Evolutionary changes in plant tolerance against herbivory through a resurrection experiment

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Abstract

Both theoretical and empirical works have highlighted the difference in the evolutionary implications of host resistance and tolerance against their enemies. However, it has been difficult to show evolutionary changes in host defences in natural populations; thus, evaluating theoretical predictions of simultaneous evolution of defences remains a challenge. We studied the evolutionary changes in traits related to resistance and tolerance against herbivory in a natural plant population using seeds from two collections made in a period of 20 years. In a common garden experiment, we compared defensive traits of ancestral (1987) and descendant (2007) subpopulations of the annual plant *Datura stramonium* that shows genetic variation for tolerance and to which the specialist herbivore *Lema daturaphila* is locally adapted. We also examined the effects of different plant genotypes on the herbivore for testing the plant genetic variation in resistance. Based on the response to the contemporary herbivore populations, results revealed a nonsignificant response in plant resistance traits (herbivore consumption, foliar trichomes and tropane alkaloids), but a significant one in tolerance. The survival of herbivores in laboratory experiments depended on the plant genotype, which suggests genetic variation in plant resistance. Although we cannot identify the selective agent for the change nor exclude genetic drift, the results are consistent with the expectation that when resistance fails to control herbivory, tolerance should play a more important role in the evolution of the interaction.

Introduction

The antagonistic interaction between plants and herbivores involves reciprocal selective pressures through host resistance traits that usually result in an escalating arms race (Futuyma & Agrawal, 2009). Although resistance traits reduce the amount of damage from antagonists, tolerance traits reduce the negative effects of damage on host fitness (Rosenthal & Kotanen, 1994; Strauss & Agrawal, 1999; Boots *et al.*, 2009; Råberg *et al.*, 2009). In contrast to resistance, host tolerance is not expected to reduce individual enemy fitness, and it

can decrease or increase attack at the population level (Espinosa & Fornoni, 2006; Miller *et al.*, 2006; Fornoni, 2011). Therefore, the coevolutionary arms race between host resistance and enemy counter-resistance traits can be modified whenever selection favours tolerance over resistance (Roy & Kirchner, 2000; Stinchcombe & Rausher, 2002; Espinosa & Fornoni, 2006).

Apart from the theoretical expectation that resource limitations influence the cost of each defence (Tiffin, 2000; Fornoni *et al.*, 2004; Restif & Koella, 2004; Wise & Abrahamson, 2005), few, if any, studies attempt to describe the ecological scenarios that affect the cost/benefit ratio of tolerance and resistance. For example, counteradaptation by natural enemies can reduce the benefits of resistance traits in the host, affecting the relative effectiveness of resistance and tolerance (Blau *et al.*, 1978; Becerra, 1994; Jokela *et al.*, 2000; Després *et al.*, 2007). It has been suggested that the degree to which the herbivore is adapted to the host resistance is

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a factor that can reduce the benefit of resistance, increasing the opportunities of selection for tolerance (Garrido *et al.*, 2012). However, the relationship between herbivore local adaptation to the host and the evolutionary response of host tolerance and resistance remains unexplored. Therefore, it was our aim to obtain direct evidence of short-term evolutionary changes in the host component of resistance and tolerance to provide insights about how these defensive strategies are simultaneously evolving in natural populations and to better understand the conditions that favour the evolution of different defensive strategies.

Studies in natural populations suggest that evolution can occur quickly – on timescales comparable with those of ecological dynamics (Thompson, 1998; Hendry & Kinnison, 1999; Grant & Grant, 2002; Hairston *et al.*, 2005). This information has shifted the perception that evolution requires vast periods of time (Darwin, 1859) and a merger of evolutionary and ecological time frames is now more accepted (Johnson & Stinchcombe, 2007; Pigliucci, 2007). Two basic factors support this notion. First, genetic changes within populations from one generation to another shape adaptive evolution. Second, feedback between ecological interactions and genetic variation can maintain biological diversity (Lankau & Strauss, 2007).

However, assessing evolution in real time has proved difficult for evolutionary biologists because they must record temporal genetic changes. A traditional approach is to monitor natural populations for a long time frame, but it cannot be assumed that phenotypic changes reflect underlying genetic changes because there is often no information or control of the effect of environment on phenotypic changes (Hendry & Kinnison, 1999; Grant & Grant, 2002). An alternative solution to this problem is the use of quiescent stages of ancient genotypes such as seeds, cysts or spores in a controlled setting to compare traits of the same population through time (Vavrek *et al.*, 1991; Hairston *et al.*, 1999; Decaestecker *et al.*, 2007; Franks *et al.*, 2007; Beaton *et al.*, 2011; Thrall *et al.*, 2012). In this way, growing past and present genotypes of the same provenance under similar conditions ensure that observed differences between temporal populations are genetic. This procedure (referred here as the *resurrection approach*) allows the capture of all the factors affecting evolution in natural populations, including genetic drift and natural selection (Franks *et al.*, 2008).

Given that traits such as resistance and tolerance are the result of the interaction between hosts and enemies (Lambrechts *et al.*, 2006), one possible coevolutionary outcome is that, even if the traits of interacting species are evolving, the result of the interaction could remain stable through time, as predicted by the Red Queen hypothesis (Jokela *et al.*, 2000). Therefore, one way of detecting an evolutionary change in a focal species is measuring the interaction outcome against a reference genotype from the interacting species (Blanquart &

Gandon, 2013). In this sense, the resurrection approach is ideal for testing the evolution of interaction traits in coevolving species (Decaestecker *et al.*, 2007; Beaton *et al.*, 2011; Rode *et al.*, 2011; Thrall *et al.*, 2012).

Here we performed a resurrection experiment with a time lapse of 20 years with the annual plant *Datura stramonium* and the contemporary population of its specialist herbivore, *Lema daturaphila*, which is locally adapted to its host (Garrido *et al.*, 2012). We explored what genetically based changes have occurred in the plant components of resistance and tolerance to herbivory. Because the herbivore is locally adapted to consume its host plant population, reducing the benefits of resistance, we expected a stronger evolutionary response on tolerance than on resistance. As genetic variation for tolerance has been already demonstrated for the studied population (Fornoni & Núñez-Farfán, 2000), we also tested whether there is genetic variance for host resistance by performing a laboratory bioassay with the herbivore.

Materials and methods

Biological system

Datura stramonium (Solanaceae) is an annual selfing plant that inhabits mainly disturbed habitats ranging from xerophytic to coniferous forest communities. In central Mexico, a specialist (*Lema daturaphila* a), a poliphagous (*Epitrix parvula*, both Coleoptera: Chrysomelidae) and the generalist herbivore *Sphenarium pupurascens* (Orthoptera: Pyrgomorphidae) are the main consumers (Núñez-Farfán & Dirzo, 1994; Carmona & Fornoni, 2013). In many populations of the central valley of Mexico, including the one studied here, *D. stramonium* is the unique host of *L. daturaphila*. This beetle spends its entire life on the plant, except for the pupal stage (Kogan & Goeden, 1970), and is responsible for most of the foliar damage, sometimes eating more than 90% of the leaf area (Núñez-Farfán & Dirzo, 1994; C. Bustos-Segura & J. Fornoni, personal observations). In the Pedregal de San Angel Reserve, *L. daturaphila* has been observed feeding consistently on *D. stramonium* plants since 1987.

Experimental design

We used seeds of *D. stramonium* that were randomly collected from 100 plants from the same population in 1987 and 50 plants in 2007 (hereafter ancestral and descendant populations) at the Pedregal de San Angel Reserve (southern Mexico City, 19°19′12″N, 99°11′24″W). In 2008, we grew seeds from different maternal plants from both population samples in a greenhouse and the resulting plants were self-fertilized. We used the new seeds from this refreshing generation to reduce the effects of seed ageing, storage and possible epigenetic

effects (Franks *et al.*, 2008). In July 2010, seeds representing ten full-sib families (genotypes) per population were sown in the greenhouse for the resurrection experiment. We randomly assigned 64 plants per population to a control group (undamaged) using 6 or 7 plants per genotype and 80 plants per population to the damage treatment with 8 plants per genotype. Plants were located inside cages just before herbivore damage treatment to prevent unintended herbivory. We used a randomized block design with each block containing the whole set of Population × Treatment combinations.

To compare defensive traits between ancestral and descendant populations, plants assigned to the damage treatment were subjected to herbivory by larvae of the specialist *L. daturaphila*. In September 2010, we collected egg clusters of *L. daturaphila* from the same study site and reared them as described in Espinosa and Fornoni (2006). Thirteen weeks after seedling germination, we placed one larva (third instar) on each fully developed leaf of plants and allowed to consume not more than 50% of the leaf area. After 9 days, larvae were removed, and 3 days later, we calculated leaf damage with a grid, to artificially clip all leaves of the damage treatment plants to 50% of original area. This procedure allowed us to include the effect that herbivore feeding could have on tolerance expression (Muola *et al.*, 2010) and for reducing the variation in removed leaf area due to differences in resistance (Stevens *et al.*, 2008). To ensure that both populations received the same amount of damage, we quantified removed leaf area at the end of the experiment, by scanning a random sample of damaged dried leaves and measuring the missing leaf area with the imaging software ImageJ version 1.46r (Ferreira & Rasband, 2012).

We counted the number of fully developed leaves on each plant before and after herbivore damage (the 12th and 15th weeks after germination, respectively). The number of leaves is a good estimate of size measured as total foliar area ($R^2 = 0.67$, $P > 0.0001$, $N = 288$). Then, relative growth was calculated as $ln(\text{Size}_2/\text{Size}_1)$. Given that *D. stramonium* expresses inbreeding depression in the number of seeds per fruit ($\delta = 0.39$) (Núñez-Farfán *et al.*, 1996; Bello-Bedoy & Núñez-Farfán, 2011), all the flowers were outcrossed with random plants from the same population to avoid this source of variation within the experiment. Because damage occurred after the onset of flowering, some fruits had already initiated their maturation and the seeds produced by them were used as a covariate. We collected the fruits when the capsule ripened, and 6 months after germination, the remaining fruits were collected. Seeds of each fruit were scanned and counted using ImageJ.

Resistance estimates

We quantified both constitutive and induced resistance against herbivory by feeding larvae with leaves of

D. stramonium. A third instar larva was placed on one fully expanded leaf of all plants and removed it 24 h later. Two weeks later this procedure was repeated to estimate induced resistance. The leaves were cut, dried and scanned to determine consumed are using ImageJ. We were able to measure leaf damage, because consumption for 24 hours did not alter the original shape of the leaves and larvae usually fed creating small holes. Consumption of the undamaged plants gives a measure of constitutive resistance, although a difference in consumption between undamaged and damaged plants indicates induction of resistance.

We measured three leaf traits associated with resistance to herbivory: trichome density, tropane alkaloids and foliar water content, using additional replicates of each genotype that grew under the same greenhouse conditions as those used for the first experiment. Previously, Valverde *et al.* (2001) have shown that plants with more trichomes were more resistant to herbivores. Tropane alkaloids are antagonists of the muscarinic acetylcholine receptor (Eich, 2008), so they can adversely affect herbivores (Krug & Proksch, 1993; Wink, 1993). Moreover, they are targets of selection for specialist herbivores of *D. stramonium* (Shonle & Bergelson, 2000). On the other hand, some plant species defend themselves against herbivores by reducing the water content of their leaves (Agrawal & Fishbein, 2006).

We counted the trichomes on a 7-mm^2 area of the adaxial side of the leaf base (ten fully developed leaves per plant and three individuals per genotype), with a dissecting microscope (Carl Zeiss, Germany). Trichome density, measured this way, is linearly related to the average trichome density for five different zones of the leaf ($R^2 = 0.73$, $P < 0.0001$, $N = 159$). To measure water content, we weighed leaves (ten fully developed leaves per plant and three individuals per genotype) immediately after cutting and again after drying for 48 h at 40 °C. To quantify the relative abundance of tropane alkaloids, we analysed extracts (11 plants per population) using gas chromatography-mass spectrometry (GC-MS). The relative abundance refers to the per cent of a single compound concentration relative to the total compound concentration in the extract, and it is related to the concentration in dry leaf matter. The extraction procedure was similar to that of Berkov and Philipov (2002).

Tolerance estimate

Tolerance to herbivory – the plant's ability to reduce the negative effects of damage on fitness – was estimated as the difference in fitness (measured as seed production) between undamaged and damaged plants in both populations (Strauss & Agrawal, 1999). A significant interaction between the population (ancestral vs. descendant) and the treatment (undamaged vs. damaged) reflects differences between populations in

the fitness response due to the presence or absence of damage (i.e. differences in tolerance).

Measure of herbivore performance in laboratory

To search for the existence of genetic variation in resistance for the ancestral population, a bioassay with the insect herbivore was performed. We measured the herbivore performance in laboratory conditions to explore the effects of nine ancestral plant genotypes on the herbivore, rearing *L. daturaphila* as described in Espinosa and Fornoni (2006). Using 87 newly hatched larvae, we placed them in individual plastic containers (250 cm^3) with 80 cm^3 of moistened soil and a 4-cm^2 leaf square from one of nine genotypes from the ancestral population. The leaf, maintained with a moistened cotton ball, was changed every 2 days with remnants dried and leaf area measured with ImageJ. We measured the larval mass and leaf consumption between days 2 and 6 after hatching and developmental time and survival until the adult stage for all the individuals.

Statistics

All data were analysed with the R system (2.15.1). Mixed models were performed with the packages *lm4* and *coxme*. Significance of fixed effects was calculated with the function *anova* type III, which performs Wald chi-square tests between the models with and without the referred effect, and significance for random effects was estimated by comparing models with and without the random factor with the function *anova* (Fry, 2004).

Resurrection experiment

We compared the resistance of the ancestral and descendant populations with mixed-effect models, using the consumed leaf area (cm^2) as the dependent variable. For the constitutive resistance model, we included only data from previously undamaged plants, with population (ancestral and descendant) as a fixed factor. In the induced resistance model, we used data from all of the plants, so we added treatment (damaged/undamaged) and Population × Treatment as fixed effects. To compare the expression of resistance traits between populations, we used generalized linear models (GLMs) with a quasi-Poisson distribution for trichome density and a quasi-binomial distribution for the foliar water content. Given that the plant produces different tropane alkaloids through the same biosynthetic pathway, their abundances are not independent. Therefore, we used a multivariate analysis of variance (MANOVA) to compare the abundances of the three tropane alkaloids. We used an arcsine-square-root transformation for analysing the alkaloid abundance.

We compared the percentage of herbivore damage between populations by means of a generalized linear mixed model (GLMM) with a binomial distribution, using population as a fixed effect. We also tested for differences in damage between plants after the compensation by artificially clipping, but for this test, we lacked enough replicates per genotype; therefore, we used a GLM with a binomial distribution. The plant size before damage (number of leaves) was compared with a GLMM using a Poisson distribution. To analyse differences in growth ($ln(Size_2/Size_1)$), we used a similar model, but using a GLM, as the relative growth is expected to distribute normally.

Differences in tolerance between populations were evaluated after controlling for the variation in plant size. Residuals from a linear regression of standardized fitness (measured as seed production) on plant size (number of leaves after damage) were used as the response variable in the mixed model. A significant Population × Treatment interaction would indicate differences in tolerance between populations. Genotype nested within population and its interaction with treatment and block were considered as random factors in all the mixed models.

Performance of L. daturaphila

We used mixed models to compare food consumption (cm^2 per day) and final larval mass (mg) of *L. daturaphila* fed the different plant genotypes. In both analyses, we included initial larval mass as a covariate. For analysing developmental time and herbivore survival, we used a proportional hazard mixed model analysis using the *coxme* function of R system. Block was a fixed factor and plant genotype was the random factor.

Results

Seeds from both collections (1987 and 2007) germinated successfully. We found no evidence of differences in constitutive resistance because consumption by *L. daturaphila* in undamaged plants did not differ between populations ($\chi^2 = 0.18$, $P = 0.67$; Fig. 1). Overall, herbivores inflicted similar levels of damage in plants from both populations (ancestral: 27.59% ± 0.90; descendant: 28.74 ± 0.81; mean ± SE; $\chi^2 = 0.66$, $P = 0.42$), whereas genotype had an effect on the percentage of herbivore damage ($\chi^2 = 31.77$, $P < 0.0001$). In the induced resistance test, neither the population nor the treatment influenced feeding and there was no significant interaction effect (population: $\chi^2 = 0.30$, $P = 0.58$; treatment: $\chi^2 = 2.82$, $P = 0.093$; Treatment × Population: $\chi^2 = 0.51$, $P = 0.47$), indicating no evidence of induced defences.

Ancestral and descendant populations had similar densities of foliar trichomes ($\chi^2 = 2.80$, $P = 0.094$; Fig. 1), and although foliar water content was lower for plants of the descendant population, the difference was only 1% ($\chi^2 = 6.06$, $P = 0.014$; Fig. 1). We identified three tropane alkaloids, namely aposcopolamine, hyosciamine and scopolamine, but found no differences
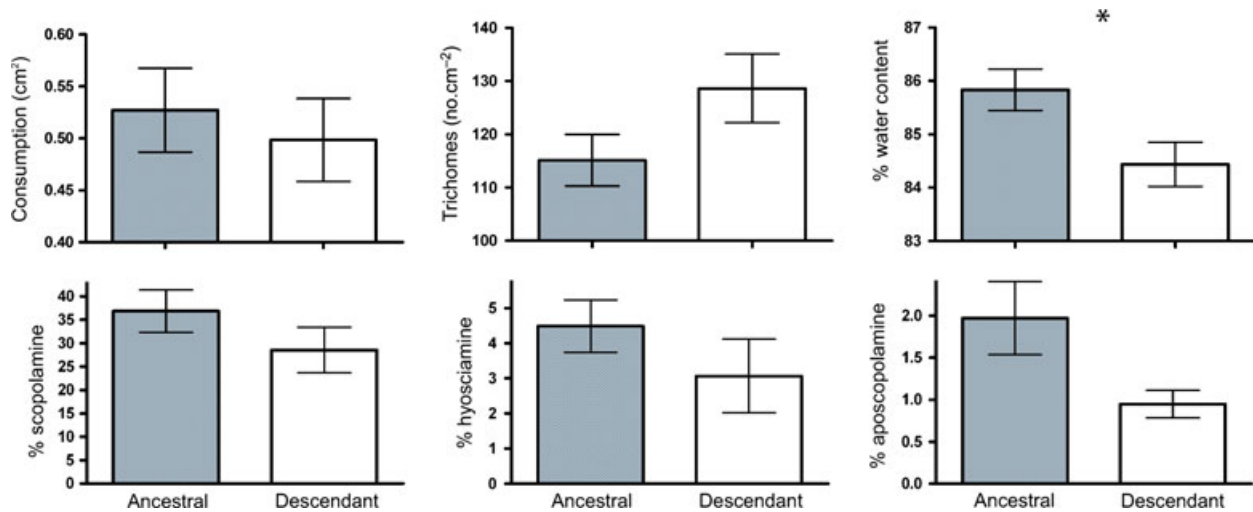
Fig. 1 Average values (± SE) of plant traits associated with constitutive resistance against herbivory in temporal populations from 1987 and 2007 seed collections of *Datura stramonium* (ancestral and descendant plants, in grey and white bars, respectively). Shown are the means of constitutive resistance traits including consumption by the herbivore *Lema daturaphila*, foliar trichome density, foliar water content (left to right, upper graphs) and abundances of alkaloids in leaves of undamaged plants (lower graphs). (*) $P < 0.05$.
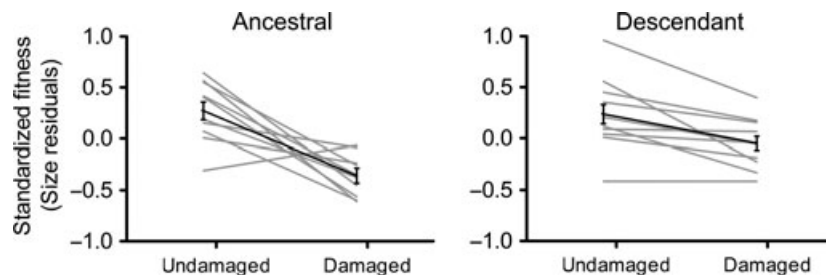
Fig. 2 Reaction norms of fitness for 10 genotypes in ancestral (left panel) and descendant plants (right panel). Plants of the damage treatment were defoliated by *Lema daturaphila* larvae and adjusted to 50% of damage by artificial defoliation. Reaction norms for each genotype (tolerance) are shown in grey and mean reaction norms in black. Error bars represent SE of the mean responses per population. Significant differences in tolerance between populations were detected ($P = 0.025$).

between the populations in their abundances ($F_{3,15} = 1.44$, $P = 0.27$; Fig. 1).

After adjusting the amount of leaf area removed by artificial clipping, we accumulated similar levels of defoliation on both populations: ~48% for the ancestral (mean ± SE: 47.46 ± 1.66) and the descendant populations (mean ± SE: 48.76 ± 1.60) ($\chi^2 = 1.56$, $P = 0.211$). The size of the plants before damage, measured as the number of leaves, did not differ between populations or damage treatments. There was, however, a significant effect of genotype ($\chi^2 = 38.49$, $P < 0.0001$). Damage negatively affected the plant growth ($\chi^2 = 9.27$, $P = 0.0023$), but there were no differences in growth between populations and no Population × Damage interaction ($\chi^2 = 0.49$, $P = 0.48$; $\chi^2 = 1.53$, $P = 0.22$, respectively). Again, genotype had a significant effect on growth, but the interaction between genotype and treat-

ment was not significant ($\chi^2 = 29.79$, $P < 0.0001$; $\chi^2 = 0.27$, $P = 0.97$, respectively).

The size of the plants had a significant effect on standardized fitness, with bigger plants producing more seeds ($F_{1,286} = 263$, $P < 0.0001$), so we used the residuals of this regression to compare fitness and tolerance to herbivory between populations and treatments. Populations did not differ in their fitness ($\chi^2 = 0.094$, $P = 0.76$), and as expected, damaged plants presented lower fitness ($\chi^2 = 10.02$, $P = 0.0015$). When damaged, plants from the descendant population showed a higher level of fitness compensation (i.e. tolerance) than the ancestral population ($\chi^2 = 5.02$, $P = 0.025$; Fig. 2). The genotype term and its interaction with treatment were not significant. There was no correlation between the average levels of tolerance and the herbivore consumption per genotype ($r = 0.084$, $P = 0.73$).

In the bioassay experiment with *L. daturaphila*, neither the block nor the plant genotype had a significant effect on consumed leaf area, mass gain or developmental time of the larvae. In contrast, the plant genotype significantly affected survival from hatching to adult emergence ($\chi^2 = 4.62$, $P = 0.032$).

Discussion

The resurrection experiment revealed that plant tolerance but not resistance to herbivory evolved during the last 20 years in a natural population of the annual *Datura stramonium*, providing evidence of an evolutionary change involved in an ecological interaction. Using the contemporary insect population as the reference genotype, we demonstrated a genetically based change in the plant compensatory ability in response to herbivore damage. In contrast to the theoretical expectation that both tolerance and resistance are mutually exclusive alternative strategies (Mauricio *et al.*, 1997), there was no correlation between tolerance and percentage of consumption per genotype; hence, we found no evidence of a trade-off between both defences (Leimu & Koricheva, 2006). Although some studies have reported evolutionary responses of resistance, mainly in invasive plant populations (Thompson, 1998; Zangerl & Berenbaum, 2005; Beaton *et al.*, 2011), our work also tracked changes in tolerance to herbivory and remarkably is the first to show that tolerance is rapidly evolving in a contemporary native plant population.

Descendant plants expressed a higher tolerance to herbivory than ancestral plants when both were damaged by the contemporary herbivore population. However, it is still possible that evolution in the herbivore population could modify the value of the interaction outcome when plants interact with the ancestral or descendant herbivore population (Thrall *et al.*, 2012). Because we could not have access to the ancestral herbivore population, we do not know the value of tolerance against the ancestral herbivores, so it is not possible to conclude as to the directionality of the observed evolutionary change in tolerance. Undetected changes in herbivore consumption in our study might also have been influenced by evolution in the herbivore population. Overall, resistance traits are not different between populations, which suggests that the absence of genetic variation in the plant population constrained an evolutionary response or that resistance traits are being maintained by other natural enemies (see Carmona & Fornoni, 2013). First, our bioassay experiment showed that survival of *L. daturaphila* depended on the plant genotype indicating that there is still variation in resistance, ruling out this potential constrain. Second, analyses of phenotypic selection in the same species and study site found negative directional selection for resistance (1 – proportion of damage) against the beetle *Epitrix sp.* and stabilizing

selection for resistance to the grasshopper *Sphenarium purpurascens* (Núñez-Farfán & Dirzo, 1994), so greater resistance is not offering an adaptive advantage in this plant population. In other location, stabilizing selection on hyoscyamine and negative directional selection on scopolamine imposed by herbivores have been reported in *D. stramonium* (Shonle & Bergelson, 2000), which could explain the lack of increase in alkaloids according to our test. Tropane alkaloids might be still useful as they are effective against generalist herbivores (Krug & Proksch, 1993; Wink, 1993; Alexander *et al.*, 2008). Hence, natural selection imposed by other natural enemies and/or other functions of secondary compounds like allelopathy (Lovett *et al.*, 1981) may explain the apparent maintenance of tropane alkaloids in the study population. Nevertheless, we cannot rule out the possibility that our test had low sensitivity to detect small changes in resistance or that changes occurred in other nonmeasured resistance traits. Even if we found 1% lower water content in leaves from descendant plants, and these traits have been related to resistance hindering digestion by herbivores (Agrawal & Fishbein, 2006), this small difference was not related to how much the larvae ate, reducing its possible biological relevance.

Although it has been hypothesized that tolerance should be favoured when resistance is no longer effective against natural enemies (Jokela *et al.*, 2000; Agrawal & Fishbein, 2008; Garrido *et al.*, 2012), there is scarce empirical data supporting this expectation (e.g. Agrawal & Fishbein, 2008), and some theoretical models predict that tolerance should evolve to fixation after invading a population (Roy & Kirchner, 2000; Boots *et al.*, 2009). However, in these models, resistance must be an effective strategy for controlling the enemy damage, but evolution of specialized enemies in the wild can lead to a reduction in the resistance efficacy. The levels of natural damage for *D. stramonium* in the field are high, and some plants lose 90% of their leaves to herbivory (Núñez-Farfán & Dirzo, 1994; personal observations). Moreover, *L. daturaphila* from our studied population (Pedregal) is locally adapted to its host (Garrido *et al.*, 2012), and preliminary results from bioassays indicate that this herbivore can tolerate even higher concentrations of tropane alkaloids than natural levels occurring in the host (Bustos-Segura & Fornoni, unpublished data). Thus, in the Pedregal population, resistance traits are not very effective in protecting the host against *L. daturaphila* (see Carmona & Fornoni, 2013). This leads to a scenario in which the benefits of resistance have been reduced, whereas those of tolerance are relatively increased (Fornoni *et al.*, 2004). Therefore, the change in plant compensatory ability found in this study (Fig. 2) is consistent with the expectation that evolution of tolerance should be favoured when resistance loses its efficiency. Interestingly, the pattern that we have uncovered supports recent evidence in another natural population of the

same species indicating that tolerance is more likely to be selected by specialized than generalized herbivores (see Carmona & Fornoni, 2013). However, as we were unable to contrast our results with a host plant population in which *L. daturaphila* is not locally adapted, our explanation could not be the only one accounting for the observed pattern.

We cannot exclude the possibility that costs of tolerance and resistance differentially changed during the last 20 years or that genetic drift had more impact than natural selection on the evolution of plant defences. Although changes in the availability of resources could affect the balance between costs and benefits of tolerance and resistance and their adaptive value (Fornoni *et al.*, 2004), we are unaware of changes in resources at the study site that could select for tolerance against herbivory. Genetic drift could also explain the results, even in this relatively large population, but we still lack an empirical method that distinguishes the results of natural selection from those of genetic drift (Pigliucci & Kaplan, 2006).

Our measure of tolerance includes seed set, to which both maternal condition and mate quality contribute. By outcrossing the flowers during the experiment, we avoided biases due to inbreeding depression (mate quality) in seed number (Núñez-Farfán *et al.*, 1996). However, selfing in the previous generation (and consequent inbreeding depression due to maternal condition) could be a mechanism by which the observed differences in tolerance to herbivory arose. Inbreeding depression on number of seeds can modify the expression of tolerance, and given that it shows variation between genotypes (Bello-Bedoy & Núñez-Farfán, 2011), evolution of reduced inbreeding depression may allow for indirect evolution of greater tolerance in a primarily selfing population such as the one studied here. Herbivore damage increases the degree of inbreeding depression (Bello-Bedoy & Núñez-Farfán, 2011); therefore, selection for plants with a less or even negative inbreeding depression would be enhanced when herbivores are present.

Disentangling the mechanistic basis of tolerance still remains as a major challenge for those studying the evolution of plant defences (Fornoni, 2011). It is still possible that the observed reduction in foliar water content was related to the increment in tolerance as part of the physiological changes needed to increase reproductive compensation; however, specific experiments need to be performed to test this possibility. Parallel to tolerance, we also measured plant relative growth, which has been one of the key traits related to reproductive compensation (Hochwender *et al.*, 2012). Herbivory negatively affected the plant growth rate, but plant populations showed similar levels of this trait. Therefore, there was no link between plant growth and differences in tolerance between populations.

The study of the evolution of tolerance is critical in the context of host–enemy interactions because it has a different ecological (demographic) effect on the enemy than resistance and thus on the evolutionary outcome of the interaction (Roy & Kirchner, 2000; Espinosa & Fornoni, 2006; Miller *et al.*, 2006; Boots *et al.*, 2009; Fornoni, 2011). Recently, rapid evolution has been defined as those genetic changes in natural populations that are fast enough to substantially modify the ecological dynamics of the focal species (Hairston *et al.*, 2005). Although resistance reduces the fitness of the enemy and its prevalence (damage), tolerance can affect or not the demographic performance of the enemy (Roy & Kirchner, 2000; Boots *et al.*, 2009). If tolerance and resistance differentially affect the demographic performance of the herbivore (Fornoni, 2011), temporal genetic changes in defensive traits may modify the dynamics of the interaction and can be considered as rapid evolutionary changes. Therefore, the use of the resurrection approach can contribute to realize how rapid evolution modifies ecological interactions and the properties of communities, to better understand the eco-evolutionary feedback of species interactions. As shown here, gathering direct proof of evolutionary change provided us with new insights into the actual evolutionary trends of the plant–herbivore interaction in a contemporary plant population.

Acknowledgments

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