido por herbívoros, así como en atributos defensivos químicos y físicos en *D. stramonium*. También mostraron una asociación geográfica positiva de la densidad de tricomas y la concentración de atropina con el daño foliar por herbívoros. Este tipo de relación es de esperar en un escenario en dónde mayor inversión en atributos defensivos no reduce los niveles de herbivoría, ya que los herbívoros son capaces de "sobreponerse" a las barreras defensivas que en el pasado pudieron ser eficaces (Janzen, 1980; Berenbaum *et al.*, 1986; Zangerl & Berenbaum, 1993; Lively *et al.*, 2004). Este resultado sugiere que el herbívoros especialista *L. daturaphila* (presente en la mayoría de las poblaciones mexicanas de *D. stramonium*) "va ganando la carrera armamentista" con la planta, de manera similar a lo que se ha reportado en otros sistemas antagonistas (see Hanifin *et al.*, 2008). Los resultados también sugieren que los herbívoros especialistas pueden usar la defensa química (atropina y escopolamina) de las plantas para identificar y seleccionar individuos en una población, como ha sido sugerido en la literatura (Agrawal & Heil, 2012). Estudios previos (Agrawal & Heil, 2012) han demostrado que la escopolamina puede actuar como fago-estimulante del herbívoro oligófago *E. parvula* (Shonle & Bergelson, 2000)*.*

En este contexto y dado que a lo largo de la distribución en México de *D. stramonium*, existen poblaciones en las que *L. daturaphila* está ausente y en las cuales el herbívoro

oligófago *E. parvula* y el generalista *S. purpurascens* son los principales herbívoros del toloache, en el capítulo II exploré las presiones selectivas ejercidas por estos herbívoros sobre la atropina, escopolamina y la densidad de tricomas foliares en diferentes poblaciones de *D. stramonium* que difieren en su herbívoro predominante. También evalué si un alcaloide más derivado (escopolamina) confiere un beneficio adaptativo más grande que su precursor (atropina) y si este efecto difiere entre herbívoros especialistas y generalistas. Los resultados revelaron una amplia variación geográfica en los patrones selectivos sobre los atributos defensivos de *D. stramonium*. A pesar de dicha variación espacial, fuimos capaces de detectar efectos particulares asociados al tipo de herbívoro sobre la defensa de *D. stramonium*. En general, todos los herbívoros estudiados seleccionan una reducción en la concentración de la atropina, el alcaloide más "viejo", sugiriendo que este metabolito secundario ya no confiere un "beneficio" como agente disuasivo de la herbivoría y que poseerlo en mayor cantidad conlleva un costo para las plantas. Además la escopolamina, el alcaloide más derivado, resultó más efectiva en contra del herbívoro generalista *S. purpurascens* lo cual apoya también nuestra expectativas iniciales. A pesar de que estudios previos en poblaciones naturales de *D. stramonium* (Fornoni et al., 2003) han encontrado selección a favor de niveles altos de tricomas foliares, en este capítulo detecté sólo un efecto marginal de la densidad de tricomas sobre la adecuación en la mayoría de poblaciones estudiadas. En general, el patrón selectivo observado en contra de la producción de atropina es consistente con los resultados del capítulo I

En el tercer capítulo de la tesis evalué la existencia de adaptación local a herbívoros generalistas y especialistas en *D. stramonium* mediante un experimento de trasplantes recíprocos. La adaptación local es un proceso central en la generación y el mantenimiento de atributos que median interacciones antagonistas (Laukkanen *et al.*, 2012). A pesar de esto, pocos estudios han evaluado el papel de la adaptación local de las plantas a sus enemigos naturales como es el caso de sus herbívoros (Ortegón-Campos *et al.*, 2009). Más aún, la mayor parte de la evidencia existente proviene de sistemas especialistas (Berenbaum, 1981; Nitao, 1989; Gandon & Michalakis, 2002), donde se espera que exista

adaptación local y muy pocos trabajos han evaluado la existencia de adaptación local en sistemas generalistas, en los cuales detectar adaptación local parecería menos probable (Gómez *et al.*, 2009; Ortegón-Campos *et al.*, 2009). Además dado que se ha reportado que el daño foliar tiene un efecto negativo en la adecuación de *D. stramonium* (Nuñez-Farfan & Dirzo, 1994), es de esperar que la adaptación a herbívoros esté mediada por los atributos defensivos exhibidos por las plantas como la densidad de tricomas foliares. Los resultados mostraron que las poblaciones de *D. stramonium* pueden adaptarse tanto a herbívoros especialistas como a generalistas. Sin embargo, no todas las poblaciones están adaptadas a su herbívoro principal. Este resultado parece reflejar la existencia de un mosaico geográfico de adaptaciones locales, como es predicho por la Teoría del Mosaico geográfico de Coevolución (Thompson & Cunningham, 2002; Thompson, 2005). Por otro lado, a pesar de que la evidencia previa indica que densidad de tricomas foliares es un carácter que está sujeto a fuerte selección por los herbívoros (Valverde *et al.*, 2001; Kariñho-Betancourt, 2009), no encontré evidencia de que la adaptación local observada sea mediada por la densidad de tricomas foliares. Esto sugiere que son síndromes de defensa (*i.e.,* conjunto de caracteres defensivos) los que están mediando la interacción planta herbívoro a nivel local, como es sugerido en la literatura (Agrawal & Fishbein, 2006; Leimu & Koricheva, 2006) y concuerda con resultados del capítulo II que indican la existencia de amplia selección correlativa actuando sobre la defensa de *D. stramonium* (en poblaciones utilizadas en el experimento de trasplantes recíprocos).

Finalmente, en el capítulo IV evalué si procesos neutros (*e. g.,* deriva génica o flujo génico) y/o las presiones selectivas están promoviendo la diferenciación fenotípica en atributos defensivos en 13 poblaciones mexicanas de *D. stramonium*. Para ello comparé el grado de diferenciación fenotípica (P_{ST}) de tres atributos defensivos de *D. stramonium* (tricomas foliares, atropina y escopolamina) con el grado de diferenciación genética neutra (F_{ST}) estimado a partir de 5 loci microsatélites. Los resultados indicaron que la diferenciación fenotípica (P_{ST}) de la escopolamina y atropina es más grande que la diferenciación en loci microsatélites (F_{ST}). Este resultado es congruente con la los patrones selectivos sobre ambos alcaloides encontrados en el capítulo II de esta tesis.

Además no encontré evidencia de una correlación entre el grado de diferenciación fenotípica de atributos defensivos y las distancias genéticas pareadas. Estos resultados son congruentes con un escenario en el que las presiones selectivas ejercidas por herbívoros están promoviendo la diferenciación de atributos defensivos, como es predicho por la teoría (Rausher, 2001; Anderson & Mitchell-Olds, 2011). Recientemente Züst et al. (2012) infirió de manera elegante que la abundancia y presión selectiva ejercida por dos herbívoros especialistas han guiado la variación geográfica de la defensa en poblaciones de *Arabidopsis thaliana*. Sin embargo, pocos trabajos han estudiado el papel de los herbívoros en moldear las diferencias geográficas en atributos defensivos de plantas.

En conjunto, esta tesis constituye un esfuerzo por entender la interacción planta herbívoro en un contexto geográfico. A lo largo de la tesis intenté hacer hincapié en la importancia de considerar diferentes atributos defensivos y a los diferentes tipos de herbívoros en el estudio de la interacción planta-herbívoro. Este trabajo resalta la necesidad de combinar experimentos de campo con evidencia obtenida de poblaciones naturales para responder preguntas acerca de la evolución de la defensa en plantas.

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