Natural selection drives chemical resistance of *Datura stramonium* ~~to its specialized seed predator~~

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Abstract

Plant resistance to herbivores involves physical and chemical plant traits that affect herbivores fitness and hence may promote coevolutionary arm-races between interacting species. *Datura stramonium* possesses tropane alkaloids that prevent and/or reduce damage by leaf beetles, but it is not known whether the specialist weevil, *Trichobaris soror* exert selection on these ~~these~~ resistance characters~~ function to reduce seed predation by the specialist weevil, *Trichobaris soror*, and are affected by natural selection~~. We measured infestation by *T. soror* as well as the concentration of the plants´two main tropane alkaloids ~~to~~ in 279 ~~plants of~~ *D. stramonium* plants ~~across~~ belonging to 31 populations in central Mexico,~~ as well as the concentration of the plants´two main tropane alkaloids~~. We assessed whether seed predators exerted preferences on the levels of both alkaloids and whether they affect plant fitness. ~~Overall, we found great variation in infestation by *T. soror* to plants of *D. stramonium*.~~ Atropine concentration relates negatively to both the proportion of infested fruits per plant, and to the intensity of infestation (weevils/fruit). In contrast, scopolamine concentration correlates negatively with the number of sound, remaining, seeds per plant. Infestation was higher in dry, high-altitude, as compared to wet lower-altitude habitats. The detection of positive selection differential of atropine's concentration in populations where the impact of seed predators is stronger indicates that higher atropine concentrations are favored regardless the average level in the population. Yet, atropine is selected against in many populations too. The among-populations variation of *D. stramonium* in infestation by *T. soror*, in the concentrations of the two main alkaloids, atropine and scopolamine, and the direction and strenght of natural selection on these, may result from a coevolutionary interaction with its specialized seed predator.

Comentario [msb1]: Since you address plant fitness and not insect fitness in this paper, saying this would be confusing. I would rewrite this sentence otherwise.

Comentario [msb2]: ?

Subjects. Ecology, Evolutionary Studies

Key-words: *Trichobaris soror*, *Datura stramonium*, natural selection, plant resistance, seed predator, tropane alkaloids, Selection differentials

Introduction

The coevolutionary process involves reciprocal selection-adaptation between interacting species through time (Dawkins & Krebs 1979; Thompson 1999; Thompson 2005). This adaptation and counter-adaptation phenomenon could result in a coevolutionary arms race, a process of offense-defense (Dawkins & Krebs 1979). A coevolutionary relationship between plants and herbivores may generate symmetrical or asymmetrical selective pressures between interacting species. These selective pressures may be different spatially and could produce a geographic structure of interactions (Forde et al. 2004; Gomulkiewicz et al. 2002; Nuismer et al. 2000; Thompson 1999; Thompson 2005). In some locations the interacting species exert reciprocal selection pressures to one another (coevolutionary hotspots), whereas in other locations reciprocal selection is highly asymmetric (coevolutionary coldspots) (Gomulkiewicz et al. 2002; Nuismer et al. 2000; Thompson 1999; Thompson 2005).

A coevolutionary arms-race between herbivores and plants may be favored in specialized interactions as in the case of insects that detoxify specific compounds (Janzen 1969; Janzen 1973; Schoonhoven et al. 2005). For instance, the aphid *Macrosiphum albifrons* is adapted to consume *Lupinus angustifolius* with a low content of alkaloids (Philippi et al. 2015); however, dietary specialist herbivore insects may also be adapted to tolerate secondary metabolites of their host plants without switching to other different host plants (Laukkanen et al. 2012). In *Arabidopsis thaliana* the abundance of two aphid herbivore species is correlated to a genetic polymorphism of the plant´s resistance locus. This polymorphism is under selection due to changes in population size of the two aphid species (Züst et al. 2012).

Host-parasite systems, that exert reciprocal selection pressures, offer the opportunity to assess the asymmetry in selection pressures and the potential to produce adaptation (Greischar & Koskella 2007). Local adaptation may produce population differentiation as a by-product of natural selection (Kawecki & Ebert 2004). This process is well illustrated by the weevil *Curculio camelliae* (Coleoptera: Curculionidae) that parasitizes the fruits of *Camellia japonica* (Theaceae) (Iseki et al. 2011; Toju 2007; Toju 2009; Toju & Sota 2006). The fruits of *C. japonica* are capsules with a thick pericarp, dehiscent, with three locules and one seed per cavity (Okamoto 1988). Females of *C. camelliae* perforate the thick pericarp with its long *rostrum*, modified labial cavity in insects, (Resh & Cardé 2009), and oviposit on the seeds (Toju 2007). A successful weevil infestation, or oviposition, depends on the phenotypic match between the rostrum length and pericarp thickness. These two traits that mediate the interaction vary geographically and are under selection (Toju 2007; Toju 2009; Toju & Sota 2006). Some evidence, however, indicates that these phenotypic characteristics may also vary according to abiotic factors, i. e., the latitude (Iseki et al. 2011). Furthermore, infestation by the weevil *C. camelliae* increases at higher-altitude localities and its obligated host plant decreases its resistance (Toju 2009). In the *C. camelliae*-*C. japonica* system, natural selection acts on pericarp thickness that is a physical barrier that prevents infestation by weevils (Toju 2007; Toju 2009; Toju 2011; Toju & Sota 2006).

In the annual herb *Datura stramonium* tropane alkaloids function either as resistance characters preventing foliar damage by herbivores and/or as phagostimulants to them (Castillo et al. 2013; Castillo et al. 2014; Shonle & Bergelson 2000). Evidence shows that alkaloid concentration in *D. stramonium* varies across populations (Castillo et al. 2014). In some populations, atropine concentration is selected against by dietary specialist and generalist folivores, whereas scopolamine is negatively selected by the dietary specialist folivore *Lema daturaphila* and positively selected by the generalist grasshopper *Sphenarium purpurascens* (Castillo et al. 2014). Fruits of *D. stramonium* are parasitized by *Trichobaris soror* (Coleoptera: Curculionidae) that reduces plant fitness by consuming the seeds (Cabrales-Vargas 1991; Cruz 2009; De-la-Mora et al. 2015). However, to what extent alkaloids of *D. stramonium* could affect infestation by the seed predator is not known. Here, we analyzed the relationship between tropane alkaloids produced by *D. stramonium* and infestation by the specialized seed predator *T. soror* across multiple populations in central Mexico. We aimed to determine whether *D. stramonium*'s tropane alkaloids prevent infestation by *T. soror*. Specifically, we addressed the following questions. 1. Are alkaloids resistance characters that prevent/reduce infestation by weevils? 2. Do seed predators exert natural selection upon plant´s alkaloids concentration? and 3. Do variation of both alkaloid concentration and infestation by weevils across populations is correlated to the localities' environmental conditions? *v.gr*. (Toju 2009).

Materials and methods

Study system

The weevil *Trichobaris soror* (Coleoptera: Curculionidae) is intimately associated to the life cycle of *D. stramonium* (Borbolla 2015; Cabrales-Vargas 1991). *Trichobaris soror* is distributed mainly in central Mexico (Barber 1935; De-la-Mora et al. 2015); adult weevils feed on leaves, calyx and floral tissues of *D. stramonium*. After mating, females oviposit at the base of developing fruits. Their larvae feed exclusively on immature seeds inside the developing fruit where they build tunnels with their own feces. Larvae pupate in the fruit and sometimes are parasitized by wasps. The weevils hibernate inside the fruit of *D.*

stramonium until the next rainy season (Borbolla 2015; Cabrales-Vargas 1991).

Besides the seed predator, *D. stramonium* (Solanaceae) is preyed upon by specialist leaf-beetles (*Lema trilineata* and *Epitrix parvula*, Coleoptera: Chrysomelidae). This weed species has been widely studied in relation to its resistance characters (alkaloids, leaf trichomes) against these leaf herbivores (Bello-Bedoy & Núñez-Farfán 2011a; Cabrales-Vargas 1991; Carmona & Fornoni 2013; Castillo et al. 2013; Castillo et al. 2014; Núñez-Farfán & Dirzo 1994; Shonle & Bergelson 2000; Valverde et al. 2001). However, it is unknow if chemical defense of *D. stramonium* prevents the infestation by the weevil *T. soror*.

Sampled populations

During the reproductive season of *D. stramonium* (September-November) in 2007, we sampled different populations across Central Mexico. We collected an average of 30 plants from 31 populations (Fig. 1, Table 1). For all plants, each mature fruit collected was bagged and labeled. Before opening, the width and length of each fruit was measured to calculate its volume with the equation:

Con formato: Resaltar

Comentario [msb3]: Explain whether all fruits per plant were collected

$$V = \left(\frac{4}{3}\right)(\pi)(lenght)\left(\left(\frac{width}{2}\right)^2\right)$$

We estimated the number of expected seeds by multiplying the volume by 0.026 (Fornoni et al. 2004). In the laboratory, we opened the fruits to determine the infestation, by counting the weevils. Also, for each fruit we counted the number of remaining, sound, seeds after predation.

Seed predator infestation measures

We measured the intensity of infestation by *T. soror* to *D. stramonium* as the number of weevils divided by the number of fruits per plant (Greischar & Koskella 2007). Also, we measured the infestation as the proportion of infested fruits per plant.

Alkaloid concentration

We quantified the concentration of leaf atropine and scopolamine per plant (Boros et al. 2010), by means of high-performance liquid chromatography (HPLC), following Castillo et al. (2013). We obtained the average leaf alkaloid concentration from a sample of 8-10 plants per population. Additionally, we measured alkaloid concentration of mature seeds in 8-10 plants of 15 populations of *D. stramonium* in order to assess if alkaloid concentration of leaves and seeds are correlated (Table S1).

Comentario [msb4]: This alkaloids in leafs and seeds were measured in the same plant individuals right?

Characterization of environmental variables

In order to control for some concomitant environmental variation, t~~T~~he values of mean annual temperature and precipitation for the 31 studied populations of the *D. stramonium* were obtained from Worldclim (Hijmans & Graham 2006). In order to characterize each population, we calculated the Lang´s aridity index (Rehman 2010) as the ratio of population mean annual precipitation and mean annual temperature. Values between 0-20 correspond to desert, 20-40 arid habitats, 40-60 wet type steppes, 60-100 wet woodland and 100-160 wet forests (Perry 1986).

Statistical analyses

The among-population variation in the intensity of infestation and the proportion of infested fruits was assessed by Wilcoxon Chi-square tests, with the null hypothesis that infestation by the seed predator does not vary among populations.

Comentario [msb5]: How so?

This should be done by generalized linear modles

We assessed the correlation between the population average of alkaloid concentration of seeds and leaves by means of Pearson's correlation. Also, we evaluated whether the intensity of infestation and the proportion of infested fruits were correlated between them. In order to assess whether alkaloids function as resistance traits against seed predation, we carried out regression analysis of the intensity of infestation and the proportion of infested fruits, on the concentration of leaf alkaloids across populations. Additionally, we tested if the concentration of (1) atropine and (2) scopolamine increases plant fitness, measured as both the mean of remaining sound seeds, and the number of non-infested fruits, by means of linear regression.

Comentario [msb6]: This should be done at the plant individual level, not the population unless measures have been taken form different individuals.

NOTE that in the data file it is not specified whether alkaloid values belong to leaves or seeds…Please specify

Comentario [msb7]: Why is it necessary to know this?

Comentario [msb8]: How come regressioin?

Did you use the population average? You should rather use the plant level measures in generalized linear mixed models in which population should be random factors

Comentario [msb9]: Again, this needs to be done at the plant individual level. Not the population llevel.

Selection differentials ~~-~~(*S*) that account for direct and indirect selection acting on a trait were calculated through Pearson correlation coefficient for each population (Lande & Arnold 1983). The concentration of atropine and scopolamine was standardized ($x' = \frac{(x-\mu)^2}{\sigma}$).

Comentario [msb10]: You did this at the plant level for each population as I can see in the dataset. This is correct.

This make me thibnk that the lines before are nor properly explained and also that wwas correctly analyzed. Is this right?

As a proxy for p~~P~~lant fitness ~~was~~ we used ~~obtained as~~ the ~~expected~~ estimated number of seeds per plant ~~(see above)~~, as an estimator of potential individual fitness ~~(Lande & Arnold 1983)~~(other examples in Kingsolver at al. 2001). The relative fitness was defined as the ratio between individual fitness and population average fitness. Selection differentials were estimated for each population by correlating alkaloid concentration standardized (atropine or scopolamine) with relative fitness (such in Sobral et al. 2015). Statistical analyzes were performed with JMP v10.0 statistical package (SAS 2012).

Comentario [msb11]: Kingsolver JG, Hoekstra HE, Hoekstra JM, Berrigan D, Vignieri SN, Hill CE et al. (2001) The strength of phenotypic selection in natural populations. Am Nat 157: 245-261.

In order to explore the effect of selection exerted by the seed predation and environmental variables on ~~mean remaining sound seeds and the proportion of infested fruits~~ across populations of *D. stramonium*, we performed two linear mixed effect models, LMM; (such in Sobral et al. 2013). In the first model, the number of sound seeds was the response variable while the population term is a random factor; the covariates were the concentration of each alkaloid, the selection differential of each alkaloid, the proportion of infested fruits together with latitude, longitude, altitude and Lang's index of populations. In the second model, the proportion of infested fruits was the response variable and the same covariates, except the proportion of infested fruits. We estimated the Variance Inflation Factor (VIF) before using the variables in each model. The VIF is a measure of multicollinearity that uses the proportion of the variance that is not shared between the variables studied. The variables with values of VIF > 10 indicate severe collinearity and therefore must be eliminated of the multivariate models (O'brien 2007). Previous analyses indicated that all variables had VIF < 5 (Table S2) and then used in the LMM. To obtain the best reduced LMM, the variables with a significance value less than 0.05 were retained (Zuur et al. 2009). We selected the models with lowest AIC values, namely those that explain better the relationship between the variables (Akaike 1974). LMM analyses were estimated using the function nlme in R Core Team v.3.2.2 (R 2011).

Comentario [msb12]: I tthis models the response variables should be the alkaloid concentration at the population level, which is the selected trait. So the one which makes sense to analyse in function of the among population selective pressures

Comentario [msb13]: Please change for the pertinent response variables (a model for scopolamine in seeds, other por leaves, other for both, other for atropine…)

If you find that selection coefficients are related to the average level of alkaloid in one or several of theise models you can conclude that selection on this traits drive their among population variation, thus they are evolving.

Con formato: Tachado

Comentario [msb14]: Also LMM are used if the response variable is normal, in the case of seeds they are not, so ther are not the correct models, for number of seeds you need to used generalized models fitted top poisson distributions.

But note that for the correct responses to be included (average alkaloids per population) it is likely that you will be ok with LMM.

Comentario [msb15]: This should be the responses

Comentario [msb16]: This does not need to be included

Comentario [msb17]: no

Results

The intensity of infestation as well as the proportion of infested fruits varied significantly among populations ($\chi^2 = 400.1647$, *d. f.* = 30, $P < 0.0001$; $\chi^2 = 383.2304$, *d. f.* =30, $P < 0.0001$, respectively) (Fig. 2). The average intensity of infestation by *T. soror* varies from populations without infestation up to those with 5 weevils per fruit, per plant. Variation in the average proportion of infested fruits ranged from zero (populations Coatepec, Huitzuco and Jalapa) up to 90% (populations Teotihuacan and Tlaxiaca; Fig. 2).

The concentration of atropine in leaves and seeds are positive and significantly correlated ($r = 0.6276$, $P = 0.0123$, $N = 15$). Similarly, the concentration of seed alkaloids, atropine and scopolamine, showed a positive correlation between them ($r = 0.5851$, $P = 0.0219$, $N = 15$). No correlation between the concentration of scopolamine in leaves and seeds was detected.

Comentario [msb18]: why did not you thi at the plant level

We found a high and positive correlation between the proportion of infested fruits and intensity of the infestation ($r = 0.8994$, $P < 0.0001$, N = 31). The mean of sound remaining seeds showed a negative correlation to the proportion of infested fruits ($r = -0.5545$, $P = 0.0012$, $N = 31$) and to the intensity of the infestation ($r = -0.5306$, $P = 0.0021$, $N = 31$). The negative correlation between leaf atropine concentration and both the proportion of infested fruits ($r = -0.4134$, $P = 0.0208$, $N = 31$, Fig. 3A) and the intensity of infestation ($r = -0.3270$, $P = 0.0725$, $N = 31$, Fig. 3B), indicates that this alkaloid reduces infestation by the seed predator. Besides, the number of uninfested fruits increases as atropine concentration increases ($r = 0.4288$, $P = 0.0161$, $N = 31$, Fig. 3C), but it is uncorrelated with the mean of remaining sound seeds ($r = 0.1147$, $P = 0.539$, $N = 31$). The scopolamine concentration is negatively correlated with the number of remaining sound seeds ($r = -0.36$, $P = 0.0467$, $N=31$, Fig. 3D).

In the first linear mixed effect model (LMM), the remaining sound seeds were negatively related to the selection differential of atropine concentration across populations and with the proportion of infested fruits (Table 2, Fig. 4A, 4B). The second LMM indicates that the proportion of infested fruits was correlated negatively to Lang’s index and positively

Comentario [msb19]: unfortunately, this models lacks any conceptual significance. Selection acts on the phenotypic trait (the alkaloid) and not on the fitness.

Please re-run this model again for the proper response variables.

to altitude of the localities (Table 2, Fig. 4C, 4D).

Comentario [msb20]: I HAVE JUST TAKEN THE LICENCE TO PERFORM THESE MODELS IN THE CORRECT DIRECTION, USING the dataset in your submission and the good news is that there is a very clear effect of the selection over scopolamine on the scopolamine population average, inm the case of atropine there is also a effect although less string. And this even when controlling for environmental variables which also have an effect on both models.

I would recommend to do this also separating scopolamine form seed s and leaves etc

Note that actually the scopolamine concentrations at the population level are not norma, so please try with different distributions in a generalized linear models and choose the best model (aicc)

When distributions have a poisson shape but variables use decimal data, a nice trick is to eliminate decoimals and d¡fit to a poisson distribution (which only accept natural numbers), in order to get a good fit

Comentario [msb21]: Same than in previous comment

Discussion

The concentration of the alkaloid atropine varied across populations of *D. stramonium* and it was negatively related to both the intensity of infestation and the proportion of infested fruits by the specialist seed predator *T. soror*. We found that the concentration of atropine in seeds and leaves was positively associated across populations. Although such pattern was not detected for scopolamine, the concentration of both alkaloids in seeds shows a positive relationship across populations. Our major finding was that atropine concentration reduces infestation by *T. soror* weevils, functioning as a chemical resistance trait against the seed predator. It has not been previously reported that atropine is linked to resistance to seed predation in *D. stramonium*. The results also showed that mean plant fitness, as estimated by uninfested fruits, increases at higher concentration of atropine across populations. Moreover, we detected positive selection differentials in populations where the negative impact of infestation was more severe (i. e., the low values of remaining seeds). Thus, natural selection may favor to increase the concentration of atropine. Such populations may constitute hot spots where an asymmetric arms race process is taking place.

Previous studies have shown that atropine plays a role in the interaction between *D. stramonium* and its main folivore insects in central Mexico. For instance, populations of *D. stramonium* with higher concentration of atropine were more damaged by dietary specialist and generalist folivore insects, acting as a chemical clue that attract them and/or as a phagostimulant (Castillo et al. 2013; Castillo et al. 2014). In this study we found the opposite pattern: populations of *D. stramonium* with higher atropine concentration had a higher number of uninfested fruits. This suggests that atropine deters the seed predator *T. soror*. A similar trend has been found in the hemiparasitic plant *Castilleja indivisa* (Adler 2000), where the alkaloid lupanine, obtained from its host plant, *Lupinus texensis*, reduces damage to its flowers by herbivores and increases visitation by pollinators thus enhancing plant fitness, measured as the number of seeds.

In contrast, we found that populations of *D. stramonium* with higher scopolamine concentrations had a lower number of sound seeds. This suggests that scopolamine acts as a phagostimulant to *T. soror*. Selection to reduce scopolamine concentration was previously demonstrated by Shonle & Bergelson (2000) in one experimental population of *D. stramonium* attacked by folivore insects. Thus, the resistance role of tropane alkaloids of *D. stramonium* vary according to the type of herbivore and the tissue they consume. While scopolamine is positively selected by specialist folivores in some populations of *D. stramonium* from central Mexico, atropine is selected against (Castillo et al. 2014). In the case of *T. soror,* atropine is positively selected while populations with higher concentration of scopolamine had lowest values of remaining sound seeds. It seems that the level of the chemical resistance traits in *D. stramonium* may be determined, in part, by the differential or even opposed, selection pressures exerted by herbivores that consume different plant tissues.

Females and males of *T. soror* feed on *D. stramonium* leaves, producing small holes on theses. Also courtship and mating occurs on the plants (J. Núñez-Farfán, *personal observation*). It is possible that *T. soror*'s females select plants to oviposit while feeding, "assessing" the chemical resistance level of the plants (i.e., atropine/scopolamine concentration in leaves). Some evidence in this line shows a close relationship between *Trichobaris* and *Datura*. For instance, *Trichobaris bridwelli* oviposits preferentially on *D. stramonium* rather than on tobacco plants (*Nicotiana tabacum*) (Cuda & Burke 1991), since *T. bridwelli* does not tolerate the pyridine alkaloids of tobacco (Cuda & Burke 1991).

On the other hand, Diezel et al. (2011) have experimentally demonstrated that *T. mucorea*, a species that burrows into the stems of *Nicotiana attenuata*, increases infestation on transgenic plants of *N. attenuata* where the chemical defenses (jasmonic acid and nicotine) were silenced.

We found evidence of natural selection on chemical resistance of *D. stramonium* since higher atropine concentrations drove down seed predator infestation. The first LLM analysis revealed that remaining sound seeds are negatively related to selection differentials on atropine, implying that in populations where “virulence” of *T. soror* is very high, selection differentials are positive (cf. Fig. 4A). In these population the negative effect of infestation promotes increases in atropine concentration. Thus, in populations of *D. stramonium* where infestation is low, selection acts to reduce atropine concentration (left side of Fig. 4A). Thus populations of *D. stramonium* vary in the direction and strengtht of selection on chemical defense, an expectation of the geographic mosaic of coevolution (i.e., hotspots and coldspots; (Thompson 2005).

The second LMM analysis indicated that infestation by *T. soror* increases in populations at higher altitudes and lower Lang´s index vales. That is to say, populations from high and dry environments suffer more fruit infestation (cf. Fig. 4C, D; Castillo et al. 2013). Similar results were found in other studies in *D. stramonium* (Borbolla 2015; Hernández-Cumplido 2009). Likewise, in the interaction between *Curculio camelliae-Camellia japonica*, infestation by the weevil was higher at high altitudes, where the fruits of *C. japonica* trees have thinner pericarps (Toju 2009). In *D. stramonium* alkaloid concentration can be lower in dry and high altitude locations. In this sense, it has been reported alkaloid production may be water limited, decreasing in concentration in dry enviroments (v.gr., populations Actopan, Teotihuacan and Valsequillo;

Fig. 2) (Baricevic et al. 1999). Thus, the evolution of resistance to herbivores in *D. stramonium* can be environmentally conditioned.

Conclusions.

In summary, the results show that the lowest proportion of infested fruits as well as the intensity of infestation by *T. soror* are related the highest concentration of atropine in *D. stramonium*. Selection differentials of atropine were high and positive in populations where seed predation is more intense. Therefore, such populations may represent coevolutionary hotspots. Finally, the higher proportion of infested fruits occurred in population inhabiting high altitude and dry environments pointing that plant resistance also interacts with physical factors of the environment (Castillo et al. 2013).

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Competing interests

The authors declare no competing interests.

Author Contributions

- Adán Miranda-Pérez conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Guillermo Castillo, Johnattan Hernández-Cumplido, María Borbolla, Laura L. Cruz, Rosalinda Tapia-López, Juan Fornoni and César M. Flores–Ortiz, conceived and performed the experiments, and revised the manuscript.
- Pedro L. Valverde, analyzed the data, wrote the paper.
- Juan Núñez-Farfán, conceived and designed the study, analyzed the data, wrote and revised the paper,.

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