UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS
INSTITUTO DE ECOLOGÍA
BIOLOGÍA EVOLUTIVA

VALOR ADAPTATIVO DE LOS MECANISMOS PLÁSTICOS DEL CRECIMIENTO EXPRESADOS EN LA TOLERANCIA AL DAÑO FOLIAR DE *Datura stramonium*

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

IVAN DARÍO CAMARGO RODRÍGUEZ

TUTOR PRINCIPAL:	DR. JUAN SERVANDO NÚÑEZ FARFÁN (INSTITUTO DE ECOLOGÍA, UNAM)
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	DR. MARK EARL OLSON ZUNICA (INSTITUTO DE BIOLOGIA, UNAM)

MÉXICO, CD, MX. **AGOSTO, 2017**

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UNAM POSGRADO
Ciencias Biológicas

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Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
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Me permito informar a usted, que el Comité Académico, en su sesión ordinaria del día 19 de junio de 2017, aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del alumno **CAMARGO RODRÍGUEZ IVÁN DARÍO** con número de cuenta 508450954 con la tesis titulada: **"VALOR ADAPTATIVO DE LOS MECANISMOS PLASTICOS DEL CRECIMIENTO EXPRESADOS EN LA TOLERANCIA AL DAÑO FOLIAR DE *Datura stramonium*",** bajo la dirección del **DR. JUAN SERVANDO NÚÑEZ FARFÁN:**

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

ATENTAMENTE
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, Cd. Mx., a 26 de julio de 2017

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Resumen

Los caracteres asociados con la tolerancia a la defoliación le permiten a las plantas sobrevivir y reproducirse en presencia del daño de tejidos. Esta tolerancia puede variar dependiendo de los ambientes abióticos. Los cambios ontogenéticos en la tasa relativa de crecimiento y sus determinantes (la tasa de asimilación neta, el área foliar específica y la asignación de biomasa a las hojas), antes y después de un evento de defoliación, en diferentes contextos abióticos, fueron estudiados en la herbácea anual *Datura stramonium*, para determinar si estos cambios maximizan el crecimiento y el fitness en respuesta a la defoliación, teniendo así un valor adaptativo. La evidencia demostró que la cantidad de tolerancia y el vigor del fitness son el resultado de un mecanismo compensatorio ontogenético en el que interactúan el RGR y sus determinantes. Entre más grande la asignación al crecimiento antes de la defoliación, menor la asignación a la tolerancia. En respuesta a la defoliación, no solo la plasticidad, también el fenotipo promedio de los determinantes del crecimiento afectó la tolerancia y el vigor del fitness. En baja disponibilidad de luz, la tolerancia a la defoliación fue menor que en alta disponibilidad lumínica; mientras en baja disponibilidad de nutrientes, la tolerancia fue mayor que en alta disponibilidad de nutrientes. Lo anterior es debido a que la variación en la disponibilidad de los ambientes abióticos puede alterar la presencia, número y el peso de los determinantes del crecimiento que incrementan o decrecen la tolerancia y el vigor del fitness. Por lo tanto, los factores abióticos determinan la evolución de la estrategia tolerante modificando la tolerancia y vigor del fitness en respuesta a la defoliación en *Datura stramonium*. Este estudio muestra por primera vez un soporte evolutivo al argumento de que las plantas responderían con adaptaciones para proteger los nutrientes de ser consumidos en ambientes con poca disponibilidad de este recurso.

Abstract

Traits associated with tolerance to defoliation allow plants to survive and reproduce in the presence of tissue damage. Tolerance can vary depending on abiotic environments. Ontogenetic changes in relative growth rate and its determinants (net assimilation rate, specific leaf area and leaf weight ratio), before and after a single defoliation event, in different abiotic contexts, were studied in the annual herb *Datura stramonium* with the aim to determine if these changes maximize growth and fitness in response to defoliation and hence would have an adaptive value. The evidence showed that the amount of tolerance and vigor were the consequence of a compensatory ontogenetic mechanism, in which RGR and its determinants interact. Additionally, the greater the allocation to growth before defoliation the smaller the allocation to tolerance. In response to defoliation, not only the plasticity but the average phenotype of growth determinants affected the tolerance and fitness vigor. Tolerance to defoliation was lower in low light availability compared with the high light environment; while in the low nutrient environment, tolerance was higher than expressed in the high nutrient environment. Thus, the variation in the availability of abiotic resources can alter the presence, number and weight of the growth determinants that increase or decrease tolerance and vigor. Then, the abiotic environments can determine the evolution of the tolerant strategy modifying the tolerance and fitness vigor in response to defoliation in *Datura stramonium*. This study supports for the first time, from an evolutionary perspective, that plants respond with adaptations to protect their nutrients from consumption in environments with low availability of this resource.

Introducción general

Diferentes teorías han proveído una estructura conceptual para entender la variación (dentro y entre especies) de la defensa en plantas en contra de sus enemigos naturales (Stamp 2003). La mayoría de estas teorías intenta explicar cómo los cambios en el ambiente físico y biótico puede cambiar la expresión fenotípica de los caracteres defensivos y por ende su valor adaptativo. Estos caracteres se han asociado a dos principales estrategias de defensa (Núñez-Farfán et al. 2007), los caracteres involucrados en la *resistencia* reducen la cantidad de daño experimentado en los tejidos; mientras en la *tolerancia*, los caracteres involucrados le permiten a las plantas sobrevivir y reproducirse en presencia del daño de tejidos. Estas dos estrategias están bien reconocidas como componentes de la defensa, con diferentes efectos sobre la adecuación de las plantas y los enemigos naturales (Rausher 1996, 2001, Stowe et al. 2000, Utsumi et al. 2009). No obstante, muchos estudios de la adaptación de las plantas se han enfocado exclusivamente en la evolución de la resistencia (Núñez-Farfán et al. 2007, Stowe et al. 2000), dejando a un lado el estudio de los caracteres que confieren tolerancia y cómo la variación abiótico dependiente de estos caracteres altera la cantidad y el patrón de la misma.

Después del reconocimiento del estudio de la tolerancia como otra estrategia defensiva para lidiar con los herbívoros (Painter 1958, Rosenhtal & Kotanen 1994), su estudio eco-evolutivo ha estado centrado en su definición operacional (Simms 2000), mientras los mecanismos detrás de la tolerancia han recibido menos atención (Tiffin 2000). En la definición operacional, la tolerancia es la pendiente (con cantidad y signo) de la norma de reacción (i.e., la función que relaciona los ambientes a los cuales un genotipo particular es expuesto y los fenotipos que pueden ser producidos por ese genotipo, Pigliucci 2001) del *fitness* de un genotipo en respuesta a un gradiente en intensidad de defoliación (Simms 2000). Así, los genotipos de plantas que pueden soportar mayor daño en sus tejidos con menos (o ninguna) pérdida del *fitness* en respuesta a la defoliación son más tolerantes al daño (Stowe et al. 2000). En consecuencia, varios patrones de tolerancia pueden emerger (sensu Camargo et al. 2015, Stowe et al. 2000): tolerancia completa (no hay diferencias en *fitness* entre los ambientes sin y con defoliación), subtolerancia (menos *fitness* en el ambiente defoliado) y sobretolerancia (más *fitness* en el ambiente defoliado). En su definición mecanicista, la tolerancia es la estabilidad del *fitness* en un gradiente de daño alcanzada por la plasticidad (pendiente en respuesta a la defoliación) de un carácter menos visible y más distante del *fitness* (i.e., un carácter subyacente) (Alpert & Simms 2002). Aquí, el término tolerancia es usado exclusivamente para referirse al daño por defoliación diferenciándolo del uso que el término ha recibido describiendo la habilidad de las plantas para lidiar con otros tipos de estrés (e.g., salinidad, sequía, metales pesados).

Mientras la definición operacional de la tolerancia ha permitido su modelamiento evolutivo (e.g., Fornoni et al. 2003) y una serie de hipótesis han intentado explicar los patrones de tolerancia en función de la variación abiótica (Herms y Matson 1992, Janzen 1974, Coley et al, 1985, Hilbert et al, 1981, Wise y Abrahamson 2005), las causas subyacentes a la variación de la cantidad y signo de la tolerancia observada en diferentes genotipos de una población y en respuesta a la variación en la disponibilidad de factores abióticos, permanecen poco abordadas (c.f., Wise et al. 2008, Hochwender et al. 2000, Juenger & Bergelson 2000, Strauss et al. 2003, Stevens et al. 2008). Así, el propósito general de este proyecto fue desarrollar unas herramientas metodológicas para el estudio de la tolerancia, a partir del análisis crítico de varios modelos conceptuales y teóricos, que permitan identificar los caracteres subyacentes a la variación observada en los patrones de tolerancia.

En general ha sido propuesta una plétora de mecanismos relacionados con la tolerancia, los cuales están estrechamente relacionados con el crecimiento en biomasa (Rosenthal y Kotanen 1994, Stowe et al. 2000, Tiffin et al. 2000, Strauss y Agrawall 1999). Las plantas pueden mitigar los efectos negativos de la defoliación a través del crecimiento compensatorio (McNaughton 1983). Este crecimiento implica una serie de cambios plásticos en diferentes caracteres (determinantes del crecimiento; Poorter & Nagel 2000) los cuales determinan a su vez el patrón de cambio en la tasa relativa de crecimiento (RGR, relative growth rate, la tasa a la cual una cantidad dada de biomasa existente puede producir nueva biomasa por unidad de tiempo; Shipley 2000) en respuesta a la defoliación:

$$RGR = NAR \times SLA \times LWR,$$

donde NAR (Net Assimilation Rate) corresponde a la tasa de asimilación neta (el incremento en biomasa por unidad de tiempo y área foliar), la cual está fuertemente correlacionada con la tasa fotosintética. SLA (Specific Leaf Area) es el área foliar específica, que refleja aspectos de la morfología foliar tales como la densidad y el grosor. LWR (Leaf Weight Ratio) es la asignación de biomasa a las hojas. La plasticidad fenotípica de estos caracteres en respuesta a la defoliación puede contribuir a tolerar la defoliación siempre y cuando alivie los efectos negativos sobre el *fitness*.

Se ha hipotetizado que las plantas con crecimiento lento cuando son defoliadas maximizarían la biomasa aérea y el *fitness* en mayor medida que las plantas con altas tasas de crecimiento al momento del daño (Hilbert et al, 1981). Lo anterior debido a que las plantas con crecimiento lento al momento de la defoliación requerirían una menor cantidad de cambio en RGR para alcanzar a las plantas sin defoliación (Hilbert, 1981). En general, una mayor NAR y LWR ha sido predicha como principal responsable del incremento en RGR en respuesta a la defoliación (Oesterheld & McNaughton 1988, 1991, Van Staalduinen & Aten 2005, Van Staalduinen et al. 2010).

Ya que las plantas en sistemas naturales se encuentran expuestas a múltiples factores ambientales simultáneamente, y la cantidad de crecimiento compensatorio puede ser afectado por el nivel de estrés impuesto por factores tales como nutrientes y luz, el RGR de plantas no defoliadas sería menor a mayores niveles de estrés y la cantidad necesaria de cambio del RGR (y sus determinantes) para compensar el daño sería menor. A su vez, la defoliación puede aliviar o agravar los efectos del estrés lo que podría modificar la cantidad de cambio necesario en RGR (Oesterheld & McNaughton 1991), o el número de determinantes del crecimiento responsables del patrón de tolerancia a la defoliación. Así, la tolerancia se ha predicho menor si la defoliación disminuye la capacidad para adquirir, almacenar o utilizar un recurso que limita el fitness en las plantas sin defoliación (modelo por limitación de recursos; Wise & Abrahamson 2005). La anterior predicción se cumple siempre y cuando las plantas estén expuestas a ambientes con baja disponibilidad de recursos arriba del suelo (e.g., luz como recurso focal), ya que la defoliación interferiría en la capacidad de las plantas para capturarlos (modelo por limitación de recursos I; Wise & Abrahamson 2005) y se predice mayor tolerancia en alta disponibilidad del recurso focal.

Cuando las plantas están expuestas a ambientes con baja disponibilidad de recursos abajo del suelo (e.g., nutrientes como recurso focal), se espera que la defoliación no interfiera en su captura (modelo por limitación de recursos II; Wise & Abrahamson 2005) y se predice igual tolerancia en bajas y altas disponibilidades del recurso.

Estas predicciones a menudo son probadas midiendo el *fitness* en un gradiente de defoliación. No obstante, el uso de una aproximación mecanicista, en la cual se pueda correlacionar un carácter subyacente al fitness con el fitness, ayudaría a explicar el porqué de la variación en los patrones de tolerancia en diferentes disponibilidades abióticas. Tradicionalmente esta correlación ha sido investigada relacionado la pendiente de la norma de reacción a la defoliación de un carácter subyacente (la diferencia entre el valor del carácter en plantas defoliadas con el valor en plantas sin defoliar) con la pendiente de la norma de reacción a la defoliación del fitness (tolerancia, la diferencia entre el fitness en plantas defoliadas con el fitness de plantas no defoliadas) (Wise et al. 2008, Hochwender et al. 2000, Juenger & Bergelson 2000, Strauss et al. 2003). No obstante, la pendiente no es la única propiedad de una norma de reacción de un carácter subyacente, la altura de la norma de reacción (el promedio entre el valor del carácter de plantas defoliadas y sin defoliar, Pigliucci 2001) también podría afectar la tolerancia. A su vez, la altura de la norma de reacción de un carácter subyacente podría afectar la altura de la norma de reacción del fitness (vigor, el promedio entre el valor fitness de plantas defoliadas y sin defoliar; Stowe et al. 2000).

Con base en lo anterior, este trabajo uso a la herbácea anual *Datura stramonium* como sistema de estudio. Ya que algunos genotipos de esta especie pueden tolerar la defoliación mejor que otros (Valverde et al. 2001, Valverde et al. 2003, Fornoni et al. 2000), la base fisiológica de estas diferencias en tolerancia puede ser examinada. En diferentes experimentos que usan una aproximación analítica al crecimiento, la cual descompone las diferencias observadas en RGR entre plantas control y plantas defoliadas en los determinantes del crecimiento, se propusieron las siguientes preguntas de investigación:

1. ¿Un genotipo de crecimiento lento (antes del daño) posee mayor tolerancia a la defoliación que uno de crecimiento rápido?
2. ¿Las normas de reacción en respuesta a la defoliación de los determinantes del crecimiento están correlacionadas con la norma de reacción del *fitness*?
3. ¿La variación en la disponibilidad de recursos abajo (nutrientes) y arriba (luz) del suelo modifica la relación entre los caracteres subyacentes y el *fitness*?

En el primer experimento de este proyecto, Capítulo 1, usando dos genotipos que difieren ampliamente en el RGR y tolerancia a la defoliación, se exploró la plasticidad fenotípica en respuesta a la defoliación de los determinantes del crecimiento haciendo uso de la estimación de una medida de plasticidad conocida como coeficiente de respuesta al crecimiento (GRC, growth response coefficient; Poorter & Nagel 2000). Para cada determinante del crecimiento (X) se calculó un GRC usando la diferencia entre el logaritmo natural (ln) de plantas defoliadas (D) y plantas control (C), su cálculo es:

$$GRC_x = \ln X_D - \ln X_c / \ln RGR_D - RGR_c$$

Se muestra como estos GRCs cambian a lo largo de la ontogenia si el RGR cambia debido a la defoliación y se le adscribe un valor adaptativo a la plasticidad observada en ellos, comparando su efecto sobre la diferencia entre el número de semillas en las plantas defoliadas y las plantas control como una aproximación operativa de la tolerancia a la defoliación.

En los subsiguientes experimentos se pone a prueba el valor adaptativo de estas respuestas en diferentes niveles de disponibilidad de recursos abajo (nutrientes, Capítulo 2) y arriba (luz, Capítulo 3) del suelo. En el Capítulo 2, se explora como la tolerancia puede estar determinada no solo por la plasticidad de los determinantes del crecimiento sino por otra propiedad de las normas de reacción en respuesta a la defoliación, su altura (el valor promedio a través de los ambientes de defoliación). A su vez se explora como el entendimiento completo de la norma de reacción del *fitness* en respuesta a la defoliación y su evolución implica indagar por la relación del vigor (el valor promedio del *fitness* a través de los ambientes de defoliación) con la norma de reacción de los determinantes del crecimiento en respuesta a la defoliación. Ya que el estrés puede afectar la magnitud del crecimiento y sus determinantes en respuesta a la defoliación, se indagó por: (i) la correlación entre los determinantes del crecimiento y la adecuación biológica en dos niveles de nutrientes (alto y bajo) haciendo uso de cruzas híbridas interpoblacionales, las cuales permitieron tener una amplia gama de variación en RGR y plasticidad en los determinantes del crecimiento en respuesta a la defoliación, (ii) los costos en *fitness* asociados a la expresión de estas respuestas plásticas a la defoliación y (iii) la existencia de una correlación genética negativa entre la expresión temprana (antes de la defoliación) de los determinantes del crecimiento y la tolerancia a la defoliación.

En el Capítulo 3 se indagó la relación cualitativa entre los patrones de variación de caracteres relacionados con el forrajeo del recurso luz y la tolerancia observada. Indagando si una menor cantidad de compensación de los caracteres subyacentes está relacionada con la menor tolerancia observada en ambientes con baja disponibilidad lumínica (ver Apéndice 1). En el Apéndice I se cuantifica la relación de la norma de reacción del *fitness* en respuesta a la defoliación y la norma de reacción de los caracteres de forrajeo del recurso luz, mostrando como la variación en estos caracteres es la responsable de la mayor tolerancia observada en ambientes de alta disponibilidad lumínica.

El Apéndice II aporta evidencia a favor del argumento que en ambientes con baja disponibilidad de recursos abióticos *D. stramonium* puede utilizar una estrategia de tolerancia al estrés, la cual asegura el éxito reproductivo.

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Capítulo 1

ECOTYPIC VARIATION IN GROWTH RESPONSES TO SIMULATED HERBIVORY: TRADE-OFF BETWEEN MAXIMUM RELATIVE GROWTH RATE AND TOLERANCE TO DEFOLIATION IN AN ANNUAL PLANT

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Research Article

SPECIAL ISSUE: Using Non-Model Systems to Explore Plant–Pollinator and Plant–Herbivore Interactions

Ecotypic variation in growth responses to simulated herbivory: trade-off between maximum relative growth rate and tolerance to defoliation in an annual plant

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Abstract. It has been hypothesized that slow-growing plants are more likely to maximize above-ground biomass and fitness when defoliated by herbivores than those with an already high relative growth rate (RGR). Some populations of the annual herb *Datura stramonium* L. can tolerate foliar damage better than others. The physiological basis of this difference is examined here in a comparative study of two ecotypes that differ in tolerance and maximum growth rate, using a growth analytical approach. One hundred and fifty-four plants of each ecotype grown under controlled conditions were suddenly defoliated (35 % of total leaf area removed) and a similar sample size of plants remained undefoliated (control). Ontogenetic plastic changes in RGR and its growth components [net assimilation rate (NAR), specific leaf area and leaf weight ratio (LWR)] after defoliation were measured to determine whether these plastic changes maximize plant growth and fitness. Different ontogenetic phases of the response were discerned and increased RGR of defoliated plants was detected at the end of the experimental period, but brought about by a different growth component (NAR or LWR) in each ecotype. These changes in RGR are putatively related to increases in fitness in defoliated environments. At the intra-specific scale, data showed a trade-off between the ability to grow under benign environmental conditions and the ability to tolerate resource limitation due to defoliation.

Keywords: *Datura stramonium*; defoliation tolerance; growth response coefficients; net assimilation rate; ontogenetic plasticity response to defoliation; plant relative growth rate.

Introduction

As sessile organisms, plants must cope with biotic and abiotic spatio-temporal environmental fluctuations by means of phenotypic plasticity (Schlichting 1986). Although plants are commonly defoliated by herbivores, they may mitigate their negative effects through compensatory growth (McNaughton 1983). Compensatory

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response implies a series of plastic phenotypic changes in different traits that can determine the pattern of change in relative growth rate (RGR, the rate at which a given amount of existing biomass can produce new biomass per unit time; Shipley 2000) after defoliation (Oesterheld and McNaughton 1988; Trumble *et al.* 1993; Strauss and Agrawal 1999; Stowe *et al.* 2000; Tiffin 2000). These plastic responses to defoliation may include an increasing photosynthetic rate (Caldwell *et al.* 1981; Wallace *et al.* 1984), changing the allocation pattern to increase the production of new leaf area (McNaughton and Chapin 1985), or nutrient uptake (Ruess *et al.* 1983; McNaughton and Chapin 1985) and improving plant water status (Toft *et al.* 1987).

The interplay between these partial plastic traits determines the plant's overall response to defoliation, which may contribute to tolerance alleviating negative effects on fitness (McNaughton 1983; Núñez-Farfán *et al.* 2007). A genotype is considered more tolerant when defoliation has a lower effect on fitness, even to a point at which it may seem that it is not perceiving the stress or overcoming it (i.e. overtolerance, higher fitness in the defoliated environment). Such apparent lack of effect on fitness could be controlled by plastic changes in morphological and physiological traits (Bradshaw 1965; Richards *et al.* 2006; Sultan *et al.* 1998), which are less visible and more distant to fitness (i.e. underlying traits *sensu* Alpert and Simms 2002). In other words, defoliation can modify underlying traits that maintain fitness stability and the stress could be perceived at the 'macroscopic' level, affecting fitness or sometimes only at a more detailed level, modifying underlying traits (Couso and Fernández 2012). Thus, the degree and pattern of plasticity in tolerance traits could result in three different patterns of plant fitness between undefoliated and defoliated environments; namely, complete tolerance (no fitness differences between environments), undertolerance (lower fitness in the defoliated environment) and overtolerance (higher fitness in the defoliated environment). Many species fail to compensate after defoliation and are presumed to be undertolerant (Bergelson *et al.* 1996; Stowe 1998; Hanley and Fegan 2007; Klimešová *et al.* 2007), while a full range of compensatory responses has been observed for complete tolerance and overtolerance (Paige and Whitham 1987; Lennartsson *et al.* 1998; Hochwender *et al.* 2000; Hawkes and Sullivan 2001).

According to Hilbert *et al.* (1981) there is a trade-off between the ability of a genotype to grow under undefoliated environmental conditions (i.e. the environment at which is achieved the maximum growth potential, RGR_{max}), and its capacity to tolerate defoliation. They specifically predict that plants growing at nearly their shoot RGR_{max} were less likely to maximize above-ground biomass if defoliated than those with shoot growth rates far below maximum. This is because genotypes with high RGR_{max} at the time of grazing require large increases in growth rate while slow growth genotypes require only small increases to reach undefoliated plants (Hilbert *et al.* 1981). Furthermore, after defoliation, genotypes with slow RGR_{max} exhibit the highest overcompensatory growth later (higher RGR in defoliated plants) (Oesterheld and McNaughton 1988).

Compensatory growth constitutes one of the most important traits related to defoliation tolerance, because in annuals it is positively related, via attainment of a larger size, with reproductive effort (Weaver and Cavers 1980; Aarssen and Taylor 1992), survival and fecundity (Crawley 1997). Most studies on compensatory growth have concluded that, to compensate for biomass lost, a defoliated plant must have a higher RGR than an undefoliated plant (i.e. a higher rate of increasing biomass per unit of biomass already present) (Hilbert *et al.* 1981). This increase in RGR is the result of comparisons often made at a single fixed point in time after defoliation (Oesterheld and McNaughton 1991); however, environmental differences in RGR are the result of plasticity in developmental trajectories (i.e. ontogenetic plasticity; Pigliucci 2001). Whether RGR increase in defoliated plants is sufficient to produce as much biomass as the undefoliated plants would depend on how long that environmental difference is maintained (Hilbert *et al.* 1981). Therefore, monitoring the growth difference of defoliated and undefoliated plants over time may be of great value for the study of the response to defoliation (Hilbert *et al.* 1981; Oesterheld and McNaughton 1991). This approach has proved successful in the study of other environmental plant stressors (Shipley 2000; Useche and Shipley 2010*a*, *b*). However, little is known about the time-course of growth, and frequently it is ignored or assumed to be monotonic in defoliated environments (but see Oesterheld and McNaughton 1988).

Plant growth analysis can be used to assess the contribution of different mechanisms of compensatory growth to RGR via its three determinants. First, the net assimilation rate (NAR)—the increase in biomass per unit of time and leaf area—is strongly correlated with the whole-plant net photosynthetic rate (Poorter and Van der Werf 1998). Second, specific leaf area (SLA, leaf area per unit of leaf biomass) is a parameter that reflects aspects of leaf morphology such as leaf density and thickness (Poorter and Nagel 2000). And third, the biomass allocation to leaves (LWR) needs to be considered. Thus, RGR can be broken down into these three leaf-based properties as follows: $RGR = NAR \times SLA \times LWR$. The product of SLA and LWR is the leaf area ratio (i.e. LAR, the ratio of leaf area per plant biomass). To quantify the degree to which plants can compensate for potential losses in RGR throughout development due to defoliation, we asked to what extent a difference in RGR caused by a difference in resource supply due to defoliation (in the case of defoliation, decreases in resource supplies such as light and CO_2 are expected; Trumble *et al.* 1993) is due to a difference in each of the growth determinants. In other words, how do the different components change if RGR changes due to defoliation? In order to answer this question we used a plasticity estimation called the Growth Response Coefficient (GRC; Poorter and Van der Werf 1998): the relative change in one of the growth determinants, scaled with respect to the relative change in RGR (Poorter and Nagel 2000; see Methods section) due to defoliation.

Time-course changes in plant growth and allocation parameters following a defoliation event have been reported (Oesterheld and McNaughton 1988; Anten *et al.* 2003; Van Staalduinen and Anten 2005). However, there is no consensus on how plants are expected to respond after biomass removal (Yoshizuka and Roach 2011). In response to defoliation, the behaviour of RGR suffers an oscillation over time (Oesterheld and McNaughton 1988, 1991) that can be classified into three main ontogenetic stages: initially decreasing after defoliation but later recovering to the values of control plants (buffering state); a relatively unchanging time behaviour with respect to control plants (steady state), but later reaching values above those of control plants (overcompensatory state). The buffering capacity of RGR has been related to strong progressive ontogenetic increases in NAR, and SLA counteracting the immediate decrease in LWR after defoliation, to reach the same LAR (new leaf area at a low carbon cost) as control plants (Oesterheld and McNaughton 1988, 1991). While this counteractive action of the growth components has been related to the buffering capacity of a plant's RGR, instances of steady and overcompensatory growth have received less attention (Fig. 1).

Here, we studied the amount and timing of ontogenetic plastic changes in RGR and its determinants following a single defoliation in *Datura stramonium* L. (Solanaceae), a colonizing annual plant distributed world-wide. We compared two ecotypes of *D. stramonium* with differences in tolerance to defoliation, associated with differences in herbivore pressure in different abiotic environments (Fornoni *et al.* 2003, 2004). Since there is a great variation in the response to herbivory at the intra-specific level in *D. stramonium*, the comparisons of growth parameters of defoliated and undefoliated plants of different ecotypes would provide insights into the different defensive strategies that plants evolve in interaction with herbivores (e.g. Oesterheld and McNaughton 1988).

The main hypothesis is that there is a trade-off between tolerance to defoliation and growth under less limiting conditions (undefoliated environments), but that this model only cover traits related directly to fitness, i.e. those at the macroscopic level (e.g. above-ground biomass and the number of seeds). The secondary hypothesis is, at a more detailed level here, that the more tolerant genotypes are more plastic in mechanistic traits (i.e. compensatory growth), those that would allow them to gain more fitness in the defoliated environment.

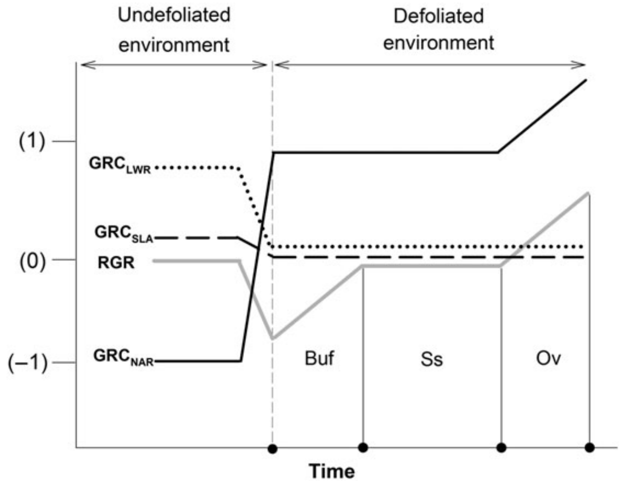

Figure 1. Hypothetical scenario relating plastic changes in RGR (signed percentages of increase or decrease of defoliated plants in relation to control plants) and the relative contribution (i.e. a GRC) of NAR, SLA and LWR to these plastic changes in a plant whose leaf-total area is highly defoliated. By definition, before defoliation (dashed line reference), the plasticity in RGR is zero. Therefore, the trajectory of RGR before defoliation is not shown, but it decreases in the model due to the negative influence of NAR. The plastic behaviour of RGR has three main ontogenetic stages (dots and vertical lines in the time axis) in the model (Oesterheld and McNaughton 1988, 1991): a buffering state (Buf), steady state (Ss) and an overcompensatory state (Ov) (see Introduction). The range of GRC values indicates the severity of a given reduction in resource supply due to defoliation (see Methods). In the model NAR plays a leading role in the RGR's buffering and Ov.

Methods

Datura stramonium is a self-compatible annual weed occurring in a wide variety of plant communities in Mexico and North America (Weaver and Warwick 1984). Although it is found in all types of soil, it prefers rich soils (Weaver and Warwick 1984) and rapidly assimilates nitrogen in the form of nitrate or ammonium (Lewis and Probyn 1978; Platt and Rand 1982). Its leaves are eaten by at least two specialist herbivorous insects, *Epitrix parvula* (Coleoptera: Chrysomelidae) (Núñez-Farfán and Dirzo 1994) and *Lema trilineata* (Coleoptera: Chrysomelidae), and other generalist species (Núñez-Farfán and Dirzo 1994; Núñez-Farfán *et al.* 1996). The average percentage of leaf area lost to herbivores in populations of *D. stramonium* from Central Mexico is 30 % (range

11–49 %; Valverde *et al.* 2001). In *D. stramonium*, unlike many plants where the adult phase begins when sexuality is first expressed, maturity and sexuality are not necessarily synonymous, as sexuality is also very important for the vegetative growth dynamics of the plant due to its particular architectural model called *Leeuwenberg* (Hallé *et al.* 1978), in which branching produces equivalent orthotropic modules, each with deterministic growth, culminating in the production of a terminal flower.

Growth conditions and harvest

Seeds from two populations (natural progenies) of *D. stramonium* from Central Mexico were collected from the Santo Domingo (SD) population in the state of Morelos (18°N, 99°W) and the Patria Nueva (PN) population in the state of Hidalgo (20°N, 99°W). Localities differ in climate, vegetation and type of soil (Table 1). Plants of both populations withstand different natural levels of defoliation by herbivores, with a difference in abiotic pressure perhaps contributing to select different growth components of RGR in response to defoliation (Poorter and Nagel 2000). Santo Domingo population has shown to be overtolerant to natural defoliation (e.g. Fornoni *et al.* 2003) while PN population to be complete tolerant (e.g. Valverde *et al.* 2001).

Full-sibs of each field's maternal plant were derived from one generation of selfing at the glasshouse. For germination, seeds were put in pots (1.5 L) and kept at a 12:12 h (light/dark) photoperiod in a Roch growth chamber, and mean temperature of 28/23 °C. When cotyledons were fully expanded, seedlings were transplanted to pots with a mixture of sand and peat moss (4 : 1), and placed at the glasshouse. We discarded half of the plants prior to transplanting to assure a reduction in the variability expressed in seed germination and to ensure that the growth conditions were as similar as possible between populations. The mean radiation was 231 W m^{-2}. The temperature regime was 20 ± 0.5 °C day and 13 ± 0.5 °C night (13 h daylength). Relative humidity was always above 60 %. The day before transplanting, the 25 % largest and 25 % smallest plants from each population were discarded (Poorter 1989). Nutrients were added in the form of 200 mL of a liquid soluble fertilizer (Peter's 20–20–20: 3.9 % NH_4-N, 5.8 % NO_3-N, 10.0 % urea-N, 20 % P_2O_5-P, 20 % K_2O-K), such that total nutrient addition over the entire growth period approximated to a nutrient supply of 400 kg N ha^{-1} $year^{-1}$ (medium nutrient level in Table 1 of McConnaughay and Coleman 1999).

The experiment consisted of 154 plants for each population. One half of the plants experienced no defoliation over the entire experiment. The other half of the plants were defoliated to the nearest 35 % of total leaf area removed. This defoliation level was chosen to simulate the upper limit of natural defoliation intensity observed in the sampled populations (~31 % of mean leaf area consumed; Table 1). Using the estimated relationship between leaf length (cm) and leaf area (cm^2) (leaf area = 0.56–0.76[leaf length] + 0.5[leaf length]^2; $r^2 = 0.99$), we were able to estimate the area to be removed with a cork-borer No. 6 (1.1 cm^2 in diameter), when plants were 61 days old (1464 h of age), after seedling emergence. The experiment was run mainly in the adult phase, but in this species sexuality is also very important for the vegetative growth dynamics of the plant; nearly the 60 % of the total vegetative growth is achieved in the adult phase (I. D. Camargo and J. Núñez-Farfán, unpubl. data).

The harvest schedule began at 59 days (1416 h) after seedling emergence and continued for 74.25 days (1782 h). To detect plastic responses to defoliation, the harvest programme was conducted with a more intense harvest frequency, bracketing the day when the treatment (i.e. defoliation) was imposed (e.g. Shipley 2000; Useche and Shipley 2010*a*, *b*). On Day 59, two plants per population per experimental condition were harvested in the morning (09:00), noon (12:00) and in the afternoon (15:00); from Day 60 (i.e. 1 day before defoliation) until Day 64, two plants per population per experimental condition were harvested at 09:00, 11:00, 13:00

Table 1. Environmental characteristics and mean relative resistance (1-natural defoliation) of two *D. stramonium* populations in Central Mexico. [1]Standard error, *n*.

Characteristics	*D. stramonium* populations		Sources
	Santo Domingo	Patria Nueva	
Habitat	Pine-oak forest	Xerophytic shrub	García (1988)
Geographic coordinates	18°N, 99°W	20°N, 99°W	García (1988)
Altitude (m a.s.l)	2050	1745	García (1988)
Mean annual precipitation (mm)	1463.2	360.5	García (1988)
Mean annual temperature (°C)	19.9	18.4	García (1988)
Relative resistance	0.559 (0.014, 30)[1]	0.816 (0.013, 18)[1]	Valverde *et al.* (2001)

and 15:00; from Day 65 until Day 67, two plants per population per experimental condition were harvested at 11:00 and 15:00; and from Day 68 until Day 74, one plant per population per experimental condition was harvested at 15:00. Plants to harvest were randomly chosen.

Plants chosen at each harvest were separated into leaves, 'support' tissues (stems, petioles and pedicels) and reproductive tissues (flowers and fruits), if present. Plant parts and their dry weight were measured after being oven-dried for at least 48 h at 80 °C. Total one-side of fresh leaf area of the plant was estimated by image analysis with Compu Eye, Leaf and Symptom Area Software (Bakr 2005).

Growth analysis and plasticity estimation

Plant traits were measured following Shipley's (2000) protocol. In general, the SLA was calculated as the whole-plant leaf area divided by whole-plant leaf dry mass. The LWR was calculated as the leaf dry mass divided by shoot dry mass. The predicted values across time of leaf area, shoot dry mass, SLA and LWR were evaluated using cubic splines by means of the *gam* function in the R package MGCV (Wood 2011).

The use of peat moss in the soil mixture precluded the estimation of the root mass ratio; however, using only above-ground tissues in the determination of maximum RGR substantially reduces the time and effort required in harvesting plants (Shipley 1989). Notwithstanding, defoliation has a minimal effect on root biomass of grasses and annual herbs that do not form rhizomes (reviewed in Ferraro and Oesterheld 2002) as *D. stramonium*. The RGR was calculated as the rate of change in the natural logarithm of shoot dry mass over time, obtained as the derivative of the cubic-spline smoother using the *smooth spline* function in R (R Foundation for Statistical Computing; http://www.R-project.org). Cubic-spline smoothers have been shown to accurately detect even subtle changes in RGR without imposing any functional assumptions on the data (Shipley and Hunt 1996). The NAR was calculated as RGR divided by the product of SLA and LWR.

All other statistical analyses testing the differences between the control and defoliated environments were done using a General Additive Model as implemented in the R package MGCV (Wood 2011) (see Appendix). We let the smooths 'interact' with the experimental conditions as a factor (control and defoliated series) and its significance was interpreted as an ontogenetic plasticity to defoliation in any trait measured. For each experimental condition, we allowed for smooths to have different smoothing parameters. The *P*-values for individual terms were calculated using the Bayesian estimated covariance matrix of the parameter estimators implemented in the MGCV package, based on a test statistic motivated by Nychka's (1988) analysis of the frequentist properties of Bayesian confidence intervals for smoothing splines (Wood 2011). When the treatment effect was significant, standard errors based on the Bayesian posterior covariance of the parameters in the fitted model were used (Wahba 1983; Wood 2011) as a visual aid to interpret the ontogenetic variation of the statistical difference between control and defoliated environments in the plot of any trait measured.

The degree of plasticity for each ontogenetic parameter was estimated as the difference between the defoliated plants and control plants divided by control plants, and therefore it reflects signed percentages of increase or decrease in defoliated plants with respect to controls. The use of signed plasticity values allowed us to evaluate the progressive ontogenetic plastic adjustments of defoliated plants to restore the trajectory of controls and, therefore, to identify whether the plastic response was active, or whether it was passive, from resource deficiency (van Kleunen and Fischer 2005). For instance, an initial decrease in LWR is expected due to defoliation (algebraically, the biomass of leaves decreases in proportion to all biomass present); the progressive recovery of defoliated plants in order to re-attain the trajectory of the controls across time in terms of this parameter is what constitutes an active response to defoliation.

To compare the relative contribution of each growth determinant to the RGR differences observed in response to defoliation, we estimated the GRC (*sensu* Poorter and Nagel 2000) for each growth determinant. Growth response coefficients are scaling (allometric) slopes, in which the natural logarithm of each growth component is regressed on the natural logarithm of RGR (Shipley 2006). Then GRCs for each growth determinant (X) were calculated using the differences between defoliated (D) and control plants (C), as follows:

$$\mathrm{GRC}_X = \frac{\ln X_\mathrm{D} - \ln X_\mathrm{C}}{\ln \mathrm{RGR}_\mathrm{D} - \ln \mathrm{RGR}_\mathrm{C}}$$

The range of GRC values indicates the severity of a given reduction in resource supply (Poorter and Nagel 2000) due to defoliation. For instance, a GRC value of 1 indicates that the proportional plastic change in the growth parameter of interest equals the proportional plastic change in RGR. A GRC value of 0 indicates that there is no plastic change in that growth parameter at all. Growth response coefficient values can be higher than 1 if the increase in the growth parameter is stronger than the increase in RGR, and can be lower than 0 if an increase in a growth parameter corresponds to a decrease in RGR (Poorter and Nagel 2000). Since the GRCs are proportional to changes in NAR, LWR and SLA relative to RGR, then these values should add up to 1 when RGR is exactly the product of them. Growth response coefficient values before defoliation were estimated as the slope of the linear regression between the natural logarithm of each growth component regressed on the natural logarithm of RGR (Poorter and Van der Werf 1998) in the time interval between 1416 and 1446 h of age. The latter interval is chosen because, by definition, there is no difference (plasticity) between the defoliated and control series in this time interval.

The contribution of ontogenetic values of each GRC to growth were evaluated with a *t*-test (when assumptions were met) or with a Wilcoxon signed-rank test (*T*), using the null hypothesis of $\mathrm{GRC}_X = 0$ (i.e. no contribution to growth). There were two negative predicted RGR values (1734 and 1782 h of age) in the final trajectory of growth in the control series of the SD ecotype; therefore, these were omitted in the estimation and plots of plasticity and GRCs.

Tolerance. As a proxy to lifetime reproductive fitness, the final reproductive output was estimated as the total seed number per plant of each ecotype, and measured in an additional sample of 60 plants at the end of the experiment (81 days after germination, 7 days after the last plant growth harvest until the last seeds were mature enough). These plants were not used for the estimation of plant growth traits described above; mainly, because plant leaves at this stage were almost absent by natural defoliation. The slope of fitness between control and defoliated plants was considered as a measure of tolerance to the defoliation. The total seed number was analysed with a general linear model with a Poisson error, and a *log* link function (JMP, Version 7, SAS Institute, Inc., Cary, NC, 1989–2007). This was conducted to investigate the relative importance of (i) ecotype (variation among ecotypes); (ii) treatment (presence of average fitness plasticity in response to defoliation regardless of specific ecotypes); and (iii) treatment by ecotype interaction (variation for fitness plasticity among ecotypes).

Results

The ontogenetic trend in plant dry mass of defoliated plants began to diverge from that of undefoliated controls once defoliation occurred, and surpassed their values at the end of the experiment, mainly in the SD ecotype (Fig. 2). Relative growth rate decreased during plant development in the control condition. The maximum RGR of the PN ecotype was ~29.4 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ at the beginning of the experiment and then decreased to around 2.45 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ at the end of the experiment (Fig. 2). However, a different trend was observed in the SD ecotype: the maximum RGR decreased by ~29 % in relation to PN; it was 16.6 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ at the beginning of the experiment and decreased with a complex growth trajectory to zero at the end of the experiment, although two sudden increases were observed between 1494–1536 and 1586–1638 h of age (Fig. 2). The RGR values of the PN-defoliated plants immediately decreased to 4.7 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ (an 81 % decrease with respect to the same value at the time of defoliation in the control plants, i.e. 25.1 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$) and slowly decreased to around 2.9 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ at the end of the experiment. As a result, the RGR of defoliated plants reached the RGR values of control plants with a marginal increase in the final phase of the experiment (Fig. 2). At the time of defoliation, the RGR values of the SD-defoliated plants immediately decreased to 4.1 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ (a 67 % decrease with respect to the same value at the time of defoliation in the control plants, i.e. 12.4 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$) and remained almost constant, slightly decreasing to around 3.4 mg $\mathrm{g}^{-1}\,\mathrm{h}^{-1}$ at the end of the experiment. As a result, the RGR of defoliated plants remained marginally lower than the control plants up to hour 1586, when defoliated plants reached the RGR values of control plants, producing an increase with respect to the values observed in the control plants at the end of the experiment (Fig. 2).

The NAR presented the same behaviour as RGR in the two ecotypes (Fig. 2). No errors are shown because NAR was estimated as a function of the means of RGR, SLA and LWR. A decrease in NAR was observed in the control condition for both ecotypes, the maximum (and minimum) values for the PN and SD ecotype were 1.2×10^{-4} g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$ (3.6×10^{-5} g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$), and 8.2×10^{-5} g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$ (-8.83×10^{-6} g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$), respectively. When the defoliation occurred, NAR values decreased to $\sim 3 \times 10^{-5}$ g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$ in the two ecotypes (a 75 % decrease with respect to the same value at the time of defoliation in the control plants, i.e. 1.2×10^{-4} g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$). After that, NAR remained almost constant in the PN ecotype, but in the SD ecotype it increased slightly (5×10^{-5} g $\mathrm{cm}^{-2}\,\mathrm{h}^{-1}$) and surpassed the values of control plants at the end.

In general, data showed evidence of ontogenetic phenotypic plasticity to defoliation in almost all traits measured; that is, the values for the defoliated series were statistically significantly different to the controls for the time frame of leaf area, shoot dry mass and LWR in the two ecotypes (Fig. 3). Specific leaf area did not express ontogenetic phenotypic plasticity to defoliation (Fig. 3). Traits displayed different patterns of ontogenetic phenotypic plasticity. Leaf area showed a monotonic increase in defoliated plants along time, re-establishing the trajectory of control plants and surpassing their values at the end, mainly in the SD ecotype. Leaf weight ratio decreased through time in control and defoliated

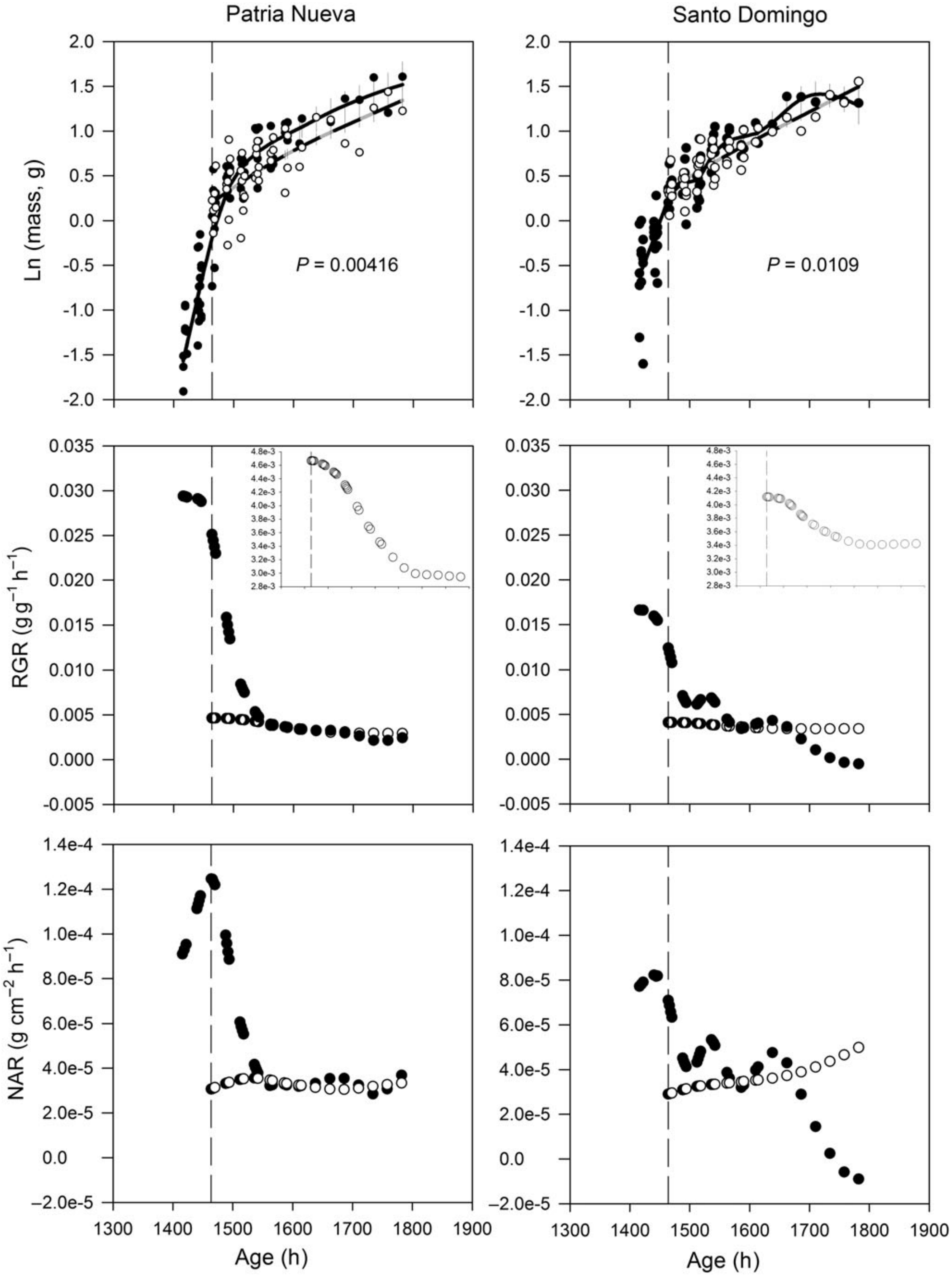

Figure 2. The time-course of shoot dry mass, RGR and NAR in two ecotypes of *D. stramonium* (PN and SD). Filled circles represent plants grown without defoliation (continuous line) and open circles represent defoliated plants (30 % of leaf removed) (dashed grey line). Standard errors are plotted in each fitted line. The dashed vertical line indicates the onset of the defoliation treatment. The inset graphs are a zoom of the time trajectory in the defoliated environment.

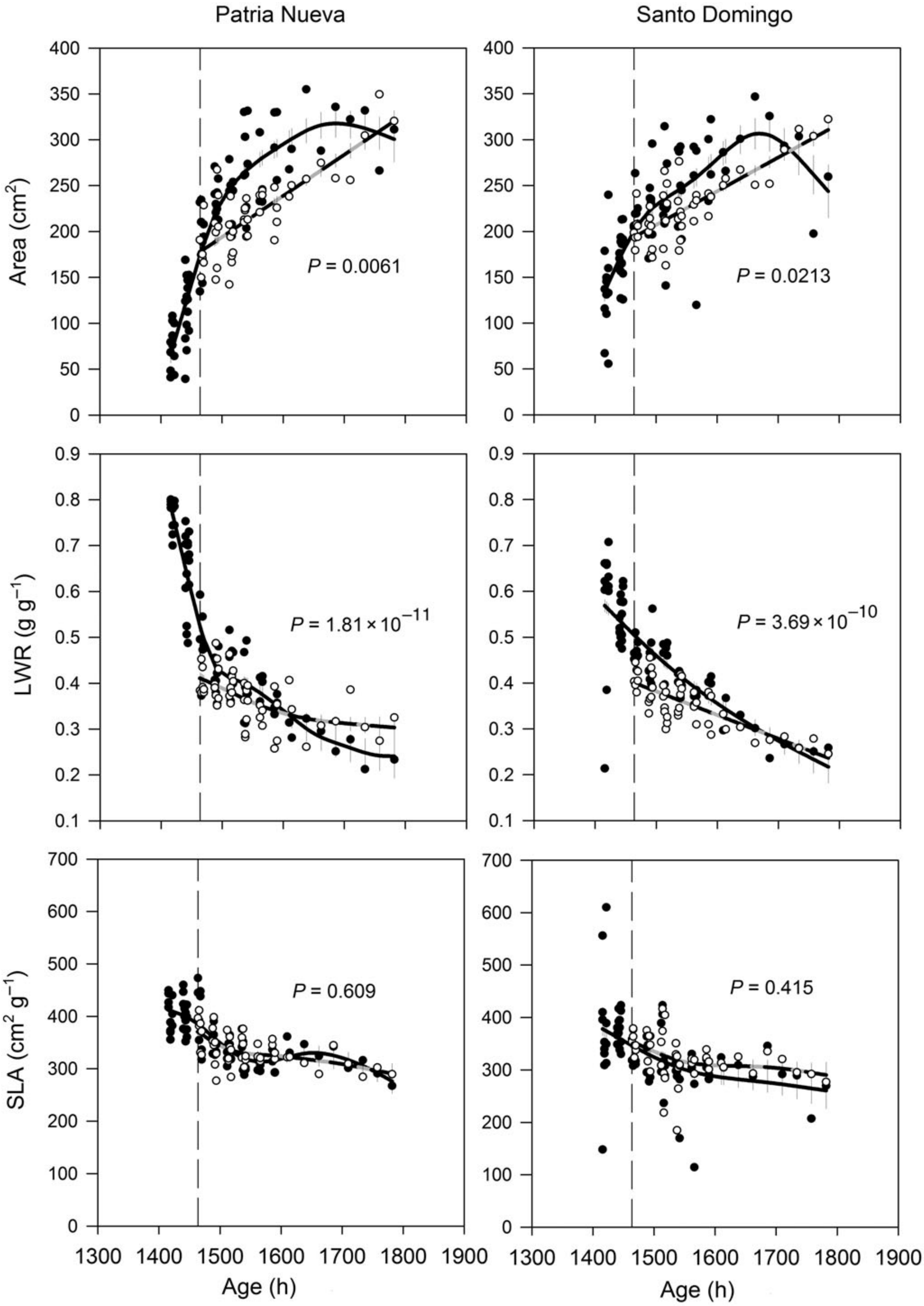

Figure 3. Area, LWR and SLA in two ecotypes of *D. stramonium* (PN and SD). Filled circles represent plants grown without defoliation (continuous line) and open circles represent defoliated plants (30 % of leaf removed) (dashed grey line). Standard errors are plotted in each fitted line. The dashed vertical line indicates the onset of the defoliation treatment.

plants of both ecotypes. When defoliation occurred, values of LWR immediately decreased to around 0.4 g g^{-1} in both ecotypes (a 21 % decrease with respect to the same value at the time of defoliation in control plants; i.e. ~0.51 g g^{-1} in both ecotypes) then progressively decreased afterwards, resulting in higher values for defoliated plants at the end mainly in the PN ecotype (Fig. 3). The time-course of SLA was qualitatively the same in both ecotypes, decreasing from ~412 cm^2 g^{-1} to around 276 cm^2 g^{-1} in the PN ecotype and from ~378 cm^2 g^{-1} to around 260 cm^2 g^{-1} in the SD ecotype (Fig. 3).

The immediate RGR decrease due to defoliation is recovered after 98 h in the PN ecotype (1464–1562 h of age). In contrast, SD-defoliated plants equalled the RGR of control plants after 122 h (1464–1586 h of age; Fig. 4, upper and lower panels). Afterwards, defoliated RGRs remained almost constant with respect to controls in PN, but with a slight increase at the end of the experiment. Whereas in SD remained nearly constant with a large increase at the end of the experiment (Fig. 4, upper and lower panels). With this evidence, changes in RGR in response to defoliation could be classified into three main ontogenetic stages: the buffering, steady and overcompensatory states.

Growth response coefficient behaviour is linked to changes in RGR (Fig. 4, upper panels). Before defoliation, the RGR decrease was achieved by an ontogenetic decrease in NAR ($GRC_{NAR} = -13.88$, PN; $GRC_{NAR} = -0.74$, SD); that is, a strong decrease in NAR occurred with a

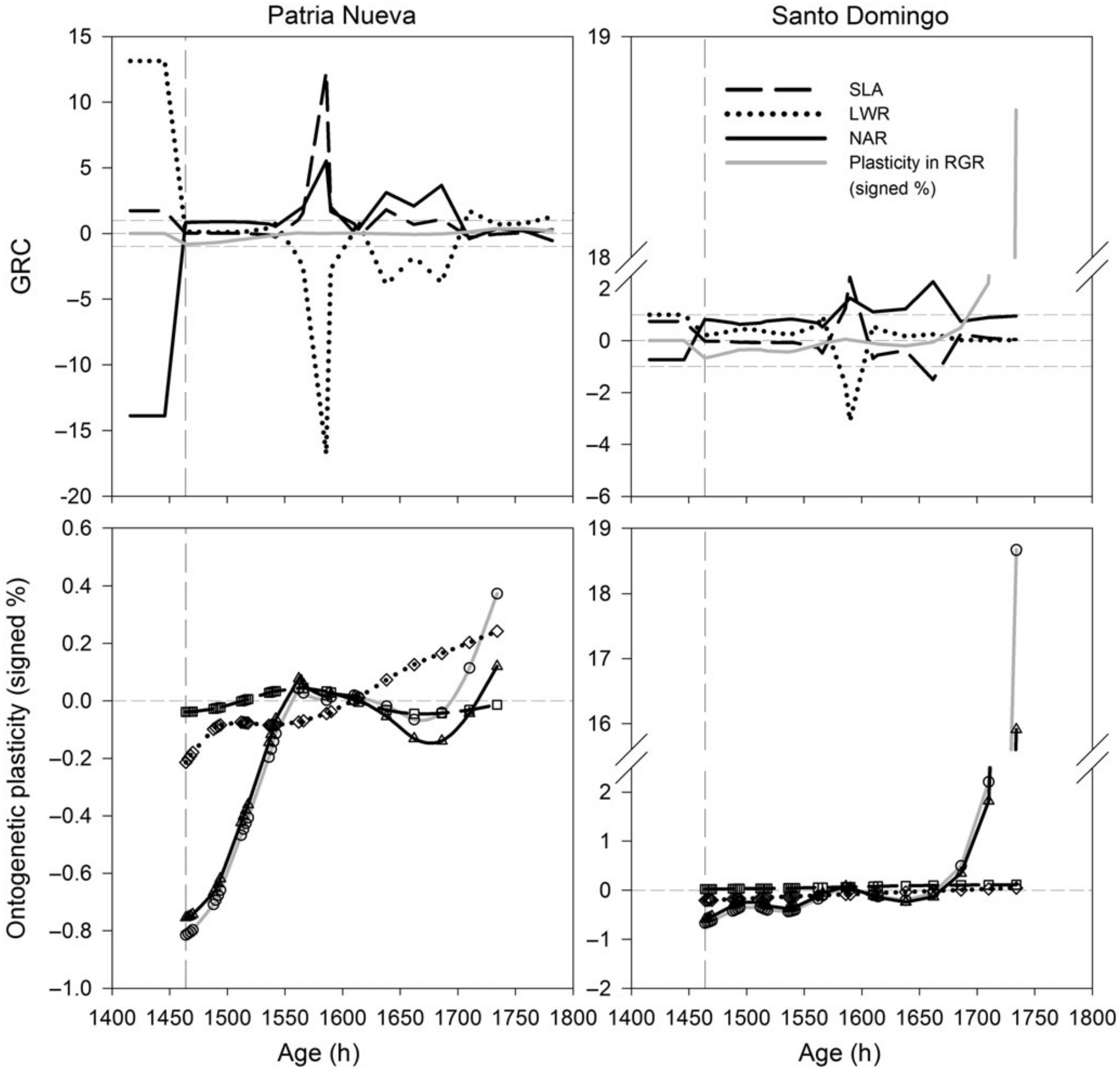

Figure 4. Time-course of GRCs and RGR in response to defoliation (vertical dashed line) in two ecotypes of *D. stramonium* (PN and SD). The higher panel shows the behaviour of plastic changes in RGR versus the changes in GRCs (horizontal dashed lines are reference values equal to 1 and −1). The lower panel is presented to show the ontogenetic plastic changes (used to estimate GRCs) in NAR, SLA and LWR underlying the plasticity in RGR.

decrease in RGR. This NAR decrease was accompanied by a positive counteracting LWR influence ($\mathrm{GRC}_{\mathrm{LWR}} = 13.14$, PN; $\mathrm{GRC}_{\mathrm{LWR}} = 1$, SD) and SLA ($\mathrm{GRC}_{\mathrm{SLA}} = 1.74$, PN; $\mathrm{GRC}_{\mathrm{SLA}} = 0.74$, SD), which prevented the RGR from falling even further. Immediately after defoliation (the buffering state), the proportional change in NAR almost equalled the proportional change in RGR; therefore, NAR was the most important trait increasing RGR in defoliated plants in both ecotypes. Nevertheless, the SD ecotype displayed an LWR contribution to the RGR increase in defoliated plants (Fig. 4, upper panels). Sizeable increases of defoliated-plant RGR (i.e. the overcompensatory state, values beyond control plants) can be achieved whenever the positive increase in a particular GRC cannot be counteracted by any other growth component. Interestingly, this overcompensation is achieved by a different GRC depending on the ecotype. For instance, the overcompensation at the end of the ontogeny observed in the RGR of the defoliated PN plants was achieved because the increase in LWR was not negatively influenced by the other growth components. At the same time, defoliated plants from the SD ecotype achieved overcompensation by NAR because other growth components did not counteract its effects.

Net assimilation rate was the most important trait (PN; $T_{17} = 76.5$, $P < 0.0001$; SD; $T_{19} = 95$, $P < 0.0001$) contributing to RGR increase in defoliated plants throughout the buffering state, followed by LWR (PN; $T_{17} = 59.5$, $P = 0.0016$; SD; $T_{19} = 76$, $P = 0.0006$) in both ecotypes (Fig. 5). Leaf area ratio (the product of SLA and LWR) is more influenced by LWR. In the RGR steady state, a strong counteractive influence between NAR ($T_8 = 18$, $P = 0.0039$) and LWR ($T_8 = -15$, $P = 0.0195$; which mainly influenced LAR) produced no RGR differences ($T_8 = -1$, $P = 0.5273$) between defoliated and control plants in PN (Fig. 5). In SD, this homeostatic transient RGR state ($T_5 = -6.5$, $P = 0.0625$) was mainly related to a trade-off between NAR ($T_5 = 7.5$, $P = 0.0313$) and SLA ($T_5 = -2.5$, $P = 0.3125$; which mainly influenced LAR) (Fig. 5). Relative growth rate overcompensation (PN; $t_4 = 4.17$, $P = 0.0126$; SD; $T_4 = 7.5$, $P = 0.0313$) is mainly achieved because growth components did not trade-off, but it was mainly accomplished by different growth components depending on the ecotype: LWR in PN ($t_4 = 4.64$, $P = 0.0094$) and NAR in SD ($t_4 = 13.58$, $P = 0.0027$) (Fig. 5).

Defoliated plants produced more seeds than controls (likelihood ratio test of treatment effect; $\chi^2 = 261.38$, d.f. = 1, $P < 0.0001$), demonstrating plasticity in fitness (Fig. 6). Ecotypes differ significantly in their mean production of seeds ($\chi^2 = 697.30$, d.f. = 1, $P < 0.0001$). The interaction between treatment and ecotype for the total number of seeds was significant ($\chi^2 = 48.47$, d.f. = 1, $P < 0.0001$), indicating ecotype variation in the tolerance capacity. Santo Domingo was the overtolerant ecotype; defoliated plants showed a 23 % increase in the number of seeds with respect to controls. In contrast, PN showed no fitness differences between environments, and then it was completely tolerant to defoliation (Fig. 6).

Discussion

Trade-off between $\mathrm{RGR}_{\mathrm{max}}$ and tolerance to defoliation

As expected from our first hypothesis, we detected a trade-off between $\mathrm{RGR}_{\mathrm{max}}$ and tolerance to defoliation. Santo Domingo was the most tolerant ecotype (i.e. overtolerant), leading to higher above-ground biomass and total number of seeds under defoliation at the end of the experiment. In addition, SD had the lowest $\mathrm{RGR}_{\mathrm{max}}$. Therefore, the slow-growing ecotype showed a positive effect of defoliation on fitness in comparison with the fast-growing PN ecotype, which showed almost constant above-ground biomass and total number of seeds (i.e. complete tolerance) between control and defoliated plants at the end. Thus, the qualitative pattern of tolerance observed in this experimental study agrees with the natural pattern of tolerance to defoliation found in *D. stramonium* (e.g. Valverde *et al.* 2001, Fornoni *et al.* 2003). No differences in total plant biomass and increases in other fitness components in response to defoliation have been reported for grazing species in final ontogenetic stages (del-Val and Crawley 2005).

Plasticity of RGR and tolerance to defoliation

Regarding our second hypothesis, greater tolerance is mediated by a higher plasticity in mechanistic traits. It was confirmed by the greater ontogenetic plasticity at the end of the experiment in overcompensatory growth expressed by the SD overtolerant ecotype (a 1866 % increase in RGR of defoliated plants with respect to controls) in comparison with the plasticity in overcompensatory growth of the PN ecotype (a 37 % increase in RGR of defoliated plants with respect to controls). This might be a case for adaptive plasticity, in which increasing plasticity in an underlying trait could increase fitness in one of a set of environments in which fitness was formerly similar, thereby increasing plasticity in fitness (Fig. 1A of Alpert and Simms 2002). If it is realized that RGR (in contrast to potential RGR) is positively related, via attainment of a larger size, to reproductive effort, survival and fecundity (e.g. Weaver and Cavers 1980; Aarssen and Taylor 1992; Crawley 1997), overcompensatory growth in the final stage of the ontogeny could constitute a selective advantage by helping plants to accumulate more biomass and produce more seeds in the presence of herbivores (e.g. *Themeda triandra*, Oesterheld and McNaughton 1988).

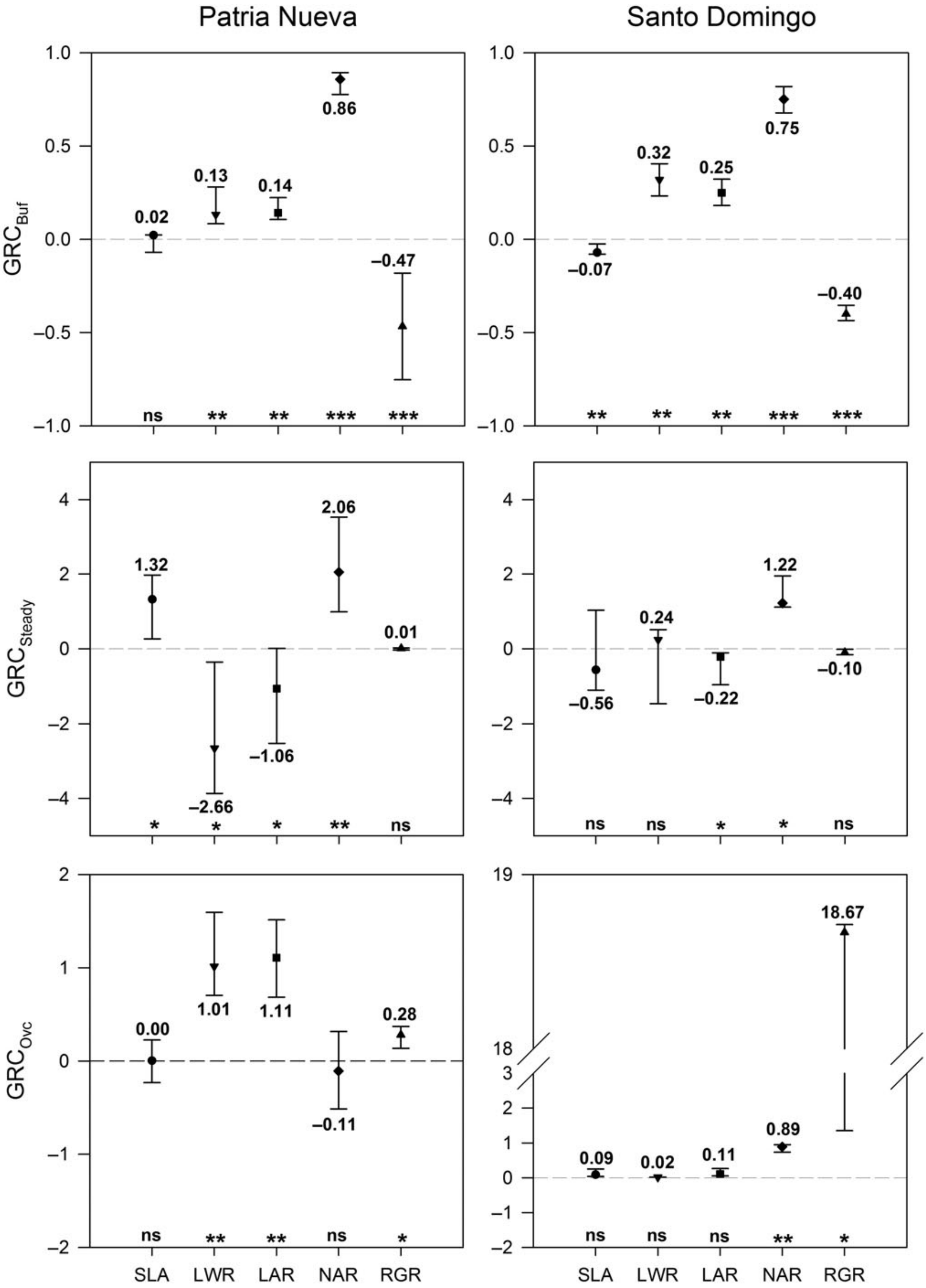

Figure 5. Error bars indicating the ontogenetic distribution by percentiles (first to third quartile) of GRCs of SLA, LWR and NAR in three main ontogenetic states of RGR (RGR_{Buf}, buffering state; RGR_{Ss}, steady state; $RGR_{ov,}$ overcompensatory state) calculated for plants under different treatments (control and defoliated plants) in two ecotypes of *D. stramonium* (PN and SD). Plasticity in RGR (signed percentages, see Methods) are plotted for reference. Asterisks at the bottom of the panels indicate the significance level under the H_o hypothesis of GRC = 0. $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$. The printed values above or below the box plots give the median GRC values.

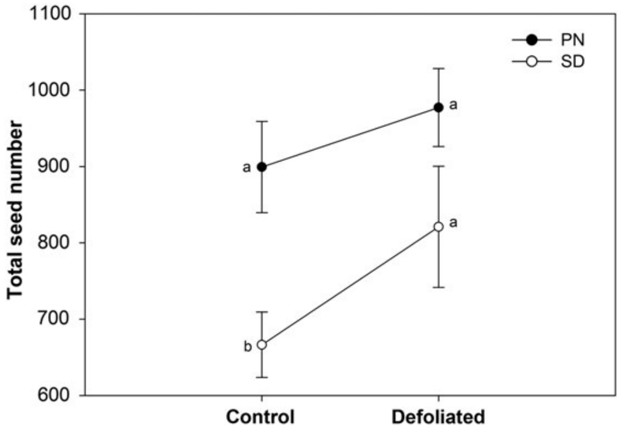

Figure 6. Norms of reactions of total seeds number for control and defoliated plants of two ecotypes of *Datura stramonium* (PN, Patria Nueva; SD, Santo Domingo) at the end of the experiment. Vertical bars represent standard errors of the mean. Different letters indicate significant differences between means at $P < 0.05$ (Tukey's HSD test).

Traits controlling ontogenetic plasticity of RGR in response to defoliation

Regarding the general question on what growth determinants are involved in the behavioural changes of RGR in response to defoliation, we have found that, throughout their ontogeny, defoliated plants of *D. stramonium* are capable of restoring, equalling or even overcompensating RGR compared with undefoliated plants. However, populations can differ in the importance of the component that controls the ontogenetic behaviour of RGR in response to defoliation.

In the buffering state. Progressive ontogenetic enhancements of NAR after defoliation contributed more to increasing RGR than enhancements of any other parameter. Increases in NAR after defoliation have been previously reported (Anten *et al.* 2003; Van Staalduinen and Anten 2005) and can be achieved through an increase in light intensity on the remaining leaves in grasses (Gold and Caldwell 1990; Senock *et al.* 1991; Van Staalduinen and Anten 2005) or by increased photosynthetic capacity (Nowak and Caldwell 1984; Zhao *et al.* 2008). However, other studies found no NAR increases (Van Staalduinen *et al.* 2010; Dobarro *et al.* 2012) after defoliation, but increases of leaf nitrogen concentration, a trait highly correlated with photosynthesis (Nowak and Caldwell 1984), suggesting that higher respiratory rates counteracted the increments in photosynthesis. This study did not measure photosynthetic capacity or levels of light intensity across the canopy. More studies are needed to evaluate the possibility of an increased photosynthetic capacity after defoliation in *D. stramonium*, as reported for other herbs (Zhao *et al.* 2008). Specific leaf area did not contribute to restoring the RGR after defoliation in both ecotypes (cf. Oesterheld and McNaughton 1988, 1991).

In the steady state of growth. A large trade-off between NAR and LAR produces no RGR differences between defoliated and control plants. However, ecotypes differed in the growth components that influence LAR. Leaf weight ratio produces a large trade-off with NAR in PN and SLA trade-off with NAR in SD.

In the overcompensatory state of growth. Leaf weight ratio was the most important trait in PN and NAR in SD. Net assimilation rate has been shown to be the most important trait conferring RGR overcompensation in *T. triandra* (Oesterheld and McNaughton 1988, 1991). However, *Dactylis glomerata* overcompensated for RGR after defoliation mainly by slowing down leaf senescence and, to a lesser extent, by increasing LWR (Dobarro *et al.* 2012). Differences in the growth component favouring overcompensatory growth may be due to a selection of one or more traits underlying RGR (Poorter 2002) in the different habitats of the ecotypes analysed. We predicted that NAR would be the trait involved in overcompensatory growth states, because of its very plastic nature, in contrast to perhaps more costly changes in biomass allocation or morphology. Considering that the PN ecotype's habitat receives very low mean annual precipitation (Table 1), it is possible that the strong water deficit in PN has favoured LWR over NAR, since decreases in photosynthetic ability due to a decrease in CO_2 assimilation can be possible even with small water deficits (Kaiser 1987). Similarly, in the understory palm *Chamaedoera elegans* (Anten *et al.* 2003) and several grass species (Van Staalduinen and Anten 2005; Van Staalduinen *et al.* 2010; Dobarro *et al.* 2012), defoliated plants allocated considerable more mass to the production of leaf laminas than control plants, favouring increases in LAR. Leaf weight ratio changes can be seen as adaptive in response to defoliation, enabling more CO_2 and light to be captured after a decrease in the available photosynthetic area. Here we assume that leaves (the organ involved directly in the acquisition of above-ground resources) have a priority over light and CO_2, demanding and limiting more of the available photosynthates (Poorter and Nagel 2000).

A higher overcompensatory growth can be observed in adult plants when each component (NAR, SLA and LWR) does not negatively affect others, so that changes in these components are not largely cancelled out. It is intriguing as to why this overcompensation is expressed at the final stage of the ontogeny in contrast to the homeostatic effect observed in early stages of the ontogenetic trajectory, in which at least one growth component experiences a large trade-off to maintain the RGR. We can tentatively conclude that this instance of overcompensation in *D. stramonium* could mainly be related to buffer the differences in total reproductive output between defoliated and control plants (see above) in the SD ecotype. It is possible that the excess of photoassimilates in this ecotype produced by a higher NAR in defoliated plants could be due to buffering differences in total biomass and, additionally, could lead to increasing seed output.

Conclusions

Using a high level of temporal resolution of growth analysis we were able to estimate the RGR_{max}, and at an intra-specific scale to detect a trade-off between the ability to grow in benign environmental conditions and the ability to tolerate resource limitation due to defoliation. In addition, the fast-growing ecotype (PN) showed a higher diminished RGR after defoliation, but also exhibited the lowest increase later. The opposite was observed in the slow-growing ecotype (SD), which was least affected early but showed the highest compensation later, not only in RGR but also in fitness. This study supports the hypothesis of Hilbert *et al.* (1981) that compensatory growth is most likely when undefoliated plants are growing at low rates because the amount of RGR change required for defoliated plants to equal the productivity of undefoliated plants is lower since RGR of undefoliated plants decreases. At the same time, if plants are growing close to their maximum capacity (i.e. RGR close to RGR_{max}), defoliation cannot increase RGR, and then compensation is unlikely (Hilbert *et al.* 1981; Oesterheld and McNaughton 1991). In line with Oesterheld and McNaughton (1988), this study showed that the ability of a genotype to tolerate defoliation will depend on the magnitude of the initial reduction in RGR, how fast the genotype reaches the equality point and how much RGR increases after that. More studies using within- and between-species comparisons are necessary to prove this prediction.

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Contributions by the Authors

I.D.C. and J.N.-F conceived and designed the experiment, I.D.C., J.N.-F. and R.T.-L performed the experiment and collected the data, I.D.C. analysed the data and I.D.C. and J.N.-F. wrote the paper.

Conflict of Interest Statement

None declared.

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Appendix

The following routine in R, using the MGCV package (Wood 2011), was used to evaluate the differences between control and defoliated plants (see Methods):

```
# loading data
dat<-read.table(file="C:/…", header=T)
# Defoliated as factor
dat$Defoliated<-as.factor(dat$Defoliated)
# Load GAM and GAMM package
library(mgcv)
# Multifactorial GAM with Damage and Hours interaction
y.fit <- gam(y ~ Defoliated + s(Hours, by=Defoliated),
data = dat, family=gaussian, bs= “cr”)
summary(y.fit)
```

Capítulo 2

EVOLUTION OF FITNESS REACTION NORMS TO DEFOLIATION IN ABIOTIC ENVIRONMENTS: GROWTH PROMOTING MECHANISMS AS PREDICTORS OF TOLERANCE AND VIGOR IN DATURA STRAMONIUM

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Evolution of fitness reaction norms to defoliation in abiotic environments: Growth promoting mechanisms as predictors of tolerance and vigor in *Datura stramonium*

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Abstract

The study of plant tolerance to herbivory has focused on the pattern of response in different abiotic environments, while the search of its underlying mechanisms has received less attention. To uncover the mechanisms of defoliation tolerance we assess the relationship between both properties of reaction norms, the slope and elevation, of RGR's components (SLA, LWR and NAR) and fitness in *Datura stramonium*. Defoliated and undefoliated plants from half-sib families were assigned to two nutrient environments (low and high), and growth traits expressed before and after defoliation were measured. Plants in low nutrients were more tolerant. We detected genetic variation for tolerance in both low and high nutrients, and for plasticity of LWR (low nutrients) and SLA (both nutrient environments). Low and high values of LWR in defoliated plants were selected for in low and high nutrients, respectively. A trade-off between growth (NAR) before damage and tolerance was detected in low nutrients. Vigor and tolerance of the fitness reaction norm was explained by reaction norms of different growth-promoting mechanisms between nutrient environments. Thus, evidence points that abiotic environmental differences in the pattern of selection acting on growth determinants would determine the evolution of tolerance and vigor of the fitness reaction norm.

Key Words: Adaptive phenotypic plasticity, Cost of plasticity to defoliation, Leaf weight ratio, Tolerance and Vigor to defoliation, Net assimilation rate, Specific leaf area

Introduction

Plant tolerance to herbivory is an across-defoliation stability in a plant fitness component achieved due to pronounced plasticity to defoliation in another phenotypic trait, less visible and more distantly connected to fitness (i.e., an underlying trait, *sensu* Alpert and Simms 2002). This mechanistic definition of tolerance differs from the representation of tolerance (the "operational" definition), as the slope (with an amount and sign) of the fitness reaction norm of a genotype in response to a gradient in intensity of defoliation (Simms 2000). Thus, different patterns of plant fitness between undefoliated and defoliated environments are possible (Stowe et al. 2000); namely, complete tolerance (no fitness differences between environments), undertolerance (lower fitness in the defoliated environment) and overtolerance (higher fitness in the defoliated environment) (Camargo et al. 2015). These patterns of tolerance emerge because defoliation can modify underlying traits that contribute to maintain stability in fitness and the imposed stress could not be perceived or detected at the 'macroscopic' level (Couso and Fernandez 2012, e.g. Camargo et al. 2015). In other words, the amount and sign of plastic changes in morphological and physiological traits (i.e., the underlying mechanisms, Alpert and Simms 2002, Bradshaw 1965, Pigliucci 2001, Richards et al. 2006, Sultan et al. 1998) can control the across-environmental fluctuations of fitness (the tolerance pattern).

While considerable effort has been made to uncover the underlying mechanisms of tolerance to herbivory (reviewed in Stowe et al., 2000), the focus of these efforts has been the relationship between the plasticity of underlying mechanisms and plasticity in fitness (e.g., Wise et al. 2008, Hochwender et al. 2000, Juenger & Bergelson 2000, Strauss et al. 2003). Notwithstanding, plasticity is not the unique property of a linear reaction norm. The across-environment expected trait value (reaction-norm elevation) of an underlying trait could have fitness consequences too (Scheiner 1993, 2002, Stinchcombe et al. 2004, Gravilets and Scheiner 2004). However, these consequences not only would impact the plasticity of fitness but its general vigor (reaction-norm elevation of fitness, Stowe et al., 2000). In other words, to uncover the mechanisms of herbivory tolerance it is necessary to assess the relationship between both properties of reaction norms, the slope and elevation, of underlying traits and fitness (see Fig. 1).

The relative growth rate (RGR, the rate at which a given amount of existing biomass can produce new biomass per unit time; Shipley 2000) before damage (i.e., intrinsic growth rate, the maximum rate experiences by plants when growth is geometric, Blackman 1919) has been shown to influence the slope and elevation of the fitness reaction norm to defoliation both theoretically (Weis et al. 2000) and experimentally (Camargo et al., 2015). Hilbert et al. (1981) proposes an allocation-cost mechanism in which an early higher investment in RGR before defoliation precludes the expression of a higher tolerance. This would create a negative genetic correlation between intrinsic growth rate and tolerance and a positive genetic correlation of intrinsic growth rate with vigor, improving performance at all damage levels (Weis et al. 2000, Camargo et al. 2015). This prediction has been shown experimentally using the total vegetative biomass as the fitness measure (Oesterheld and McNaughthon 1988, 1991) and, recently, with the numbers of seeds as the fitness proxy (Camargo et al. 2015).

While these findings strengthen the argument of tolerance as a response with constitutive components (Hochwender et al. 2000, Weis et al. 2000, Camargo et al. 2015), defoliation-induced responses has been the major interest of herbivory studies, and these plastic responses to defoliation may include an increasing photosynthetic rate (Caldwell et al. 1981; Wallace et al. 1984), changing the allocation pattern to increase the production of new leaf area (McNaughton and Chapin 1985), or nutrient uptake (Ruess et al. 1983; McNaughton and Chapin 1985) and improving plant water status (Toft et al. 1987). Traits involved in the active response to damage have been considered not directly involved with growth before defoliation (Weis et al. 2000). However, the contribution of different mechanisms to relative growth rate can be quantified via its three determinants (traits directly related with resources capture), using a growth analytical approach. First, the net assimilation rate (NAR)—the increase in biomass per unit of time and leaf area—is strongly correlated with the whole-plant net photosynthetic rate (Poorter and Van der Werf 1998). Second, specific leaf area (SLA, leaf area per unit of leaf biomass) is a parameter that reflects aspects of leaf morphology such as leaf density and thickness (Poorter and Nagel 2000). And third, the biomass allocation to leaves (LWR) needs to be considered. Thus, RGR can be broken down into these three leaf-based properties as follows: RGR = NAR × SLA × LWR. The product of SLA and LWR is the leaf area ratio (i.e. LAR, the ratio of leaf area per plant biomass). These growth-promoting mechanisms have been shown to influence plant tolerance, as the plant's active response to damage (Oesterheld and McNaughton 1988, 1991; Anten et al. 2003; Van Staalduinen and Anten 2005, Van Staalduinen et al. 2010; Dobarro et al. 2012, Camargo et al. 2015) and directly involved in growth before damage (Poorter and Nagel 2000, Shipley 2006, Camargo et al. 2015).

Growth determinants can control the rate of growth/regrowth, increasing RGR before defoliation and buffering or increasing the differences of growth in the ontogenetic response to defoliation (Camargo et al. 2015). The contribution of these mechanisms differs at interspecific (e.g., Van Staalduinen & Anten 2005), and intraspecific levels (e.g., Camargo et al. 2015) and between plants growing in different abiotic conditions (Coughenor et al. 1990, Oestrheld and MacNaughton 1991, Van Staalduinen and Aten 2005, Van Staalduinen et al. 2010). Camargo et al. (2015) have shown that the contribution of these mechanisms to regrowth ability change throughout the ontogeny in the annual herb *Datura stramonium* and that the type of growth determinant that influence overcompensatory growth (higher values in defoliated plants) at final stages of the ontogeny can be associated with the environments inhabited by the ecotypes. In general, NAR and LWR have been associated with compensatory growth in herbs (Oesterheld and Mcnaughton 1988, Oestrheld and MacNaughton 1991, Van Staalduinen and Aten 2005, Van Staalduinen et al. 2010, Camargo et al. 2015).

Very often growth determinants vary in a greater extent than RGR because their main role is buffering the differences in RGR due to differences in resources supply (Poorter and Nagel 2000, Shipley 2000) and, in this way, are the best candidates to test hypotheses regarding the causes of variation in tolerance to different abiotic environments. If growth determinants behind tolerance to defoliation change with the abiotic environment, then these would constitute the driving force behind plant-defense evolutionary strategies and, thus, set the template for the type of fitness reaction norm that can evolve. Nutrient availability may directly affect the plants' physiological state at the time of damage (Tiffin 2002), promoting a greater tolerance when nutrients required to generate new tissue are readily available or optimal (e.g. Maschinsky and Whithman 1989, Belsky 1993). However, the interaction between defoliation and nitrogen availability is controversial, allowing the co-existence of different models (Semmartin and Oesterheld 2001). An alternative model proposes that high nutrient decreases tolerance to defoliation because genotypes would be growing at maximum intrinsic growth rates, requiring large increases in growth rate in response to defoliation, while slow growth genotypes require only small increases to reach undefoliated plants (Hilbert et al. 1981). Hence, it is expected that genotypes with slow intrinsic growth rate would exhibit the highest overcompensatory growth later (higher RGR in defoliated plants) (Oesterheld and McNaughton 1988) and thus achieving higher tolerance in other fitness components than vegetative biomass (Camargo et al. 2015).

Stowe et al. (2000) emphasized that distinguishing between general vigor and tolerance may be important to understand the evolutionary response of plants to consumer-imposed selection. Selection gradient analysis is one of the best available tools to infer which traits can predict tolerance to defoliation (e.g., Juenger & Bergelson 2000). Notwithstanding, evolution of adaptive plasticity requires that plastic genotypes have the highest global fitness averaged across environments (i.e., the highest vigor) rather than the highest fitness in each environment (Releya 2002, van Kleunen and Fisher 2005). Analyses in which the fitness of individuals is regressed on the trait of interest separately for different environments (Lande & Arnold, 1983) are powerful in determining whether a plastic response per se would be beneficial, but they do not unequivocally prove that the plastic response is selected for (van Kleunen and Fisher 2005). Thus, inferring the underlying mechanisms of a fitness reaction norm requires looking for a correlation between tolerance and the different properties (slope and elevation) of a putative trait, and besides we need to look for a relationship of the putative trait with the vigor of the fitness reaction norm. Such an approach would permit to determine the causal structure behind a correlation between tolerance and the intrinsic growth rate. If such a correlation exists, it may be due to the pleiotropic action of the same underlying mechanisms, and direct selection on these mechanisms to increase intrinsic growth rate or to buffer differences in RGR in response to defoliation, would cause an indirect evolutionary response in plant tolerance and general vigor (Weis et al. 2000).

Here, we used a plant growth analytical approach to investigate the underlying mechanisms of tolerance to defoliation in low and high nutrient availabilities. We conducted a greenhouse experiment generating a hybrid experimental population of *D. stramonium*, from parental populations that differ in tolerance to defoliation, and associated with differences in herbivore pressure in different abiotic environments (Fornoni et al. 2003, 2004). The species is largely known to prefer rich soils (Weaver and Warwick 1984) and rapidly assimilates nitrogen in the form of nitrate or ammonium (Lewis and Probyn 1978; Platt and Rand 1982). We address the following questions: What are the traits most related to RGR before and after defoliation in different nutrient availabilities? Are these growth-promoting mechanisms before and after defoliation influencing the tolerance and vigor of the fitness reaction norm depending on the nutrient environment? Is there a negative genetic correlation between intrinsic growth-promoting mechanisms and tolerance and a positive genetic correlation of these mechanisms with vigor? Finally, is there a nutrient context-dependent expression of these correlations?

Material and Methods

EXPERIMENTAL SYSTEM

Study species

Datura stramonium is a self-compatible annual weed occurring in a wide variety of plant communities in North America (Weaver and Warwick 1984). It is found in all types of soil, but often prefers rich soils (Weaver and Warwick 1984), and rapidly assimilates nitrogen as nitrate or ammonium (Lewis and Probyn 1978; Platt and Rand 1982). Its leaves are eaten by at least two specialist herbivorous insects, *Epitrix parvula* (Coleoptera: Chrysomelidae) and *Lema trilineata* (Coleoptera: Chrysomelidae), and by one generalist species (Núñez-Farfán and Dirzo 1994; Núñez-Farfán et al. 1996). The average of leaf area lost to herbivores in populations of *D. stramonium* from Central Mexico is 30 % (range 11–49 %; Valverde et al. 2001). In *D. stramonium*, unlike many plants where the adult phase begins when sexuality is first expressed, maturity and sexuality are not necessarily synonymous, as sexuality is also very important for the vegetative growth dynamics of the plant due to its architectural model, called Leeuwenberg (Hallé et al. 1978), in which branching produces equivalent orthotropic modules, each with deterministic growth, culminating in the production of a terminal flower. Nearly 60 % of total vegetative growth is achieved in the adult phase (unpubl. data).

Crossing design

Seeds of *Datura stramonium* were originally collected from two populations of central Mexico, the Santo Domingo population in the state of Morelos (18°N, 99°W) and the Patria Nueva population in the state of Hidalgo (20°N, 99°W). Localities differ in climate, vegetation and type of soil (Table 1). These populations of *D. stramonium* withstand different natural levels of defoliation by herbivores (Valverde et al., 2001) and express differences in the contribution of growth components to RGR in response to defoliation, associated with differences in abiotic pressures (Camargo et al. 2015). The Santo Domingo population displays a range of tolerance that includes under-, complete- and overtolerance levels (e.g., Fornoni et al. 2003, Camargo et al. 2015), while the Patria Nueva population shows complete tolerance (e.g., Valverde et al. 2001, Camargo et al. 2015). Full-sibs of each field's maternal plant were derived from two generations of selfing in common garden conditions in a glasshouse at the Instituto de Ecología of the Universidad Nacional Autónoma de México. Experimental hybrids (F_1) between both populations were then generated using a North Carolina design II (Comstock and Robinson 1952). Thirty F_2 plants were grouped into five sets of six plants each. In each set, three plants from Santo Domingo (the population with the higher range of tolerance) were assigned to function as males and three from Patria Nueva as females. Each male was crossed to each of the three females, yielding 9 crosses in each of the five sets. Therefore, a total of 45 crosses were made. To diminish environmental maternal effects and to increase genetic identity within each half-sib family (Geber 1990, Hochwender et al. 2000b), F_3 half-sib hybrids were selfed to obtain F_4 inbred hybrids. Two males failed to produce progeny, as well as three crosses from different sets. Hence, replicated seeds of 36 F_4 hybrid-inbred families (HIF, hereafter) were used in the experiment. To ensure the expression of a large range of tolerance among the hybrid population, we selected some parent lines based on knowledge from a previous study (Camargo et al. 2015).

Since our aim was to exploit the phenotypic variance of all crosses, all HIF (36) were used to detect predictors of the reaction norm in fitness (see below; e.g., Tiffin 2002, Chaney and Baucom 2014). To infer genetic variation on growth components we analysed the maternal breeding values (i.e., as a proxy we used the mean value of each maternal line; Tiffin 2002, Chaney and Baucom 2014), because the method used to estimate RGR and growth components gives no replications of these traits for all HIF. Notwithstanding, we present the analysis of genetic variation using maternal lines and compare with all HIF in fitness. Previous analyses showed evidence of genetic variation for tolerance to defoliation in both, maternal lines and all HIF but not for paternal lines. Thus, in the case of all HIF, the estimate of genetic variance is the upper bound estimation that includes non-additive components of genetic variance (Falconer 1989). However, albeit some maternal influence would be present, maternal breeding values are closer to additive genetic variance because in this design the ratio of variances between paternal and maternal lines is expected to be 1 (i.e., every progeny family has half-sib relationships through both common male and common female) (Nduwumuremyi et al. 2013).

Growth conditions and harvest

Plants were grown in the glasshouse. Germination was timed so that all plants began growing within a 3 days period. The day before transplanting, the 25% largest and the 25% smallest plants from each HIF were discarded to assure a reduction in variance expressed in seed germination and to ensure that environmental effects were minimized (Poorter 1989). Therefore, the growth conditions were as similar as possible between HIF, allowing us to compare plants at the same age but quantifying the growth trajectory at different intervals (see growth conditions and harvest). The experiment included 3168 plants from 36 HIFs. Nineteen days after seedling emergence, when the true leaves are full expanded, individuals from each HIF were randomly assigned to one of four treatments: 0% defoliation/low nutrients, 0% defoliation/high nutrients, 50% defoliation/low nutrients, 50% defoliation/high nutrients. For simplicity, treatments without defoliation were designed as control series, while treatments with defoliation as defoliated series. Plants were transplanted to pots of 1.2 L with a mixture of water-washed-fine silica sand and expanded perlite (3:1). Nutrients were added to each pot prior to transplant in the form of Scotts Osmocote 14-14-14 slow-release nitrogen-phosphorous-potassium fertilizer. Under the low nutrient treatment, the soil mix was fertilized with 4 g/pot, representing a total N addition of 0.6 g N per plant. Under the high nutrient treatment, the soil mix was fertilized with 13 g/pot, for a total N of 1.8 g N per plant. On day 61, we removed 50% of total leaf area per plant in the defoliated treatments, by cutting longitudinally (without cutting the central vein) half of each leaf. This defoliation level represents the upper limit of average defoliation in populations of *D. stramonium* from Central Mexico (range 11-49%, Valverde et al. 2001). In a previous study (Camargo et al. 2015) that employed some parental genotypes used here, we identified that ca. 61 days after seedling emergence the upper limit of geometric growth in *D. stramonium* is reached (cf. Fig. 2 in Camargo et al. 2015). During this period, the species only accomplish vegetative growth and therefore is less prone to reductions in growth rate due to self-limitations. Since all experimental environments (defoliation and nutrients) could be controlled at the pot level and because the glasshouse space allowed 792 plants per room, we used 4 rooms (blocks) for the entire experiment; plants of each HIF were assigned completely random to each of the 792 spatial positions equally spaced within 8 benches in each glasshouse room, to prevent any confounding effect due to spatial heterogeneity. We controlled day and night temperature in the four glasshouses; water was provided twice a day by an automatic irrigation system. The mean radiation was 230 W m^{-2}. The temperature regime was 20 ± 0.5 °C day and 13 ± 0.5 °C night (13 h day length). Relative humidity was always above 60 %.

We conducted three harvests along the plants' growth trajectory at 35 (after a 16-day period of acclimation in pots), 61 and 110 days of age. The first and second harvests were aimed to estimate the intrinsic growth potential (ρ) before defoliation and the corresponding growth determinants. The second and third harvests were used to estimate growth determinants in response to defoliation and fitness components (third harvest, see below). 3-4 plants were harvested in the first two harvests, and 9-11 plants per HIF/treatment combination were used to estimate fitness and growth determinants in the third harvest. A total of 216, 441 and 1562 plants were sampled for the first, second, and third harvest, respectively. Plants to be harvested at a given age were randomly chosen from among the 4 blocks (e.g., Meziane and Shipley 1999) and from each treatment combination. Harvested plants were separated into leaves, 'support' tissues (stems, petioles and pedicels), roots, and reproductive tissues (flower and fruits), if present. Plant parts and their dry mass were measured after being oven-dried for at least 48h at 80 °C (Camargo et al. 2015). Total one-side of fresh leaf area of the plant was estimated by image analysis with Compu Eye, Leaf and Symptom Area Software (Bakr 2005). At the third harvest, we measured two correlates of maternal fitness per plant: total vegetative biomass dry weight (i.e., without including reproductive biomass), and total lifetime numbers of seeds.

DATA ANALYSIS

Growth rate and growth determinants estimation

The RGR and its growth determinants were estimated using the classical method (Venus and Causton, 1979, Causton and Venus 1981) as implemented in the Microsoft Excel macro for its proper calculation (*sensu* Hunt et al. 2002). The expected value of RGR was estimated, following Fisher (1921), as the difference in biomass between harvest means [ln(W)]: between the harvest interval 35-61 days for control series (i.e., ρ before defoliation) and between the interval 61-110 days for control and defoliated series (i.e., RGR after defoliation) (Hoffman and Poorter 2002, Hunt et al. 2002). Each growth determinant of control and defoliated series was estimated as the average value, with variance, across the harvest interval 61-110 days. Instantaneous RGR and growth determinants are defined and related as follows (Hunt et al. 2002):

$$\underset{\text{RGR}}{\left(\frac{1}{W}\right)\left(\frac{dW}{dt}\right)} = \underset{\text{NAR}}{\left(\frac{1}{L_A}\right)\left(\frac{dW}{dt}\right)} \times \underset{\text{SLA}}{\frac{L_A}{L_W}} \times \underset{\text{LWR}}{\frac{L_W}{W}} \quad (e.1),$$

where *t* is time, W is total dry weight per plant, L_A is total leaf area per plant and L_W is total leaf dry weight per plant. The product of SLA and LWR, defined as L_A/W and known as leaf area ratio (LAR) was also derived. Additionally, the allometric coefficient for root/shoot development was determined using the Excel macro by Hunt et al. (2002; see their article for equations of derived quantities and associated variances in the Excel macro).

From growth determinants, we calculated growth response coefficients (GRC, Poorter and Nagel 2000) with the aim of comparing the relative contribution of each growth determinant to the RGR differences observed in response to defoliation (Camargo et al. 2015). These coefficients are very valuable for defoliation studies because they can explore the relationship between compensation in growth determinants and compensation in RGR. Given that RGR = [NAR × SLA × LWR], the relative differences in RGR between defoliated (D) and control (C) plants should equal the relative difference in their respective [NAR × SLA × LWR], modified from Poorter and Nagel (2000):

$$\frac{\text{RGR}_\text{D}}{\text{RGR}_\text{C}} = \frac{\text{NAR}_\text{D} \times \text{SLA}_\text{D} \times \text{LWR}_\text{D}}{\text{NAR}_\text{C} \times \text{SLA}_\text{C} \times \text{LWR}_\text{C}} \quad \text{(e.2).}$$

In their original contribution, Poorter and Nagel (2000) aimed to explain a given reduction in resource supply and therefore use the ratio of high resource RGR to low resource RGR. Hence, we modified equation e.2 to estimate the amount of compensation to defoliation using Ln-transformation of both sides of equation (see Camargo et al. 2015), which gives:

$$\ln \mathrm{RGR_D} - \ln \mathrm{RGR_C} = (\ln \mathrm{NAR_D} - \ln \mathrm{NAR_C}) + (\ln \mathrm{SLA_D} - \ln \mathrm{SLA_C}) + (\ln \mathrm{LWR_D} - \ln \mathrm{LWR_C}) \quad \text{(e.3)}.$$

Therefore the compensation to defoliation (i.e., the difference between defoliated and control plants), in ln-transformed RGR values, is the sum of the compensation in ln-transformed values of NAR, SLA and LWR. To avoid collinearity, e.3 can be converted to:

$$1 = \frac{\ln \mathrm{NAR_D}\text{-}\mathrm{NAR_C}}{\ln \mathrm{RGR_D} - \ln \mathrm{RGR_C}} + \frac{\ln \mathrm{SLA_D}\text{-}\mathrm{SLA_C}}{\ln \mathrm{RGR_D} - \ln \mathrm{RGR_C}} + \frac{\ln \mathrm{LWR_D}\text{-}\mathrm{LWR_C}}{\ln \mathrm{RGR_D} - \ln \mathrm{RGR_C}} \quad (\mathrm{e.}4),$$

resulting in what Poorter and Nagel (2000) define as Growth Response Coefficients (GRCs); these coefficients add up to 1:

$$GRC_{NAR} + GRC_{SLA} + GRC_{LWR} = 1 \ (\mathrm{e.}5).$$

Here, GRC value of 1 indicates that compensation in a growth determinant equals the compensation in RGR. A GRC value of 0 indicates that there is no change in the growth determinant at all. GRC values can be higher than 1 when compensation in the growth parameter is stronger than the compensation in RGR, or lower than 0 if compensation in a growth determinant goes with a decrease in RGR (Poorter and Nagel 2000). Thus, we replaced the GRCs by the slopes estimated from a linear regression with ln(NAR), ln(SLA) or ln(LWR) as the dependent variable, and ln(RGR) as the independent variable (Poorter and Van der Werf 1998). Testing for differences in the pattern and the intensity of the contribution of compensation in each growth component to compensation of RGR between nutrient environments was accomplish by means of an ANCOVA.

Variation in ρ and growth components before defoliation

We performed a mixed model ANOVA of maternal line families because the method used to estimate RGR and growth components gives no replication of these traits for all HIF but exact values of its sample mean and variance (Causton and Venus 1981). All traits were log transformed before analyses to improve normality and homoscedasticity. Thus, we determine the relative importance of: Maternal line (presence of genetic variation); (ii) Nutrients (presence of average plasticity in response to nutrient environments regardless of specific maternal line and nutrients); (iii) Maternal by Nutrients (genetic variation for plasticity to nutrients environments).

Variation in fitness after defoliation among treatments (general patterns of tolerance)

Maternal line and HIF families were used to test the relative importance of the nutrient environment on tolerance to defoliation. The number of seeds and vegetative biomass were square root and log transformed, respectively, to improve normality and homoscedasticity. The means and ANOVA results for the raw data of the number of seeds are presented since qualitative results of significant test did not differ from the transformed data. A two-way ANOVA was used to determine the relative importance of: (i) Defoliation (presence of average plasticity in response to defoliation regardless of nutrient environment); (ii) Nutrients (presence of average plasticity in response to nutrient environment regardless of specific HIFs and defoliation); (iii) Defoliation by Nutrients (variation for plasticity to defoliation among nutrient environments). Subsequent Highly Significant Tukey tests were used when necessary.

Genetic variation for fitness, RGR and growth components after defoliation

We explored genetic variation in fitness, RGR and growth components within each nutrient environment (Hochwender et al. 2000). The number of seeds and vegetative biomass were square root and log transformed, respectively; RGR and its components were log transformed to improve normality and homoscedasticity. The full model included the main effects of HIF/maternal line (random effects), defoliation (fixed effect), and HIF/maternal line by defoliation interaction. In the case of RGR and its components the analysis used the same model but only with maternal lines (see above). A significant interaction between HIF/maternal line and Defoliation indicates the presence of genetic variation in plasticity (i.e., tolerance to defoliation for fitness components).

Plasticity to defoliation and trait means estimation

We estimate individual plasticity for all traits and fitness components (i.e., estimate of the tolerance slope) as the difference in mean trait values between defoliated and control plants (Strauss and Agrawal 1999) within each nutrient environment. A correction was applied to make plasticity dimensionless to ease comparisons across traits and nutrient environments, by dividing the plasticity estimate relative to the mean population response (i.e., mean plasticity of the population = 1) (Falconer, 1990). We used the absolute value of the mean population response for this correction, retaining the plasticity sign because families varied from negative to positive responses in various traits (see Fig. 4). The method allows determining if any genetic correlations between mean and plasticity are of biological significance rather than numerical/statistical artefacts (Stinchcombe et al. 2004, e.g., Tiffin et al. 1999). Higher and positive values depict greater compensation/tolerance to damage than smaller or negative values. Trait means for all reaction norms were estimated as the grand mean value across control and defoliated plants for each nutrient environment.

Predictors of the fitness reaction norm

To test whether trait mean value and plasticity of growth components in response to defoliation affect (1) the average fitness (vigor) or (2) the fitness plasticity of a given HIF (j), we used a mixed stepwise regression analysis (Strauss et al. 2003; Wise et al. 2008) to control for correlations between independent variables. We regressed relative values of either seed set or vegetative biomass grand mean ($\overline{W}_j$) (averaged over control and defoliated plants) (Weis and Gorman 1990, Stinchcombe et al. 2004), and their plasticity (plW_j), over our estimates of trait means (i.e., elevation of the reaction norm, $\bar{X}_j$) and plasticities (i.e., steepness of the reaction norm, plX_j) for all traits, thus:

$$1.\ \bar{W}_j = Constant + \ \alpha_0\overline{NAR}_j + \alpha_1\overline{LWR}_j + \alpha_2\overline{SLA}_j + \beta_0 plNAR_j + \beta_1 plLWR_j + \beta_2 plSLA_j$$

$$2.\ plW_j = Constant + \ \alpha_0\overline{NAR}_j + \alpha_1\overline{LWR}_j + \alpha_2\overline{SLA}_j + \beta_0 plNAR_j + \beta_1 plLWR_j + \beta_2 plSLA_j$$

The quadratic terms and the cross-product of elevation and slope did not were included in the models because there was no evidence of significant effects in prior analyses. A mixed stepwise model in JMP (Version 9. SAS Institute Inc., Cary, NC, 1989-2007) was used to calculate the multiple regressions; the *F* statistic for a variable to be added had to be significant at the entry level of $P = 0.15$ and deleted from the model if the trait did not produce an *F* statistic significant at the "stay" level of $P = 0.10$ (Strauss et al. 2003). After the selection process, a regression was run on selected traits to determine their significance as predictors of the properties (i.e., vigor or fitness plasticity) of the fitness reaction norm (Wise et al. 2008). A comparison between regression coefficients for different traits was possible standardizing them in units of 1 SD. To be as liberal as possible in our search for fitness reaction norm predictors, controlling the increase in the probability of committing Type II error, we did not correct for multiple test (Moran 2003, e.g., Banta et al. 2010).

The contribution of growth and growth components, before defoliation, to fitness-component reaction norms, was analysed too. In which case equation 1 and 2 were modified without plasticities of growth components, because plasticity is absent before defoliation. Hence, mean values of growth components were used for each HIF, and separate regressions were used for low and high nutrient availabilities before and after defoliation. Due to the strong correlation of RGR with NAR, before and after defoliation (see results), the RGR was not included as an explanatory variable and conclusions regarding its role influencing fitness reaction norms were performed with the analysis of GRCs (see above).

Costs of the plastic responses to defoliation in each environment were evaluated for the traits identified as predictors of the fitness reaction norm, with the aim to support its adaptive value. Detection of costs is most likely for adaptive plasticity because there will be strong selection against costly nonadaptive plasticity (DeWitt 1998). A cost of plasticity is found when a more plastic family exhibits lower fitness in a focal environment than a less plastic genotype with the same trait value (DeWitt 1998). In both, control and defoliated environments (*k*), we tested for plasticity costs by regressing the mean fitness (seed set) of HIF (*j*) in the focal environment ($\overline{W}_{j,K}$) on the HIF mean trait value in the focal environment ($\bar{X}_{j,K}$) and estimates of plasticity over both environments (plX_j, van Tienderen 1991; Scheiner and Berrigan 1998):

3. $\overline{W}_{j,K} = constant_K + \alpha_K \bar{X}_{j,K} + \beta_K plX_j.$

The analyses were carried out separately for each growth component and nutrient environment; one-tailed significance tests were performed given that negative selection gradients are expected if plasticity is costly (e.g., van Kleunen et al. 2000). Traits were standardized in units of 1 SD prior to analyses.

Results

Variation in ρ and growth components before defoliation

The results of the mixed model ANOVA showed that plants growing in higher nutrients grew faster than plants in low nutrients ($F_{1,\ 20.03}$ = 4.46, *P* = 0.048; mean ± SE, low nutrients: 70.28 mg $g^{-1}d^{-1}$; mean ± SE, high nutrients: 77.96 mg g^{-1} d^{-1}). A significant effect of maternal line variation across nutrient environments was also detected ($F_{13,\ 13}$ = 2.69, *P* = 0.043). However, genetic variation for plasticity to nutrients in RGR was not detected. The growth components not showed significant effects to any of the terms tested.

Contributions of growth determinants to variation in ρ and regrowth

The only trait highly related to variation in RGR before defoliation was NAR; however, its contribution did not differ between environments ($F_{1,\,68}$ = 0.36, P = 0.553; common slope 1.27 ± 0.25, $F_{1,70}$ = 25.69, P = <0.0001).

NAR (slope 1.64 ± 0.21, $F_{1,\,34}$ = 60.65, P < 0.0001) and LWR (slope 0.47 ± 0.20, $F_{1,\,34}$ = 5.52, P = 0.025) were the traits related to variation in regrowth after defoliation in low nutrients. That is, families with high values of these traits in defoliated plants had also smaller reductions of RGR in response to defoliation. In high nutrients, families with high values of NAR (slope 1.21 ± 0.18, $F_{1,\,34}$ = 46.61, P < 0.0001) in defoliated plants had smaller reductions of RGR in response to defoliation. However, the contribution of NAR did not differ between nutrient environments ($F_{1,\,68}$ = 2.46, P < 0.1215; common slope 1.43 ± 0.14, $F_{1,\,70}$ = 108.15, P < 0.001).

Variation in fitness components after defoliation among treatments (general patterns of tolerance)

The ANOVA model (Table 1) revealed a significant effect of nutrients and defoliation on fitness components; the average phenotype was higher in high nutrients and lower in defoliated plants. The mean number of seeds decreased in low nutrient environments ca. 16% with respect to high nutrients. Defoliation decreased seed number ca. 21% in relation to control plants. Mean vegetative biomass decreased in low nutrients ca. 12% with respect to high nutrients. Likewise, defoliation decreased vegetative biomass about 12% relative to control plants. Variation in tolerance to defoliation in response to nutrient environments was detected in seed number but not in vegetative biomass. Plants in the low nutrient environment had more tolerance to defoliation (i.e., seed number of defoliated plants decreased 15% and 21% in low and high nutrients, respectively, in relation to control plants (Table 1, Fig. 2).

Genetic variation of fitness, RGR and growth components after defoliation

Fitness components

In the low nutrient environment, we did not detect a significant effect of defoliation in the average number of seeds and vegetative biomass but significant genetic variation among maternal lines in vegetative biomass was detected. Genetic variation for tolerance to defoliation was detected only for the number of seeds (Table 2, Fig. 3). In the high nutrients environment, the number of seeds decreased significantly with defoliation, but not for vegetative biomass. Genetic variation for maternal lines and genetic variation for tolerance to defoliation was detected in both fitness components (Table 2, Fig. 3).

RGR and growth components

In the low nutrient environment, RGR and growth components were plastic; trait values significantly decrease in response to defoliation (Table 3, Fig. 4). We found no evidence of both maternal line effect in RGR and NAR, or genetic variation for plasticity in these traits. However, genetic variation of plastic responses of LWR and SLA was detected (Table 3, Fig. 3).

In the high nutrient environment, RGR and growth components significantly decrease trait values in response to defoliation providing evidence of plasticity in these traits (Table 3, Fig. 3). All traits but SLA showed a significant maternal line effect. However, we found evidence of genetic variation for SLA plasticity (Table 3, Fig. 4).

Predictors of the fitness-component reaction norms

Before defoliation

In the low nutrient environment, the only trait negatively correlated with the tolerance of both seed-number and vegetative-biomass was NAR (seed-number tolerance, $\alpha = -0.7 \pm 0.29$, $P = 0.022$, $r^2 = 0.15$; vegetative-biomass tolerance, $\alpha = -1.32 \pm 0.45$, $P = 0.0062$, $r^2 = 0.2$). None of the traits were correlated with general vigor of both, seed-number or vegetative-biomass. In high nutrients, there were no traits explaining the variation of tolerance and vigor of both fitness-component reaction norms.

After defoliation

In the low nutrient environment, seed-number general vigor was negatively correlated with mean LWR and LWR plasticity, showing no differences in magnitude (Table 4). Together, these traits explained 30% of variation in seed-number general vigor and did not explain the variation of vegetative-biomass general vigor but average NAR explained 53%. Tolerance in seed number was negatively correlated with mean LWR and SLA plasticity. Together, these traits explained 28% of variation in seed number tolerance. Thus, mean LWR was the only trait with pleiotropic influence on both, vigor and tolerance of the seed number reaction norm. Mean NAR, NAR plasticity, and mean SLA were positively correlated with tolerance of vegetative biomass and together explained 52% of its variation. Thus, mean NAR was the only trait with pleiotropic influence on both, vigor and tolerance of the vegetative biomass reaction norm. In general, all traits tended to have more influence (i.e., greater gradients) on tolerance than vigor for both fitness components (Table 4). Mean trait value or its plasticity that significantly influenced vigor or tolerance had similar magnitude of selection gradients (Table 4).

In the high nutrients environment, a qualitatively different pattern of genetically correlated traits with both vigor and tolerance of seed number was detected. In contrast to the low nutrients, plasticity in LWR is positively correlated with vigor of seed number (Table 5), and together with plasticity in SLA and mean NAR, accounted for 29% of variation in seed number vigor; in turn, these two traits were negatively and positively correlated with LWR plasticity, respectively.

Mean NAR and LWR were positively and negatively correlated, respectively, with vigor of vegetative biomass, and together explained 77% of its variation (mean LWR explained just 3% of this variation). Mean NAR and NAR plasticity were positively correlated with seed number tolerance showing no differences in magnitude and explaining 28% of its variation. Thus, mean

NAR was the only trait with pleiotropic influence on both, vigor and tolerance of the seed number reaction norm. NAR plasticity was the only trait positively correlated with tolerance of vegetative biomass. As in low nutrients, all traits tended to have more influence (i.e., estimates were higher) on tolerance than vigor of both fitness components (Table 5).

Cost of plasticity in growth components

Within-environment analysis of the cost of plasticity revealed that LWR and RGR plasticity were costly in control plants in the low nutrient environment (selection gradient, -0.098, -0.055, respectively; Table 6, Fig. 5). In the defoliated environment, with low nutrients, families with higher mean values of allocation to leaves had lower fitness. This confirmed that an increased allocation to leaves, either intrinsic or environmental induced, is highly constrained and had higher fitness costs in this environment.

In defoliated environments plasticity in total biomass (both nutrient environments), had positive effects in the number of seeds (values in italics in Table 6). These positive effects were significant when we used two-tailed test ($P < 0.05$), and suggest selection acting on total biomass in these experimental environments.

Discussion

This study reinforces the argument that the fitness-reaction norm to defoliation is a complex property of genotypes composed of constitutive and damage induced components (Weis et al. 2000, e.g. Hochwender et al. 2000, Stevens et al. 2000). Previous theoretical models show that ρ would change the vigor (Stowe et al. 2000, Weis et al. 2000) and tolerance of the fitness reaction norm to defoliation (Weis et al. 2000). Our experimental results showed that growth-promoting mechanisms before damage were not related to the vigor but the tolerance of the fitness reaction norm to defoliation only in low nutrient environments, and that these mechanisms would affect vigor only when expressing plasticity in response to defoliation; that is, the vigor of the fitness reaction norm is controlled by after-defoliation mechanisms and it is not constrained by early ontogenetic expression (before defoliation) of the same mechanisms. In contrast, the amount of tolerance of the fitness reaction norm to defoliation would be controlled by constitutive (only in low nutrients) and damage-induced growth promoting mechanisms. Here, we discuss the correlation among growth promoting mechanisms and the fitness reaction norm, and then how context-depended expression of these mechanisms shapes its form and influence its evolution.

Impact of defoliation on fitness

Our study shows that the expression of genetic variation for tolerance was constrained by the nutrient environment at least in vegetative biomass. In low nutrients, only seed number expressed genetic variation whereas in high nutrients both fitness components showed genetic variation for tolerance. Previous studies in *Datura stramonium* have demonstrated genetic variation for tolerance in the number of seeds in natural populations (Fornoni et al. 2003), and in controlled conditions using inbred lines (Fornoni and Núñez-Farfán 2000).

We found that tolerance to defoliation in response to nutrient environments can change depending on the fitness component analyzed. Plants in low nutrients were more tolerant in defoliation, as assessed through the number of seeds, than plants in high nutrients. However, for total vegetative biomass, plants were equally tolerant irrespective of the nutrient environment. Hence, this study underscores the need of estimating fitness with traits closely linked to it (Hanley and Fegan 2007), and demonstrated for *Arabidopsis thaliana* (Banta et al. 2010). Notwithstanding, we contend that marked across-environment phenotypic stability in response to defoliation, observed in low nutrients for the number of seeds, was related in part to reductions in vegetative biomass in response to defoliation. This response might result if mother plants adjust allocation to vegetative biomass, diminishing it, while maintaining seed provisioning. In fact, fitness of defoliated plants in low nutrients was related to the plasticity of vegetative biomass (see cost of plasticity below, Table 6).

Intuitive ecological thinking has long held that plants growing in relatively high nutrient environments should be better able to tolerate herbivory than plants growing in low nutrient environments (e.g., Janzen 1974, Whittaker 1979). Notwithstanding, the magnitude and generality of tolerance responses has spurred a great deal of discussion (McNaughton et al. 1983, Belsky 1986, Crawley 1987, Bergelson 1992, McNaughton 1993, Painter and Belsky 1993, Maschinski and Whitman 1989, Wise and Abrahamson 2005). Based on analysis of seed number, our data support the model of Hilbert et al. (1981), in which, at high levels of resources, plants were growing at their highest growth rate and defoliation would not promote a higher tolerance (Camargo et al. 2015). In contrast, based on analysis of vegetative biomass, our data supports the limited resource model (LRM; Wise and Abrahamson 2005), in which, it is expected equal tolerance at both nutrient levels because nutrients would limit plant fitness but defoliation would not limit soil nutrients or the organs to harvest these (roots; model LRM II in Wise and Abrahamson 2005). Part of this controversy can be solved if one considers the fitness proxies assessed in different experiments, particularly those that focused on RGR and total biomass, which contribute to fitness but less directly related to it (e.g. Belsky 1986, Oesterheld and McNaughton 1991, Maschinski and Whitman 1989). Then, although we have shown that the response to defoliation by *Datura stramonium* will depend on nutrient availability (Georgiadis et al. 1989), the relationship between tolerance and nutrient level is complex and still not readily predictable (Banta et al. 2010) since across-environment stability of a fitness component (i.e., seed number) might be achieved by, among other traits, marked plasticity in other fitness component (i.e., vegetative biomass) (Bradshaw 1965, Sultan 1995, Pigliucci 2001, Richards et al. 2006). Thus, while tolerance of vegetative biomass and seed number were not correlated ($r = 0.27$, $P = 0.10$), plasticity in vegetative biomass was a main determinant of fitness in defoliated plants in low nutrients (see above).

Role of growth determinants influencing the fitness reaction norm

An expected response to defoliation is a decrease in growth components due to a decrease in resource supply (Camargo et al. 2015). This instance of lower trait levels in defoliated plants due to a decrease in resources supply is called *passive plasticity* (van Kleunen and Fisher 2005). Notwithstanding, how to differentiate passive plasticity due to resource limitation from *active plasticity* due to changes in allocation is not an easy task (van Kleunen and Fisher 2005). For *D. stramonium*, lower values of growth components in defoliated plants of a genotype, are restored throughout the ontogeny by means of active plasticity (i.e., a progressive increase of growth components in defoliated plants; see figure 4 in Camargo et al. 2015) and, at any moment, genotypes could differ in their ability to compensate defoliation. We found that the relative contribution of different traits to fitness varied with the fitness component analyzed and with the nutrient environment. For instance, higher vigor and tolerance in seed number were related, among other traits, to passive plasticity in growth determinants (i.e., genotypes with lower values in defoliated environments for LWR and SLA had higher fitness; see negative selection gradients in low nutrients in Table 4), but with active plasticity of growth determinants (i.e., genotypes with higher values in defoliated environments for NAR and SLA in defoliated plants, had higher fitness) for both vigor and tolerance of vegetative biomass (see positive selection gradients in low nutrients in Table 4).

Our data showed that nutrient environments change the contribution of growth components to fitness. For instance, active plasticity in LWR was selected against in low nutrients but selected for in high nutrients (Table 5). This reflects a high control of biomass allocation to leaves in low nutrients, and supported by the fitness cost associated with active plasticity in control plants (Table 6, Fig. 5). We interpret this pattern as an adaptive strategy to deal with a decrease in resource use. At low level of nutrients, the ability of plants to tolerate defoliation is affected by the removal of stored nutrients and a reduced capacity for nutrients uptake (McNaughton and Chapin 1985). Then, since nutrients limit growth and fitness, we may expect herbivores to evolve mechanisms for an efficient extraction of nitrogen from plant tissue, whereas plants respond with adaptations to protect their nitrogen from consumption (Thao and Hunter 2011). Other studies have shown how damaged organs transfer resource away from leaves and herbivory (Henkes et al. 2008, Babst et al. 2005), as a strategy to escape and for future growth (Karban and Baldwin 1997, Briske et al 1996). These causes, and the question if passive plasticity in LWR is truly expressed in response to resource limitation or if it is favored because transfers old (stored) and/or new resources to other organs, remain unanswered in *D. stramonium*; further research is needed to better understand its adaptive value. Notwithstanding, *D. stramonium* possesses substantial genetic variation for tolerance to defoliation itself, and plasticity of LWR in low nutrients. Thus, *D. stramonium* has the potential to evolve passive plasticity in response to natural selection for an increased vigor to defoliation.

Tolerance in terms of number of seeds was highly regulated in low nutrients. It is constrained by active plasticity in SLA and the height of LWR. Thus, the investment of a higher leaf area per leaf biomass in this environment would entail a higher cost because the value of each leaf to the plant is high (Janzen, 1974), and make the leaves more apparent to herbivores. Tolerance in terms of vegetative biomass was less prone to be constrained by plasticity in growth components in both nutrient environments. These results highlight the plastic nature of *D. stramonium* to nutrient availability and reinforces the evidence that in low nutrient environments, it would express a cascade of responses to deal with stress; this has been demonstrated for other reproductive traits like herkogamy, which reduces along flowers of successive branches, increasing the probability of self-pollinated flowers, and favoring reproductive assurance (Camargo et al. 2017).

The opposite pattern for LWR was observed in high nutrients, were active plasticity in LWR increases the vigor of the fitness reaction norm. We found that LWR can play a role, under high nutrient availability (e.g., Van Stalduinen et al. 2010), decreasing reductions of RGR in response to defoliation but in a lesser extent compared to NAR (cf. Van Stalduinen et al. 2010). The increase biomass allocation to leaves in higher nutrients might come from carbon mobilization from roots since root:shoot ratios were lower in defoliated plants (i.e., control plants 0.94 ± 0.01, defoliated plants 0.74 ± 0.01; $t_{1,\,140} = 8.17$, $P < 0.0001$; unpublished data). Carbon mobilization to growing leaves in response to defoliation has been showed in other species (e.g., Rivera-Solis et al. 2012, reviewed by Stowe et al. 2000, Tiffin 2000).

Evidence shows that NAR is the main contributor to differences in RGR before and after defoliation in both nutrient environments. Previous studies in *Datura stramonium* corroborates this finding (Camargo et al. 2015), and studies in other herbs (Anten et al. 2003, Van stauldinien and Anten 2005). However, NAR only can play a role affecting the tolerance of the fitness reaction norm in high nutrients. NAR reflects the balance between carbon gain in photosynthesis and carbon losses via respiration and other processes, per unit leaf area per unit time (Poorter and Van der Werf 1998, Lambers and Poorter 1992, McKenna and Shipley 1999). It might be possible that in low nutrients all variation in NAR was diverted to growth while in high nutrients the excess of variation in NAR (slopes were higher than one, see results) would be used to impact the fitness reaction norm. Thus, compensatory growth favoring the fitness reaction norm would only be possible when the nutrients required to produce additional reproductive tissue are readily available (Maschinski and Whitham 1989). Genetic variation in growth (using leaf absolute growth as a proxy) and compensatory growth in response to natural herbivory has been showed in *D. stramonium* (Valverde et al. 2003)

The nutrient environment altered the relationship between the plasticity of growth components and the properties of the fitness reaction norm, altering the relative importance of the traits themselves to natural selection. This has important consequences when assessing the tolerance mechanisms: it implies that the *fitness-correlate* approach to estimate the type and the strength of relationship with tolerance and vigor of the fitness reaction norm, may depend on the integration of the plastic response of growth components to defoliation. While we found a trade-off between an intrinsic (before defoliation) mechanism of plant growth (i.e., NAR) and tolerance to defoliation in the low nutrients environment, we did not found such a relationship between the early expression of these components and the vigor of the fitness reaction norm (cf., Weis et al. 2000). Nutrient limitation is hypothesized to increase the likelihood of trade-offs, because decreasing resource availability would constrain the potential phenotypic trait space of the fitness landscape, increasing the penalties for high trait expression levels (Züst and Agrawal 2017). This suggests that when growth conditions are good, the genetic constitution of an individual and the phenotype it expresses matter less and less (Pigliucci 2001). Notwithstanding, in traits expressed after defoliation, the increase in vigor due to active plasticity in LWR observed in higher nutrients was buffered in the population by a selection against active plasticity in SLA (Table 5). This reflects that even in environments that lack specific selection pressures, there are regulatory mechanisms that preclude the evolution of a *"Darwinian Monster"*, an organism able to perfectly adapt by plasticity to all environmental conditions (Pigliucci 2001).

Finally, this work shows the importance of considering the fitness reaction norm to defoliation as a complex response integrated by constitutive and damage induced growth mechanistic components. Its full understanding makes necessary to assess the correlation between growth mechanistic components with tolerance or vigor, and the different properties (slope and elevation) of a putative trait. The advent of the analysis of mechanistic components of plant growth to study the evolution of plant defense to herbivores (Camargo et al. 2015, Züst et al. 2015) would be benefited by performing quasi-natural experiments (Scheiner 2002), and would reinforce the argument that the expression of plant-mechanistic growth components is constrained by natural abiotic conditions and can determine the form and evolution of the fitness reaction norm.

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Table 1. Two-way ANOVA testing variation in tolerance to defoliation in response to two levels of nutrient availability in *Datura stramonium* fitness components.

	Number of seeds			**Vegetative biomass**		
Source	MS	df	*P*	MS	df	*P*
Nutrients	3883303.63	1	**<0.0001**	3.0411292	1	**0.0005**
Defoliation	6930990.54	1	**<0.0001**	2.9535062	1	**0.0006**
Nutrients ✕ Defoliation	654449	1	**0.0317**	0.228153	1	0.3363
Error	141538	1558		0.24662	1558	

Table 2. Mixed-Model ANOVA testing genetic variation for tolerance to defoliation in two environments of *Datura stramonium* fitness components.

	Low nutrients						**High nutrients**					
	Seed number			**Vegetative biomass**			**Seed number**			**Vegetative biomass**		
Source	MS	df	*P*	MS	df	*P*	MS	df	*P*	MS	df	*P*
Defoliation	733.21	1	**0.005**	0.57444	1	0.1229	1821.4	1	**<0.0001**	2.99007	1	**0.002**
HIF	143.294	35	0.0674	0.67694	35	**0.0012**	352.314	35	**<0.0001**	0.872	35	**0.0005**
Defoliation ✕ HIF†	85.8786	35	**0.0006**	0.23544	35	0.1143	87.4562	35	**0.0195**	0.27852	35	0.0945
Error	42.61	704		0.18	704		55.488	714		0.208225	714	

†. Hybrid-inbred family

Table 3. Mixed-Model ANOVA testing genetic variation to defoliation at two levels of nutrient availability in *Datura stramonium* growth components.

	RGR			**NAR**			**LWR**			**SLA**		
Source	MS	df	*P*	MS	df	*P*	MS	df	*P*	MS	df	*P*
Low Nutrients												
Defoliation	0.46528	1	**<0.0001**	0.45674	1	**0.0082**	0.91554	1	**<0.0001**	0.32539	1	**0.0405**
Maternal line	0.01818	13	0.3062	0.08024	13	0.2174	0.03408	13	0.4406	0.04775	13	0.7499
Defoliation ✕ Maternal line	0.01364	13	0.5555	0.0515	13	0.4147	0.03132	13	**0.0209**	0.06998	13	**0.0167**
Error	0.015087	44		0.048497	44		0.013713	44		0.029559	44	
High Nutrients												
Defoliation	0.85728	1	**<0.0001**	1.74901	1	**<0.0001**	0.9815	1	**<0.0001**	0.22522	1	**0.0476**
Maternal line	0.03552	13	**0.0423**	0.13948	13	**0.0113**	0.04876	13	**0.0113**	0.06476	13	0.3591
Defoliation ✕ Maternal line	0.01314	13	0.7548	0.03675	13	0.551	0.01284	13	0.1045	0.05279	13	**0.0031**
Error	0.018830	44		0.040428	44		0.007728	44		0.017544	44	

Table 4. Stepwise regressions of properties (plasticity and vigor) of fitness-component reaction norms on properties (plasticity and height) of growth-component reaction norms in *Datura stramonium* growing in low nutrients. Linear selection gradients (estimate) with cumulative R^2 (Cum. R^2) and *P*-values (*P*) are presented.

	Fitness component vigor				**Fitness component tolerance**			
Trait	Estimate	Cum. R^2	*P*	Step	Estimate	Cum. R^2	*P*	Step
A) Seed number								
Height LWR	-0.1	0.3	**0.004**	2	-0.81	0.2	**0.005**	1
LWR Plasticity	-0.1	0.09	**0.003**	1	–	–	N.S.	–
SLA Plasticity	–	–	N.S.	–	-0.52	0.28	**0.065**	2
B) Biomass								
Height NAR	0.15	0.53	<**0.0001**	1	1.35	0.47	**0.001**	2
Height SLA	–	–	N.S.	–	0.66	0.52	0.089	3
NAR Plasticity	–	–	N.S.	–	1.98	0.32	<**0.0001**	1

N.S. indicates that the trait had not significant effects

Table 5. Stepwise regressions of properties (plasticity and vigor) of fitness-component reaction norms on properties (plasticity and height) of growth-component reaction norms in *Datura stramonium* growing in high nutrients. Linear selection gradients (estimate) with cumulative R^2 (Cum. R^2) and *P*-values (*P*) are presented.

	Fitness component vigor				**Fitness component tolerance**			
Trait	Estimate	Cum. R^2	*P*	Step	Estimate	Cum. R^2	*P*	Step
A) Seed number								
Height NAR	0.09	0.29	**0.042**	3	0.44	0.09	**0.031**	1
Height SLA	–	–	N.S.	–	–	–	–	–
NAR Plasticity	–	–	N.S.	–	0.44	0.21	**0.031**	2
LWR Plasticity	0.11	0.09	**0.015**	1	–	–	N.S.	–
SLA Plasticity	-0.11	0.18	**0.024**	2	–	–	N.S.	–
B) Biomass								
Height NAR	0.2	0.74	<**0.0001**	1	–	–	N.S.	–
Height LWR	-0.05	0.77	**0.037**	2	–	–	N.S.	–
NAR Plasticity	–	–	N.S.	–	1.26	0.74	<**0.0001**	1

N.S. indicates that the trait had not significant effects

Table 6. Regression analysis of fitness (number of seeds) on both mean value and plasticity to defoliation of growth components in *Datura stramonium,* growing at two levels of nutrient availability. Here mean values are HIF (j) trait values in a focal environment (k) with or without defoliation $(\overline{X}_{j,K=1}, \overline{X}_{j,K=2})$, and plasticities are HIF trait plasticities (plX_j). Cost of plasticity are indicated by negative regression coeficients of plX_j. Linear selection gradients (estimate) with associated standard error (SE) and *P*-values (*P*) are presented.

	Control			**Defoliated**		
	Estimate	SE	*P*	Estimate	SE	*P*
Lower nutrients						
Mean NAR	0.05	0.035	0.081	0.088	0.068	0.101
Mean LWR	-0.036	0.042	0.202	**-0.188**	0.055	0.001
Mean SLA	-0.066	0.062	0.1483	-0.022	0.068	0.373
Mean RGR	-0.036	0.032	0.136	-0.079	0.07	0.133
Mean Biomass	0.011	0.03	0.357	0.01	0.059	0.432
NAR plasticity	-0.015	0.035	0.339	-0.048	0.068	0.242
LWR plasticity	**-0.098**	0.042	0.013	0.037	0.055	0.25
SLA plasticity	-0.034	0.062	0.292	-0.051	0.068	0.229
RGR plasticity	**-0.055**	0.032	0.049	-0.011	0.07	0.44
Biomass plasticity	0.006	0.03	0.422	*0.155*	0.059	0.007
Higher nutrients						
Mean NAR	0.028	0.053	0.299	0.16	0.08	0.027
Mean LWR	-0.024	0.043	0.286	-0.031	0.084	0.358
Mean SLA	-0.071	0.067	0.149	-0.073	0.074	0.165
Mean RGR	-0.005	0.045	0.454	0.143	0.105	0.093
Mean Biomass	0.052	0.039	0.097	0.013	0.056	0.353
NAR plasticity	-0.023	0.053	0.331	-0.036	0.08	0.328
LWR plasticity	0.054	0.043	0.108	0.135	0.084	0.059
SLA plasticity	-0.091	0.067	0.091	-0.083	0.074	0.133
RGR plasticity	-0.046	0.045	0.157	-0.073	0.105	0.248
Biomass plasticity	-0.014	0.039	0.365	*0.297*	0.056	<0.0001

The analyses were carried out separately for each trait, and each included linear terms for mean (in a focal environment, control or defoliated) and plasticity to defoliation. Cost of plasticity are in bold when significant using a one-tailed test. Positive selection gradients are in italics when significant using a two-tailed test ($P < 0.05$).

Figure Legends

Figure 1. Four scenarios of one-to-one changes between the reaction-norm properties (slope and elevation) of an underlying trait (UT) and fitness, in response to defoliation. Two genotypes are depicted (solid and discontinuous lines), which vary in slope and elevation. Upper left, a UT increase in elevation for a genotype (discontinuous line) is related to an increase in the general vigor (elevation) of the fitness reaction norm. Lower left, a UT increase in plasticity for a genotype (slope of discontinuous line) is related to an increase in its general vigor. Upper right, a UT increase in elevation is related to an increase in tolerance (fitness plasticity). Lower right, a UT increase in plasticity is related to an increase in tolerance.

Figure 2. Least squares mean values of seed number in plants of *Datura stramonium* from a hybrid population growing in high (open symbols) and low (closed symbols) nutrient levels. The error bars are the 95% confidence intervals.

Figure 3. Norms of reaction to defoliation in fitness components (seed number and vegetative biomass) of 36 hybrid-inbred families of *Datura stramonium* growing in low and high nutrient environments.

Figure 4. Norms of reaction to defoliation for growth components of 36 hybrid-inbreed families of *Datura stramonium* growing in low and high nutrient environments.

Figure 5. Cost of plasticity in LWR and RGR of 36 hybrid-inbreed families of *Datura stramonium* plants growing in control conditions of the low nutrient environment. The slopes of the lines observed were obtained from separate regressions of the relative number of seeds in control plants, on the plasticity of either LWR or RGR after controlling for the values of these traits in control plants. Reference vertical-dashed line indicates cero plasticity in unstandardized values.

Figure 1

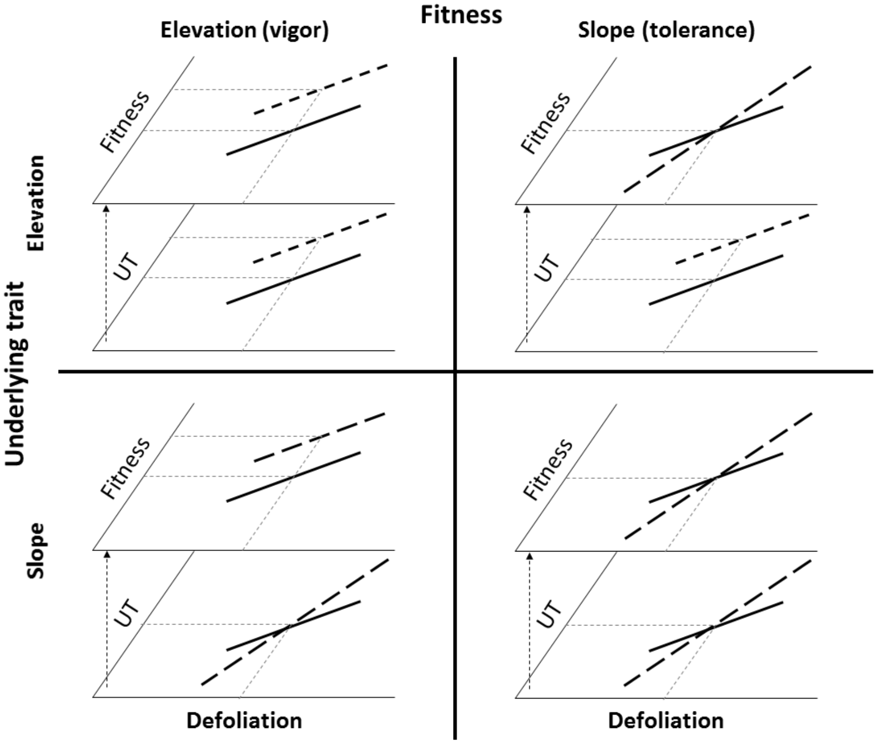

Fitness
Elevation (vigor)
Slope (tolerance)
Underlying trait
Elevation
Slope
Fitness
UT
Fitness
UT
Fitness
UT
Fitness
UT
Defoliation
Defoliation

Figure 2

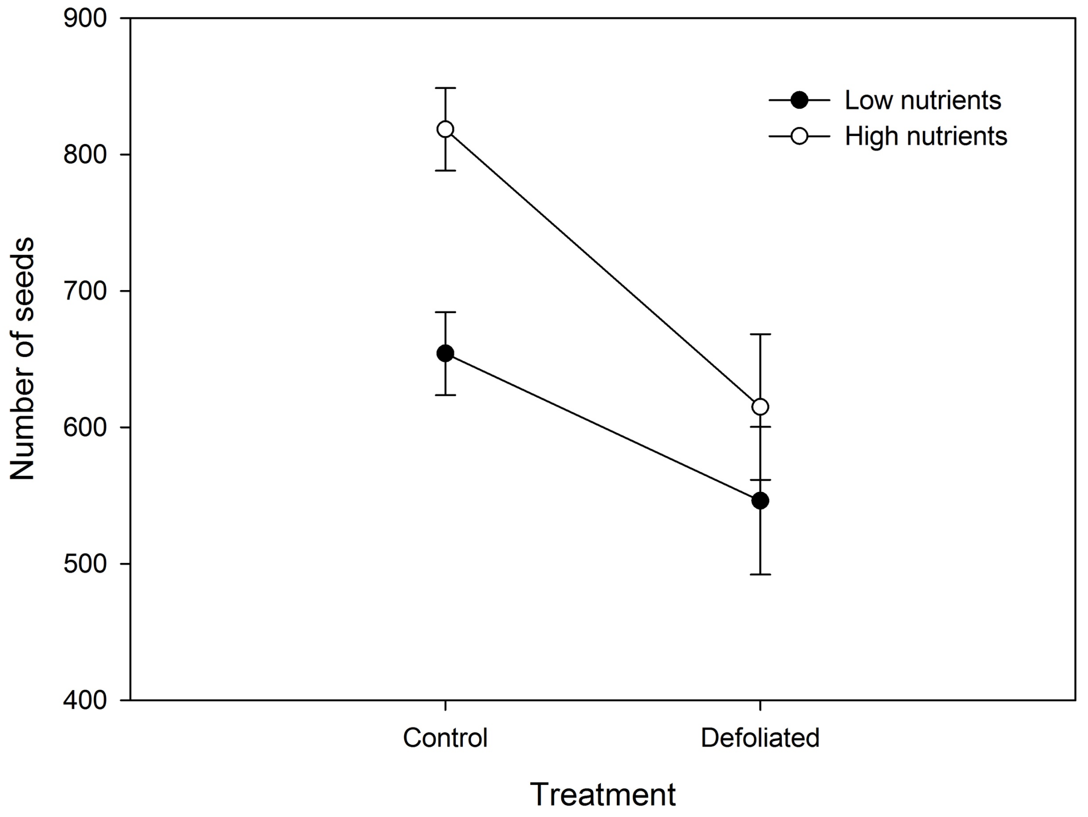
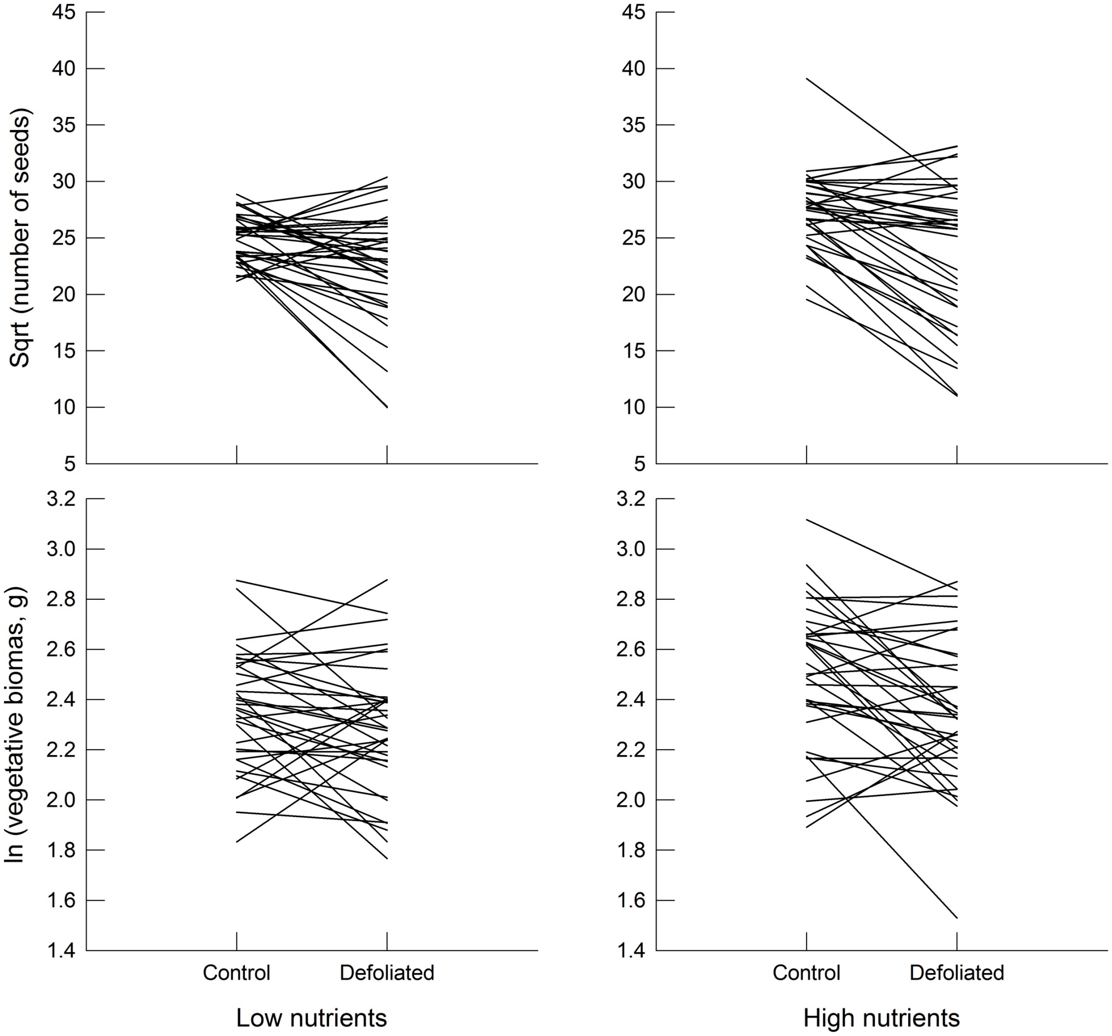

900
800
700
600
500
400
Number of seeds
Control
Defoliated
Treatment
Low nutrients
High nutrients

Figure 3



Sqrt (number of seeds)
45
40
35
30
25
20
15
10
5
ln (vegetative biomas, g)
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
Control
Defoliated
Low nutrients
High nutrients

Figure 4

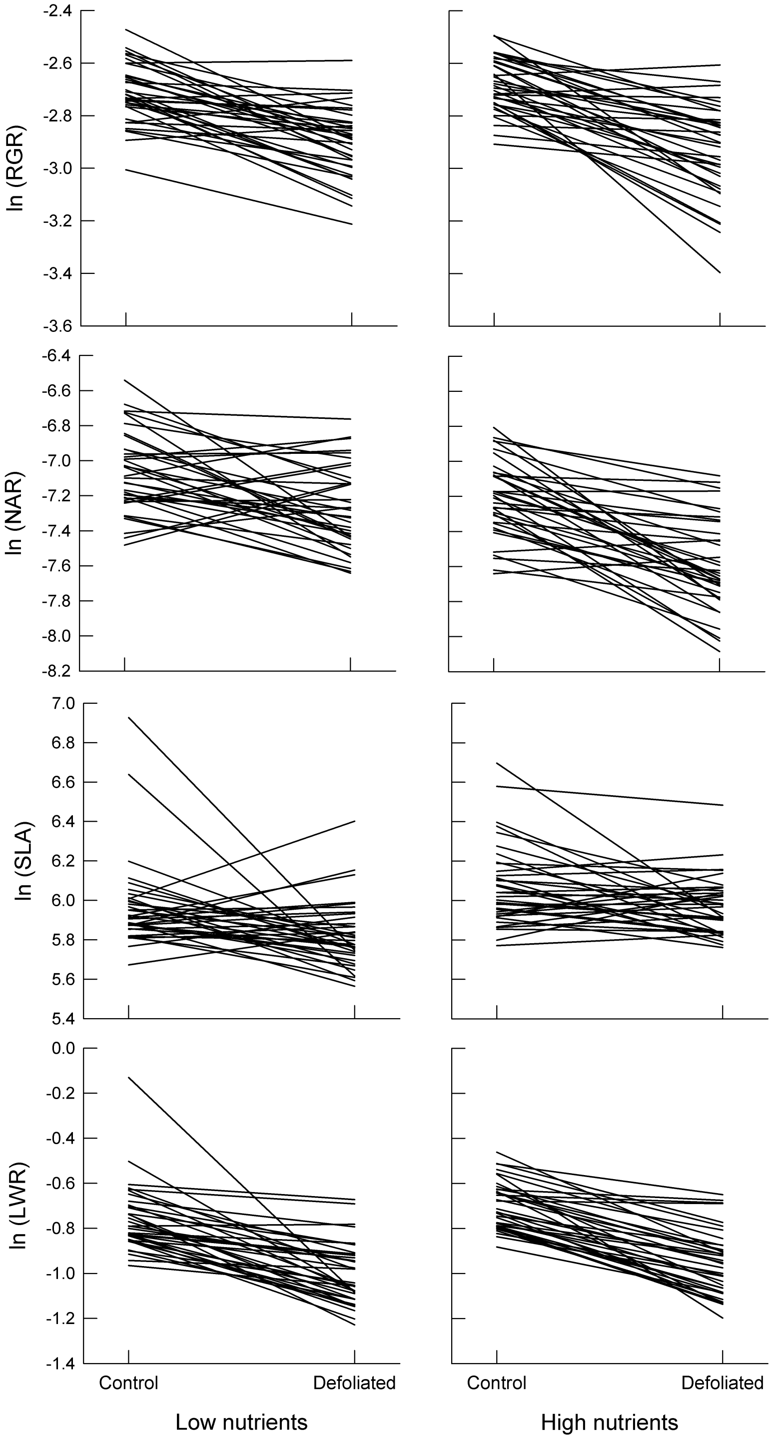
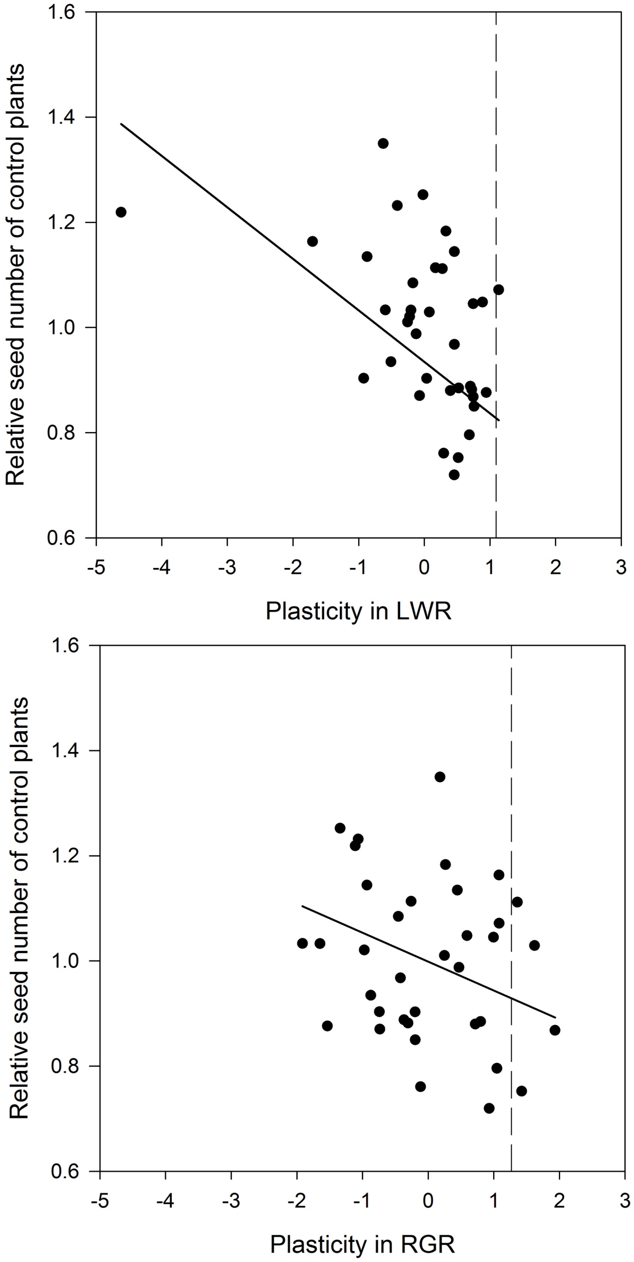

ln (RGR)
-2.4
-2.6
-2.8
-3.0
-3.2
-3.4
-3.6
ln (NAR)
-6.4
-6.6
-6.8
-7.0
-7.2
-7.4
-7.6
-7.8
-8.0
-8.2
ln (SLA)
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
ln (LWR)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Control
Defoliated
Control
Defoliated
Low nutrients
High nutrients

Figure 5


Relative seed number of control plants
1.6
1.4
1.2
1.0
0.8
0.6
-5
-4
-3
-2
-1
0
1
2
3
Plasticity in LWR
Relative seed number of control plants
1.6
1.4
1.2
1.0
0.8
0.6
-5
-4
-3
-2
-1
0
1
2
3
Plasticity in RGR

Capítulo 3

LIGHT LIMITATION REDUCES TOLERANCE TO LEAF DAMAGE IN *DATURA STRAMONIUM*

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Light limitation reduces tolerance to leaf damage in *Datura stramonium*

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ABSTRACT

Question: Does light limitation reduce tolerance to leaf damage in the annual herb *Datura stramonium*?

Hypothesis: Tolerance to leaf damage should be lower under light limitation (shade) than under full sunlight, because a reduction in leaf area and less exposure to light will limit carbon photo-assimilation and plants' ability to maintain fitness.

Organism: Jimsonweed, *Datura stramonium* L. (Solanaceae).

Methods: Damaged and undamaged plants of ten full-sib families ($N = 471$ plants) were grown under two different light treatments, full sunlight (control) and 35% light reduction (shade). Total seed number produced per plant was used as an estimate of maternal plant fitness. To compare the effect of light reduction on tolerance, we used the index of tolerance (the difference in seed production between the damaged and undamaged treatment of each genetic family). We also evaluated the effect of defoliation and light limitation on photosynthetic activity (estimated as the chlorophyll content index) and leaf growth compensation (i.e. total leaf area, mean area per leaf, and leaf number), compensatory characters related to tolerance.

Conclusions: Light limitation exacerbates the negative effect of leaf damage on plant fitness, producing a two-fold average reduction in tolerance. Moreover, compensatory growth was lower in the light-limited environment. Under high damage and light limitation, the evolution of higher tolerance to damage can be highly constrained.

Keywords: index of tolerance, leaf damage, light limitation, Solanaceae, tolerance mechanisms.

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INTRODUCTION

Plants in their natural environment grow in a heterogeneous matrix of biotic interactions (e.g. herbivores and pollinators) and abiotic resources (e.g. minerals, water, and light) that determine their reproductive success. Resource availability can affect development and performance of plants if resource limitation compromises plant vital functions (Valladares *et al.*, 2007). Resource availability may also reduce plants' ability to respond to the biotic environment, modifying their interactions with other organisms (Fornoni *et al.*, 2003; Wise and Abrahamson, 2007; Sun and Ding, 2009). Such is the case in plant–herbivore interactions, where resource availability plays an important role in the evolution of defensive strategies (Coley *et al.*, 1985; Strauss and Agrawal, 1999; Agrawal and Fishbein, 2006; Núñez-Farfán *et al.*, 2007; Wise and Abrahamson, 2007).

Plant tolerance to damage by herbivores is a defensive strategy that buffers losses in individual fitness (Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999). When damage is experimentally imposed at a single level (e.g. damaged or undamaged plants), tolerance is defined as the difference in fitness between related damaged and undamaged plants or the proportional fitness of damaged individuals relative to undamaged ones (Strauss and Agrawal, 1999). In fact, for continuous damage environments, tolerance can be treated as the reaction norm of fitness as a function of leaf damage (Simms, 2000). Because individual plant fitness cannot be examined in both damaged and undamaged states, tolerance must be estimated from a group of related plants (genotypes) (Strauss and Agrawal, 1999; Simms, 2000; Stowe *et al.*, 2000).

Tolerance to herbivory can be affected by the amount of genetic variance present in a given population, environmental deviations, potential trade-offs with resistance (defensive strategy that prevents herbivory through defensive traits) to herbivores (Tiffin and Rausher, 1999; Weinig *et al.*, 2003), and by genotype × environment interactions resulting from variation in resource availability and/or different type and abundance of herbivores (Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999; Stowe *et al.*, 2000; Hawkes and Sullivan, 2001; Núñez-Farfán *et al.*, 2007; Banta *et al.*, 2010). The Limiting Resources Model (LRM), proposed by Wise and Abrahamson (2005), states that tolerance will be limited if herbivory reduces plants' ability to acquire a limiting, focal resource in a given environment. A prediction of this model is that when herbivory reduces the acquisition of a limiting resource, such as minerals (by damaging roots) or CO_2 and light (by consuming leaves), plants will experience a drastic fitness reduction compared with conditions where these resources are not limited. Following this prediction, we hypothesized that plant tolerance should be reduced in light-limited environments because both leaf damage and reduced photosynthetic active radiation (PAR) decrease carbon sequestration (focal resource). Since a genotype's tolerance to damage is itself a reaction norm that can be expressed in different environments, say light and shade, we can ask if tolerance to damage has the potential to evolve (i.e. family × damage × light interaction). According to Falconer (1952), the interaction between genotype and environment can be regarded as a genetic correlation of the same character between two environments. Via and Lande (1985) considered that plasticity of a trait can evolve if selection acts in each environment. However, when character states are tightly genetically correlated, the evolution of new reaction norms may be limited. In this regard, the increase of tolerance to damage in two light environments would depend on the genetic variance within environments, and the genetic covariance between environments (Via and Lande, 1985; Via, 1987, 1994).

Here, we assess experimentally if light limitation reduces plant tolerance to leaf damage in the annual herb *Datura stramonium*. We hypothesized that the capability to maintain tolerance should be reduced under light limitation (shade treatment) because a reduction in leaf area and lower exposure to light will limit plants' ability to grow and reproduce.

To achieve this, we exposed artificially damaged and undamaged (control) plants of ten different genotypes (genetic families) to full sunlight and partial shade treatments. In order to evaluate the effect of light limitation on the tolerance response after damage, we measured total seed production in all treatment combinations. Then, we obtained average tolerance in each treatment by estimating the fitness differences between damaged and undamaged plants. We further assessed photosynthetic activity and leaf growth compensation (total leaf size, mean leaf size, and number of leaves per plant) in response to damage and light limitation, characters associated with tolerance (Strauss and Agrawal, 1999).

METHODS

Study system

Datura stramonium L. (Solanaceae) is an annual herb dispersed exclusively by seeds produced by selfing or outcrossing (Motten and Stone, 2000). This plant grows in old fields, roadsides, and disturbed areas in Mexico, USA, Canada, and Europe (Weaver and Warwick, 1984; Núñez-Farfán and Dirzo, 1994). The leaves of this plant are persistent, allowing insects to consume up to 100% of leaf area (J. Núñez-Farfán, personal observation). In Mexico, the main consumers of *D. stramonium* are the specialist folivores *Lema trilineat*a (syn. *Lema daturaphila*) and *Epitrix parvula* (Coleoptera: Chrysomelidae), and the generalist *Sphenarium purpurascens* (Orthoptera: Pyrgomorphidae). Leaf damage caused by these herbivores reduces seed production, imposing selection on traits of resistance and tolerance to leaf damage (Núñez-Farfán and Dirzo, 1994; Valverde *et al.*, 2001, 2003; Fornoni *et al.*, 2004). Studies both in greenhouse and in natural conditions have found genetic variation for tolerance within and between populations (Fornoni and Núñez-Farfán, 2000; Fornoni *et al.*, 2003). Increased growth rate of leaves and stems have been related to tolerance to herbivores (Valverde *et al.*, 2003; Bello-Bedoy and Núñez-Farfán, 2011). Furthermore, *D. stramonium* has shown genetic variation in resistance traits such as tropane alkaloids (hyoscyamine and scopolamine) and leaf trichomes (Shonle and Bergelson, 2000; Kariñho-Betancourt, 2009; Castillo *et al.*, 2013, 2014).

Greenhouse experiment

A greenhouse experiment was performed from June to December 2007. The plants belonged to ten genetic families of *D. stramonium* previously obtained from an autogamous cross-design experiment. Therefore, individual plants of each family shared at least 50% additive genetic variance (full-sibs) (Falconer and Mackay, 1996). Parental plants were collected from a natural population located in Patria Nueva, State of Hidalgo, in central Mexico (20°22′12.46″W, 99°3′1.98″N). To assess the effect of light limitation on plant tolerance to damage, we randomly assigned 48 plants from each of the ten genetic families to the following damage and light treatments (12 plants per combination):

1. Plants without foliar damage + full sunlight (control; $n = 118$)
2. Plants without foliar damage + shade ($n = 115$)
3. Plants with 50% foliar damage + full sunlight ($n = 119$)
4. Plants with 50% foliar damage + shade ($n = 119$).

Nine seedlings died during the experiment, leaving a total sample size of 471 plants. We distributed plants in a randomized block design to control environmental variation within the greenhouse (Steel and Torrie, 1980).

Leaf damage and light treatments

Using scissors, we removed 50% of the foliar tissue from plants assigned to the damage treatment by cutting along their main veins without damaging the veins. To assess tolerance at the same phenotypic stage, the leaf damage treatment was applied when the first floral bud appeared (*c.* 6–7 leaves) (Fornoni and Núñez-Farfán, 2000). Any new leaves that appeared during the experiment were damaged in the same way. The shade treatment consisted in covering the plants with shade cloth that reduced photosynthetic active radiation (PAR) by about 35%.

Data collection

To obtain the total number of seeds produced by each plant, all fruits were individually collected into paper bags; fruits were then opened to count the seeds. To minimize variation in seed number between plants due to differences in herkogamy, all flowers were manually self-pollinated at dusk when the stigmas of *D. stramonium* are fully receptive (Motten and Antonovics, 1992). Total seed number has been shown to be an important component of fitness in annual plant species that possess a high degree of self-fertilization; therefore, seed number was used to estimate differences in tolerance between families and treatments (Fornoni and Núñez-Farfán, 2000; Valverde *et al.*, 2003; Mostafa *et al.*, 2011). Following Simms and Triplett (1994), we defined the index of tolerance (T) of each family (i) as

$$T_i = \frac{\bar{S}_{damage} - \bar{S}_{no\text{-}damage}}{\bar{S}_{no\text{-}damage}} = \left(\frac{\bar{S}_{damage}}{\bar{S}_{no\text{-}damage}} - 1\right),$$

where $\bar{S}_{damage}$ and $\bar{S}_{no\text{-}damage}$ are the mean seed number of a family in the damaged and undamaged (control) treatment respectively. When calculating the average index of tolerance ($\bar{T}_E$) in a light environment, all ten families were included, i.e.

$$\bar{T}_E = \frac{1}{n}\sum_{i=1}^{n} T_i.$$

Thus, $\bar{T} = 0$ indicates complete tolerance, $\bar{T} > 0$ over-tolerance, and $\bar{T} < 0$ under-tolerance.

We estimated the relationship between tolerance of genotypes (families) in the two light environments (Via and Lande, 1987) by means of a Pearson correlation of family mean values [i.e. a genetic correlation between environments (Falconer, 1952)]. We compared the average tolerance between light environments using Student's t-test.

To evaluate leaf responses to artificial damage and shade, we measured chlorophyll content on five fully expanded leaves per plant using a portable chlorophyll meter (CCM-200 model, Opti-Science). The chlorophyll meter measures the chlorophyll content index (CCI), which indicates the amount of total chlorophyll for a given unit area. Previous studies have shown that the CCI is a good indirect proxy of photosynthetic activity (Buttery and Buzzell, 1977; Naumann *et al.*, 2008; Hu *et al.*, 2009; Mostafa *et al.*, 2011). We counted leaf number and collected all the leaves to obtain total leaf area and mean leaf size per plant. To do this we used the image analyser software Image Pro Plus v.6.0.0.260 (Media Cybernetics, Inc.).

Statistical analyses

To assess the effect of light limitation and leaf damage on relative seed number, we performed a mixed-model analysis of variance (ANOVA). The model included the random effect *family*, and the fixed effects *damage* and *light availability*, as well as the interactions between them. We compared mean tolerance of plants in full light and partial shade using a *t*-test.

A mixed-model ANOVA (as described above) was also performed to calculate the CCI, mean leaf size, total leaf area, and number of leaves produced (in independent models). Whenever an ANOVA was significant, we performed a Tukey-Kramer LSD *post hoc* test to highlight significant differences between treatments. All analyses were performed in JMP v.9 (SAS Institute, 2007). Following Garrido *et al.* (2010), prior to analyses, seed production was relativized per plant genotype to control for differences in vigour among genetic families. Thus we expressed average seeds produced by a family relative to the average seed production across environments.

RESULTS

Effect of light limitation and leaf damage on seed production and tolerance

Seed production in *D. stramonium* was significantly reduced in the leaf damage and light limitation treatments (Table 1a). In both treatments, seed production was reduced relative to the control treatment (Fig. 1a). Plants in the leaf damage and light limitation treatments showed the lowest seed production (Fig. 1a). Moreover, the relative seed number of undamaged plants exceeded that of damaged plants (Fig. 1a). The shade × damage interaction was also significant; plants with no damage and full light attained the highest mean seed production, whereas plants in the damage + shade treatment showed the lowest mean seed production (Fig. 1a). The family × damage interaction was significant, indicating between-family variation in tolerance to damage (Fig. 2, Table 1a). However, the interaction family × shade × damage was not significant, indicating that the combination light level plus damage affected all families equally (Table 1).

The mean index of tolerance of plants grown in full sunlight was 2.1-fold higher than plants grown in low light ($T_{\mathrm{FL}} = -0.3260 \pm 0.022$ vs. $T_{\mathrm{LL}} = -0.1550 \pm 0.022$, respectively; $t = -3.54$, $P = 0.0022$, $N = 10$) (Fig. 1b). The genetic correlation between family tolerance in the two light environments was high ($r = 0.685$, $N = 10$, $P = 0.028$). The level of tolerance in one environment (full light) predicts well tolerance in the other (shade).

Chlorophyll content index

Chlorophyll content was significantly affected by leaf damage and light reduction (Table 1b). Shade reduced mean CCI in relation to control plants (Fig. 3a). The interaction shade × damage was significant; in full sunlight damaged plants had higher CCI values than undamaged plants, whereas both groups of plants had similar and lower CCI values in the shade (Fig. 3a, Table 1b). Finally, we detected genetic variation in CCI expression in response to the shade treatment (family × shade interaction; Table 1b).

Table 1. Results of ANOVA of (a) relative seed number, (b) chlorophyll content index (CCI), (c) mean leaf area, (d) total leaf area, and (e) number of leaves in response to family, damage, and shade treatments, in a greenhouse experiment with *Datura stramonium*

	(a) Relative seed number				(b) CCI				(c) Mean leaf area			
Source of variation	d.f	SS	*F*	*P*	d.f	SS	*F*	*P*	d.f	SS	*F*	*P*
Family	9	3.8771	1.6506	0.2097	9	2452.25	2.6115	0.0810	9	24925.2	1.5877	0.4278
Damage	1	8.8471	44.9669	**<0.0001**	1	1042.96	24.5397	**0.0008**	1	119790	99.5462	**<0.0001**
Shade	1	5.7129	51.5161	**<0.0001**	1	21660.8	243.58	**<0.0001**	1	498144	193.855	**<0.0001**
Family × Damage	9	1.7713	4.2148	**0.0217**	9	382.564	1.5658	0.2573	9	10822.7	0.5928	0.7760
Family × Shade	9	0.9979	2.3746	0.1069	9	800.771	3.2775	**0.0459**	9	23134.3	1.2671	0.3651
Damage × Shade	1	0.7110	15.2030	**0.0036**	1	257.083	9.4676	**0.0132**	1	3426.76	1.6893	0.2259
Family × Damage × Shade	9	0.4202	0.3659	0.9508	9	244.321	0.7674	0.6468	9	18258.3	1.0692	0.3844
Error	430	54.8684			430	15211.9			424	804463.2		

	(d) Total leaf area				(e) Number of leaves			
Source of variation	d.f	SS	*F*	*P*	d.f	SS	*F*	*P*
Family	9	184.5444	3.0726	**0.0014**	9	271.36291	3.6577	**0.0002**
Damage	1	2277.2492	341.2437	**<0.0001**	1	740.55597	89.8378	**<0.0001**
Shade	1	2642.4132	395.9632	**<0.0001**	1	127.62005	15.4817	**<0.0001**
Family × Damage	9	43.9205	0.7313	0.6803	9	65.31586	0.8804	0.5427
Family × Shade	9	105.9168	1.7635	0.0732	9	63.3194	0.8535	0.5672
Damage × Shade	1	65.5005	9.8152	**0.0019**	1	42.9622	5.2118	**0.0229**
Family × Damage × Shade	9	49.0037	0.8159	0.6017	9	56.11856	0.7564	0.6570
Error	424	2829.5134			430	3544.601		

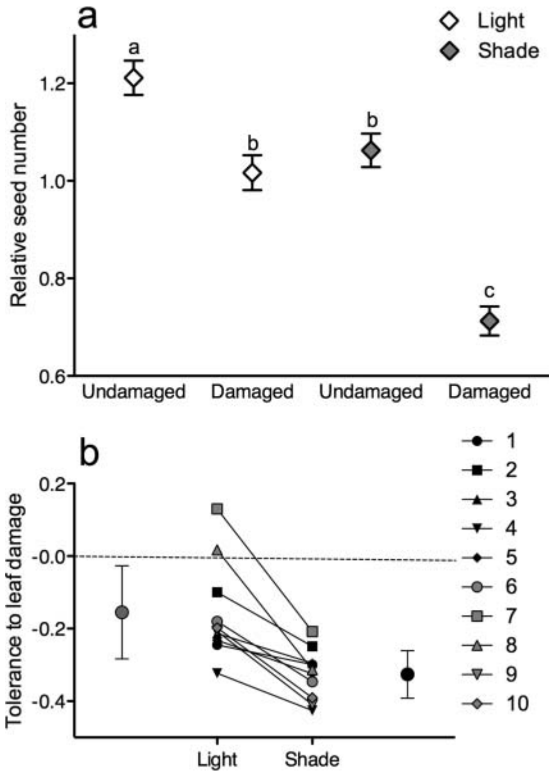

Fig. 1. (a) Average relative seed number (± 1 SE) produced per plant of *Datura stramonium* grown under two light conditions (light and shade) and with or without foliar damage. Averages with different letters differ significantly after a Tukey-Kramer LSD *post hoc* test; $N = 471$. (b) Index of tolerance (see Methods) of ten genetic families of *Datura stramonium* in control and light-limited environments. Circles represent the average index of tolerance (± 1 SE) in light environment.

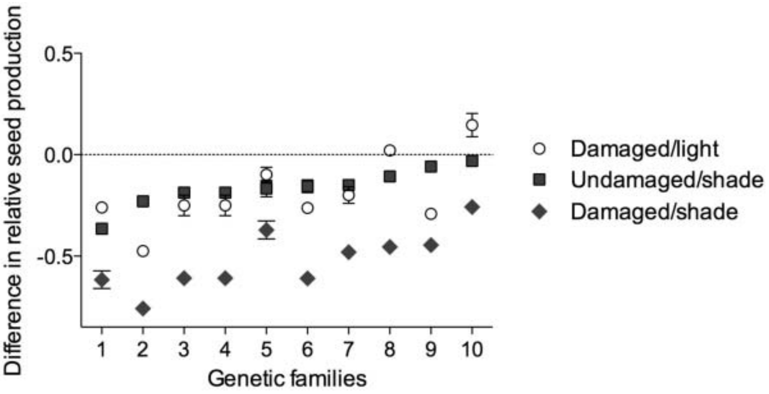

Fig. 2. Differences in relative seed production of ten genotypes of *Datura stramonium* exposed to a combination of leaf damage (0 and 50%) and light limitation (0 and 35%) treatments. Dotted line represents relative seed production of plants (a genotype) in the control treatment (undamaged/natural light). Whiskers represent ± 1 SE.

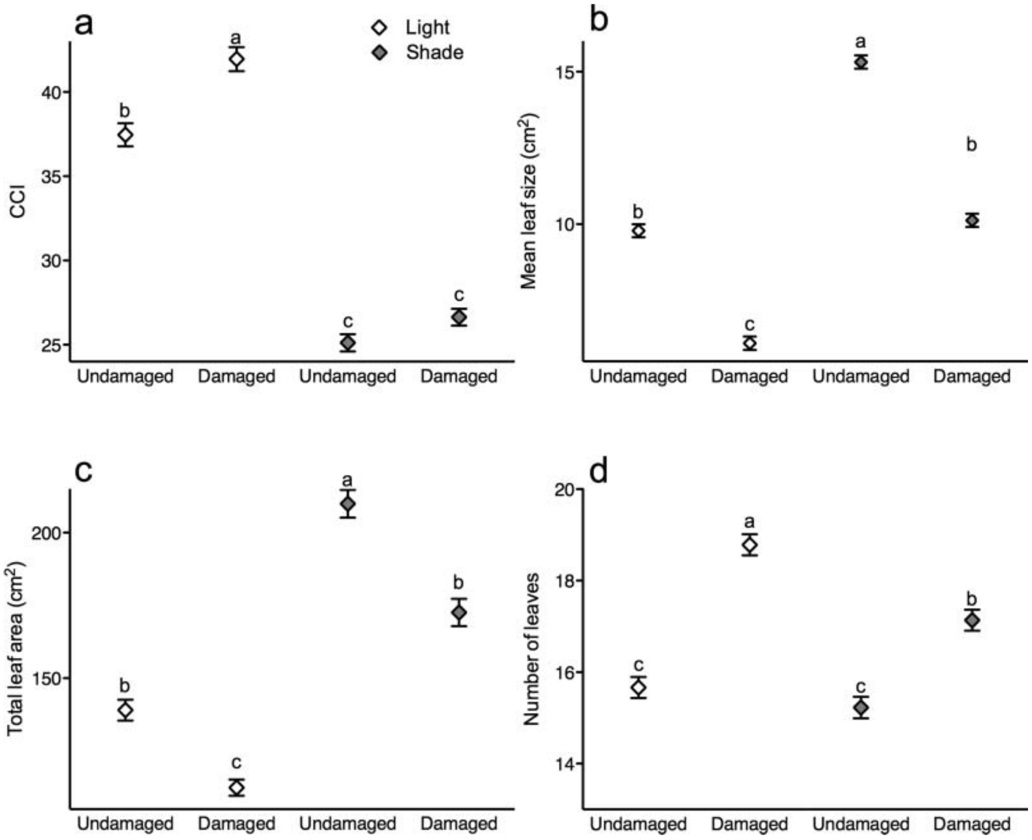

Fig. 3. Averages (± 1 SE) of (a) chlorophyll content index, (b) mean leaf size, (c) total leaf area, and (d) number of leaves produced by plants of *Datura stramonium*, in a greenhouse experiment under damage and light availability treatments. Averages with letters differ significantly after a Tukey-Kramer LSD *post hoc* test; $N = 471$.

Leaf traits

Leaf damage reduced both mean leaf size and total leaf area, both of which were larger under light limitation (Table 1c, d and Fig. 3b, c). Furthermore, the analyses detected a significant damage × shade interaction and genetic variation for total leaf area and number of leaves (Table 1d, e). Within each light environment, number of leaves was higher for damaged plants, and plants in the shade produced a lower number of leaves than plants in full sunlight (Fig. 3d, Table 1e).

DISCUSSION

Foliar damage negatively affected seed production in *D. stramonium*. Likewise, plants growing in partial shade showed reduced seed production compared with plants in full sunlight. The tolerance response is expected to buffer fitness losses due to foliar damage. In the present study, we found that damaged plants – both in full sunlight and partial shade –

showed lower fitness compared with undamaged plants. Furthermore, the ability of plants to tolerate leaf damage was on average two-fold lower in plants grown in partial shade than plants grown in full sunlight. Thus, light-limited environments constrain tolerance.

In line with our expectations, damaged plants growing under light limitation showed the lowest seed production, whereas undamaged plants in full sunlight showed the highest seed production, and although genetic variance for tolerance occurs across light environments, the interaction family × damage × light was not significant. Our results show that the light-limiting condition exacerbates the negative effects of leaf damage on seed production in *Datura stramonium*, resulting in a reduced tolerance to damage. Moreover, the strong negative genetic correlation of tolerance in the two light environments suggests limits to the evolution of tolerance in light-limited environments (see Via, 1994).

Resource availability, light exposure (full or partial), and soil nutrients help explain the level of tolerance of plants when damaged (Coley *et al.*, 1985; Núñez-Farfán *et al.*, 2007). It has been hypothesized that the tolerance response to damage by herbivores should decrease if a resource relevant for tolerance to damage is not freely available, and if damage by herbivores to plant tissues exacerbates such limitation (Wise and Abrahamson, 2007). Thus, individuals would incur an extra fitness cost. In this study, tolerance was lower in light-limited conditions compared with natural light (see Fig. 1b). A reduction in leaf area owing to damage and light limitation could diminish CO_2 fixation and photosynthetic rate, and thus the compensatory response (Mabry and Wayne, 1997; Rand, 2004; Salgado-Luarte and Gianoli, 2011).

Although a previous experiment in *D. stramonium* found no changes in tolerance at similar levels of defoliation in different light environments (Aguilar-Chama and Guevara, 2012), our results indicate that light availability *per se* has a negative effect on the tolerance of plants. Differences between experiments might be due to differences in experimental conditions or studied populations. For instance, we measured the index of tolerance by family to assess genetic variation in tolerance (cf. Table 1a) because it has been documented for different populations of *D. stramonium* (Fornoni and Núñez-Farfán, 2000; Valverde *et al.*, 2003; Fornoni *et al.*, 2004). In contrast, Aguilar-Chama and Guevara (2012) used different plants ($n = 6$) from different populations ($n = 8$), so tolerance could not be assigned to a given family or genotype.

Understanding changes in plant physiology and leaf traits induced by herbivore damage may help predict the potential for tolerance to evolve in heterogeneous environments (Fornoni *et al.*, 2003; Núñez-Farfán *et al.*, 2007). Chlorophyll content is a good indirect proxy of photosynthetic activity (Buttery and Buzzell, 1977; Naumann *et al.*, 2008; Hu *et al.*, 2009; Mostafa *et al.*, 2011), which has been suggested to be an important mechanism of plant tolerance to herbivore damage (Strauss and Agrawal, 1999; Fornoni, 2011). However, it is unclear whether plants should increase or maintain chlorophyll content as an adequate response to herbivore damage. We found evidence of genetic variation in the chlorophyll content index in shade conditions, indicating the potential for this trait to evolve in light-limited environments (Coley *et al.*, 1985; Wise and Abrahamson, 2005; Stevens and Kruger, 2008). Furthermore, the increment in chlorophyll content in damaged plants in full light indicates that plants responded to leaf damage by increasing chlorophyll content. This increase was small in this as in previous studies (Huang *et al.*, 2013) and limited by shade (low light negatively affected chlorophyll content). Both increased and constant leaf chlorophyll content can form part of the compensatory response to tolerate damage, since remaining leaf tissue is photosynthetically active after damage (Zangerl *et al.*, 2002), contributing to plant growth and reproduction. Thus, increasing the level of chlorophyll in damaged plants in full light could contribute to tolerance of damage, whereas this response is absent when light is limited.

Variation in tolerance can also be explained by differences in total leaf area between plants growing under full light compared with plants growing in shade. Our previous studies in *D. stramonium* have found that plants with large leaves are more tolerant than plants with small leaves (Fornoni *et al.*, 2003; Valverde *et al.*, 2003; Bello-Bedoy and Núñez-Farfán, 2011). These results showed that shaded plants had both higher total leaf area and mean leaf size than plants exposed to natural light levels. This response, however, did not increase their tolerance. Instead, plants in shaded conditions with larger leaves produced fewer leaves and less seeds, indicating that the increase in leaf area did not buffer the negative impact on seed number in *D. stramonium*. Thus, there is evidence to suggest that light limitation reduces tolerance persistence in populations under strong and constant pressure by herbivores. The plastic responses of plants to shade are not always adaptive and can even be maladaptive when negatively related to other fitness components (Valladares and Niinemets, 2008). Our results show a contrasting pattern of total and mean leaf area with seed number in undamaged and damaged plants, suggesting a potential trade-off between these traits. This trade-off may arise from the costs associated with the production of new leaf area to capture light, reducing the resources available for reproduction (Valladares and Niinemets, 2008). The fitness cost associated with the plastic response to light limitation could partially explain the difference in tolerance to defoliation between damaged and undamaged plants growing under different conditions of light availability.

The lower tolerance observed in shaded plants was related to the lower amount of compensation in the putative traits measured, compared with plants in full light. Thus, this study highlights the importance of measuring not only the pattern of tolerance but the mechanisms associated with the tolerance response (Camargo *et al.*, 2015). Finally, these results offer insights to better understand the evolution of tolerance to herbivory in natural environments.

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Discusión general y conclusiones

Los factores ecológicos que pueden influenciar la evolución de la tolerancia a la defoliación son variados, a la fecha los más importantes reportados son entre otros, la disponibilidad de los recursos abióticos (Wise and Abrahamson 2007), el tiempo y la magnitud del daño, el momento ontogenético del daño y el tipo de daño (e.g., Oesterheld and MacNaughton 1988, Maschinski & Whithman 1989, Strauss & Agrawal 1999, Tiffin 2002, Steven et al. 2007, Suwa & Maherali 2008). Esta tesis aporta nueva evidencia de otros factores mecanicistas que influencian la respuesta tolerante como la presencia, número y el peso (magnitud) de los determinantes del crecimiento, los cuales pueden incrementar o decrecer no solo la tolerancia sino el vigor de la norma de reacción del *fitness* dependiendo del contexto abiótico y explican en alguna medida el porqué de la evidencia contrastante de los patrones de tolerancia reportados en la literatura. A continuación, discutiré las conclusiones relevantes del estudio.

La cantidad de tolerancia y el vigor de la norma de reacción son el resultado de un mecanismo compensatorio a lo largo de la ontogenia (Capítulo 1)

La evidencia aquí reportada muestra que plantas defoliadas de *D. stramonium* presentan valores menores en los componentes del crecimiento inmediatamente después del daño (plasticidad pasiva debido a una disminución en el suplemento de recursos, van Kleunen & Fisher 2005) y que estos valores iniciales son restablecidos por incrementos progresivos (plasticidad activa), a lo largo de la ontogenia hasta incluso superar los valores presentados por las plantas control. Así, este estudio reporta por primera vez las fases ontogenéticas de este mecanismo compensatorio (c.f. Oerterheld and McNaughton 1988) y permite establecer que en cualquier momento diferentes genotipos pueden diferir en la habilidad para compensar el daño. Esta habilidad está relacionada con la velocidad del crecimiento antes del daño. A una escala intraespecifica se sugiere un compromiso (*Trade-off*) entre la habilidad para crecer en condiciones ambientales favorables y la habilidad para tolerar la limitación de recursos debida a la defoliación. No obstante, un mayor crecimiento antes del daño favorecería el vigor de la norma de reacción del *fitness*.

La tolerancia es afectada por la contingencia ontogenética en la expresión de los determinantes del crecimiento (Capítulo 2)

Este estudio muestra como la norma de reacción del *fitness* a la defoliación en *D. stramonium* es afectada por caracteres relacionados con el crecimiento que pueden ser expresados de forma constitutiva (antes del daño) e inducidos (después del daño). Mientras el vigor no es afectado por la expresión temprana de estos caracteres, la tolerancia es afectada negativamente por un crecimiento rápido antes de la defoliación solo en bajos nutrientes. Es decir, genotipos con altas tasas de asimilación neta (fuertemente correlacionadas con el RGR, ver Resultados Capítulo 2) tienen menor producción de semillas y biomasa en respuesta a la defoliación. Lo anterior implica un costo de asignación de recursos, entre más grande la asignación al crecimiento intrínseco menor la asignación a la tolerancia. Mientras otros estudios han encontrado la manifestación de una correlación genética negativa entre el *fitness* de plantas dañadas y control (e.g., Manzaneda et al. 2007, Simons & Johnston 1999), este es el primer estudio en demostrar un *Trade-off* de asignación a lo largo de la ontogenia.

Se ha predicho que la defoliación parcial podría ser beneficiosa para las plantas (Crawley 1987), ya que removería muchos obstáculos como el mayor acceso a la luz y otras restricciones arquitecturales, lo cual haría de la interacción planta-herbívoro una asociación mutualista (i.e., hipótesis mutualista, Simmons & Johnson 1999). La existencia de un *Trade-off* en ambientes con baja disponibilidad de nutrientes en *Datura stramonium* aporta evidencia en contra de esta hipótesis ya que la defoliación puede acarrear un costo para los genotipos que crecen rápido antes del daño, el cual implicaría aumentar los efectos negativos de la defoliación sobre el *fitness*. No obstante, en ambientes con alta disponibilidad de recursos las restricciones a la evolución de una mayor tolerancia no se observan en *Datura stramonium*.

El vigor no es solo el resultado de plasticidad adaptativa (Capítulo 2)

En nuestro estudio detectamos variación genética para la plasticidad en LWR (bajos nutrientes) y SLA (bajos y altos nutrientes). Esta plasticidad incremento el vigor de la norma de reacción del número de semillas cuando los genotipos expresaron plasticidad pasiva. No obstante, cuando los genotipos expresaron plasticidad activa esta resulto maladaptativa (decreció el vigor de la norma de reacción del número de semillas), LWR en bajos nutrientes y SLA en altos nutrientes. Mientras otro estudio ha reportado plasticidad adaptativa de la floración temprana y el número de ramas en respuesta a la defoliación para *Ipomopsis agregata* (Juenger & Bergelson 2000), este es el primer estudio que muestra plasticidad maladaptativa en los determinantes del crecimiento.

La detección de costos (bajos nutrientes de plantas control) en la expresión de plasticidad activa en LWR y el RGR demuestra que *Datura stramonium* posee un mecanismo fuertemente regulatorio de la asignación de biomasa a las hojas y muestra que en bajas disponibilidades de nutrientes la especie posee una estrategia conservadora en la eficiencia del uso de los recursos (c.f., Valladares et al. 2002). Lo anterior, aunado a la capacidad de *D. stramonium* de incrementar la auto-polinización como una medida de aseguramiento reproductivo en ambientes con baja disponibilidad de nutrientes (Apéndice II), demuestran su habilidad de comportarse como una especie tolerante al estrés (Grime 1977).

Otros estudios han mostrado como órganos dañados transfieren recursos lejos de las hojas de los herbívoros (Henkes et al. 2008, Babst et al. 2005) como una estrategia de escape o para crecimiento futuro (Karban & Baldwin 1997, Briske et al. 1996). No obstante, este estudio muestra por primera vez un soporte evolutivo al argumento de que las plantas responderían con adaptaciones para proteger los nutrientes de ser consumidos en ambientes con poca disponibilidad de recursos (Janzen 1974, Thao & Hunter 2011). En este caso las familias de *Datura stramonium* con mayor plasticidad pasiva en LWR y RGR (menores valores del carácter en plantas defoliadas) se verían favorecidas por la selección natural.

Las diferencias en tolerancia entre ambientes abióticos no son solo el resultado de plasticidad de un carácter subyacente (Capítulo 2 y 3, Apéndice I)

Este estudio mostró que no solo la plasticidad en caracteres subyacentes afecta la tolerancia, sino que la altura de la norma de reacción de estos caracteres contribuye a la variación de la tolerancia observado (Apéndice I y tablas 4 y 5 Capítulo 2). La variación en la disponibilidad de los ambientes abióticos puede alterar la presencia de caracteres que favorezcan la tolerancia (plantas de baja luminosidad, Apéndice 1), el número y el peso (magnitud) de los caracteres que la incrementan o decrecen, y, por tanto, determinan el perfil de tolerancia que se puede observar.

La diferencia en tolerancia medida como el número de semillas en respuesta a la disponibilidad de nutrientes no soportó el modelo II de limitación de recursos (Wise & Abrahamson 2005), este modelo predice igual tolerancia en bajas y altas disponibilidades de nutrientes ya que se espera que la defoliación no interfiera en su captura. A pesar de que la tolerancia fue favorecida por caracteres que tuvieron un efecto positivo en alta disponibilidad de nutrientes, este ambiente presento menor tolerancia a la defoliación comparado con el ambiente de baja disponibilidad de nutrientes.

La mayor tolerancia en baja disponibilidad de nutrientes se podría explicar siempre y cuando las planta en bajas disponibilidades de nutrientes no estuvieran limitadas por la pérdida de carbono de la defoliación, ya que aun cuando la tasa fotosintética pueda disminuir, el nitrógeno es suficiente para la demanda (Wise & Abrahamson 2005). Lo contrario ocurriría en altas disponibilidad de nutrientes, en donde las plantas siempre estarían limitadas por carbono, aquí los nutrientes son suficientes para alcanzar tasas máximas en *fitness,* pero la defoliación haría limitante la disponibilidad de carbono, en cualquier cantidad de defoliación la pérdida del fitness sería más grande relativo a las plantas de baja disponibilidad de nutrientes (Wise & Abrahamson 2005). Es por esto que plantas con crecimiento lento antes del daño se verian favorecidas en bajas diponibilidades de nutrientes cuando son defoliadas, maximizando la tolerancia en mayor medida que las plantas con altas tasas de crecimiento al momento del daño (Capítulo 2, Hilbert et al. 1981), por ende, se observa una mayor tolerancia en bajas disponibilidades de nutrientes (Capítulo 2).

A pesar de lo anterior, la respuesta obtenida en gran medida depende del componente del *fitness* usado, la biomasa vegetativa soportó el modelo II de limitación de recursos (Wise & Abrahamson 2005), encontrándose igual tolerancia en bajas y altas disponibilidades de nutrientes. Otro estudio en *Arabidopsis thaliana* muestra como la variación en los patrones de tolerancia en respuesta al daño apical en diferentes ambientes de nutrientes depende de las poblaciones de origen usadas (Banta et al. 2010).

La tolerancia en respuesta a la defoliación en ambientes con variación en la disponibilidad lumínica soportó el modelo I de limitación de recursos (Wise & Abrahamson 2005). En ambientes con baja disponibilidad de luz, la defoliación interferiría con la capacidad de las plantas para capturar luz para la fotosíntesis y por tanto se predice mayor tolerancia en alta disponibilidad de luz (Wise & Abrahamson 2005). Las diferencias en tolerancia debidas a la variación en la disponibilidad de luz son en gran medida debidas a la ausencia de caracteres que la favorezcan en ambientes de baja disponibilidad lumínica (Apéndice I). La exacerbación de la limitación de recursos arriba del suelo debidos a la defoliación evitaría la acción de la selección sobre los caracteres forrajeros de luz. Sin embargo, otros estudios en *Ipomea purpurea* que concuerdan con el modelo I de limitación de recursos (Tiffin 2002), no han encontrado una alteración en el patrón de la selección dependiente de la disponibilidad de los recursos abióticos, en parte la discordancia de resultados se debe a la disparidad de caracteres subyacentes al *fitness* usado en los diferentes estudios, forrajeros de luz (este estudio), caracteres reproductivos y de resistencia (Tiffin 2002).

Otras Implicaciones Ecoevolutivas

En altos nutrientes (Capítulo 2), *D. stramonium* no presenta restricciones a la evolución de la tolerancia, varios caracteres contribuyen positivamente a incrementarla, principalmente una alta tasa de asimilación neta. No obstante, la adaptación a niveles de defoliación mayores sería posible si el incremento en vigor debido a una plasticidad activa en LWR no fuese contrarrestado por una selección en contra de la plasticidad activa en SLA. Lo anterior es claramente una restricción evolutiva, en este ambiente abiótico la selección no favorece genotipos con altos valores de plasticidad activa para LWR y SLA. Mientras existe una priorización para el incremento de la asignación de biomasa foliar en respuesta a la defoliación en ambientes ricos en nutrientes (mayor LWR), la cual favorece el vigor, esta mayor biomasa foliar no es usada en la expansión de una mayor área foliar (mayor SLA), ya que esta resultaría redundante. En caso de no existir esta restricción, incrementos del área foliar en respuesta a la defoliación favorecerían mayor captación de luz para la fotosíntesis e incrementos aún mayores del vigor (e.g., Verhoeven et al. 2004). Esta restricción es posible en altos nutrientes donde el aumento de biomasa foliar de cara a la defoliación no resulta costoso en comparación con ambientes pobres en nutrientes, en los cuales la pérdida foliar sería más difícil de reemplazar (Janzen 1974).

Datura stramonium se comporta como invasora en varios países nórdicos y en Sur-África (van Kleunen et al. 2007), este potencial invasor podría estar ligado a la ausencia de una regulación entre la plasticidad activa entre LWR y SLA, lo cual facilitaría la evolución de un “*monstruo darwiniano*” en la especie, un organismo capaz de adaptarse por plasticidad a incrementos en la defoliación (Pigliucci 2001). Mientras el potencial de auto-fertilización es menor en altos nutrientes en la especie (Apéndice II), poblaciones invasoras que colonicen ambientes con alta disponibilidad de nutrientes incrementarían su potencial invasor si resultan sitios con mayor abundancia de polinizadores (Apéndice II). El estudio del potencial invasor de la especie, contrastando poblaciones nativas y foráneas, podría beneficiarse de las herramientas para el estudio de la tolerancia desarrolladas en este trabajo, lo cual permitiría poner a prueba estas hipótesis.

Perspectivas

Central al mejoramiento de nuestro entendimiento de la variación en la tolerancia en función de la variación abiótica, es enfocarse en examinar la relación genética entre la altura de la norma de reacción y la plasticidad de los caracteres que confieren tolerancia. Es de particular importancia para estudios futuros considerar las bases genéticas de la norma de reacción a la defoliación, incluyendo como la expresión genética de la altura y la plasticidad cambia con los ambientes abióticos. Si la plasticidad en un carácter subyacente al fitness es influenciada por “genes de plasticidad” que son distintos de los genes que afectan la expresión de la altura de la norma de reacción a la defoliación (Scheiner 1993), esto indicaría que el perfil de expresión génica podría cambiar con el ambiente abiótico modificando la tolerancia a la defoliación, ya que esta es afectada por la altura y la plasticidad de la norma de reacción como se demostró en este estudio. Lo anterior, explicaría en buena medida la variación del patrón de tolerancia en respuesta a los ambientes abióticos. La recompensa de este trabajo sería poder aumentar nuestra habilidad de predecir en qué condiciones abióticas podría evolucionar una mayor tolerancia.

Estudios de selección artificial que seleccionen por genotipos con diferentes combinaciones entre alturas y plasticidad para la norma de reacción a la defoliación en los determinantes del crecimiento, serian el primer paso para ayudar a descubrir los mecanismos genéticos subyacentes a la tolerancia a la defoliación. La expresión de módulos de genes, en genotipos altamente plásticos a la defoliación, pero con alturas bajas, se podrían comparar con genotipos con grandes alturas y poca plasticidad (Schlichting & Wund 2014, Ehrenreich & Pfennig 2016). Realizar cruces recombinantes entre estos extremos fenotípicos permitiría hacer un mapeo genético para identificar los loci que controlan los recombinantes no plásticos con grandes alturas de los plásticos con bajas alturas (Ehrenreich & Pfennig 2016).

Conclusión general

Los determinantes del crecimiento en *Datura stramonium* mostraron tres propiedades necesarias para el cambio evolutivo (Lewontin 1970, West-Eberhard 2005). Primero, presentan variación en la plasticidad a la defoliación que puede ser modificada en respuesta a condiciones abióticas. Segundo, estas condiciones abióticas causan una reorganización del fenotipo que conduce a una nueva población de fenotipos, proveyendo nuevo material para la selección, en la cual, diferentes caracteres son los responsables de la variación en las propiedades de la norma de reacción del *fitness*. Tercero, alguna de esta variación fenotípica tiene un componente genético, lo cual puede conducir a evolución adaptativa.

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Apéndice I

RELACIÓN ENTRE LA NORMA DE REACCIÓN (PLASTICIDAD Y ALTURA) A LA DEFOLIACIÓN DE CARACTERES FORRAJEROS DE LUZ Y LA NORMA DE REACCIÓN DEL *FITNESS* (TOLERANCIA Y VIGOR) A LA DEFOLIACIÓN, EN AMBIENTES CON ALTA DISPONIBILIDAD LUMÍNICA†

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†. Este apéndice usa los datos del capítulo 3:

Light limitation reduces tolerance to leaf damage in *Datura stramonium*

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***Evolutionary Ecology Research*, 2017, 18: 351–362**

Este apartado tiene como objetivo aportar evidencia a favor del argumento de que la tolerancia es un carácter complejo, cuyos mecanismos subyacentes no sólo dependen de la *plasticidad* (*cf*., Alperts & Simms 2002), sino de la *altura* de la norma de reacción en respuesta a la defoliación. Las plantas de *Datura stramonium* son más tolerantes a la defoliación cuando crecen en ambientes de alta disponibilidad lumínica (grupo testigo) que cuando crecen en ambientes de baja luminosidad (ver Capítulo 3). Éstas diferencias en tolerancia se deben, en parte, a que en los ambientes de baja luminosidad ninguno de los caracteres medidos contribuye a la tolerancia observada, mientras en alta luminosidad dos de cinco caracteres contribuyen positivamente al incremento de la tolerancia observada (*i.e*., en orden de importancia: la plasticidad en el índice del contenido de clorofila –CCI-, y la altura del área foliar total –TLA-, Tabla I, Figura I.A, B).

La plasticidad del número de hojas (NL) y del área foliar total, y la altura del índice del contenido de clorofila, amortiguan (estimados negativos en Tabla I, Figura I.A, B) el efecto positivo en tolerancia debido a la plasticidad en el índice del contenido de clorofila, y la altura del área foliar total. La producción de un mayor número de hojas o en últimas la producción de una mayor área foliar total como respuesta a la defoliación en ambientes con alta disponibilidad lumínica podría ser redundante con otros caracteres como la plasticidad en el contenido de clorofila (mayores valores en plantas defoliadas), que favorece una eficiencia fotosintética con la misma biomasa foliar (ver Discusión Capítulo 3). No obstante, un incremento en la plasticidad del número de hojas favorece un mayor vigor de la norma de reacción del *fitness* (Tabla I, Figura I.C). Por tanto, un genotipo de *D. stramonium* puede confrontar una disyuntiva si el incremento en vigor debido a la plasticidad en el número de hojas (incrementa su número en el ambiente con defoliación) es costoso al incrementar su susceptibilidad a la herbivoría (tolerancia). Sin embargo, la tolerancia es el producto de varios caracteres, por lo que el peso negativo de la plasticidad en el número de hojas puede ser contrarrestado.

Finalmente, familias con altos contenidos intrínsecos (altura de la norma de reacción) de clorofila no alcanzan una mayor tolerancia que aquellos que responden plásticamente aumentando el contenido de clorofila en respuesta a la defoliación (Tabla I, Figura I.A, B).

Table I. Stepwise regressions of properties (plasticity and vigor) of fitness-components reaction norms on properties (plasticity and height) of growth-components reaction norms in *Datura stramonium* growing in a high light environment. Significant linear selection gradients (estimate) with associated standard error (S.E.) and *P*-values (*P*) are presented in bold type.

	Seed-number vigor ($R^2_{\text{adj}} = 0.37$)				**Seed-number tolerance** ($R^2_{\text{adj}} = 0.96$)			
Trait	Estimate	S.E.	*P*	BIC	Estimate	S.E.	*P*	BIC
Height CCI	–	–	N.E.†		**-0.87**	0.092	0.0025	29.41
Height TLA	–	–	N.E.		**0.87**	0.094	0.0027	30.16
Height NL	–	–	N.E.		**–**	–	N.S. ‡	0.47
CCI Plasticity	–	–	N.E.		**2.00**	0.172	0.0014	28.91
TLA Plasticity	–	–	N.E.		**-1.02**	0.133	0.0047	29.21
NL Plasticity	**0.66**	0.265	0.0369	28.45	**-0.93**	0.113	0.0037	28.33

†. N.E., indicates that the trait did not enter in the final model

‡. N.S., indicates that the trait has not a significant effect

Note: To test whether trait mean value and/or plasticity of growth-components in response to defoliation affect (1) the average fitness (vigor) or (2) the fitness plasticity of a given half-sib family (*j*), I used a stepwise regression analysis (Strauss et al. 2003; Wise et al. 2008) to control for correlations between independent variables. The Bayesian Information Criterion (BIC) was used, introducing a penalty term for the number of parameters in the overall models (†), to find the traits that explain the greater variance in it. We regressed relative values of seed set grand mean ($\overline{W}_j$) (averaged over control and defoliated plants), and their plasticities (plW_j), over our estimates of trait means (i.e., elevation of the reaction norm, $\overline{X}_j$) and plasticities (i.e., steepness of the reaction norm, plX_j) for all traits (*sensu* Camargo et al. unpublished manuscript, see Methods in Capítulo 2):

a). $\overline{W}_j = Constant + \alpha_0\overline{CCI}_j + \alpha_1\overline{TLA}_j + \alpha_2\overline{NL}_j + \beta_0 plCCI_j + \beta_1 plTLA_j + \beta_2 plNL_j$

b). $plW_j = Constant + \alpha_0\overline{CCI}_j + \alpha_1\overline{TLA}_j + \alpha_2\overline{NL}_j + \beta_0 plCCI_j + \beta_1 plTLA_j + \beta_2 plNL_j$

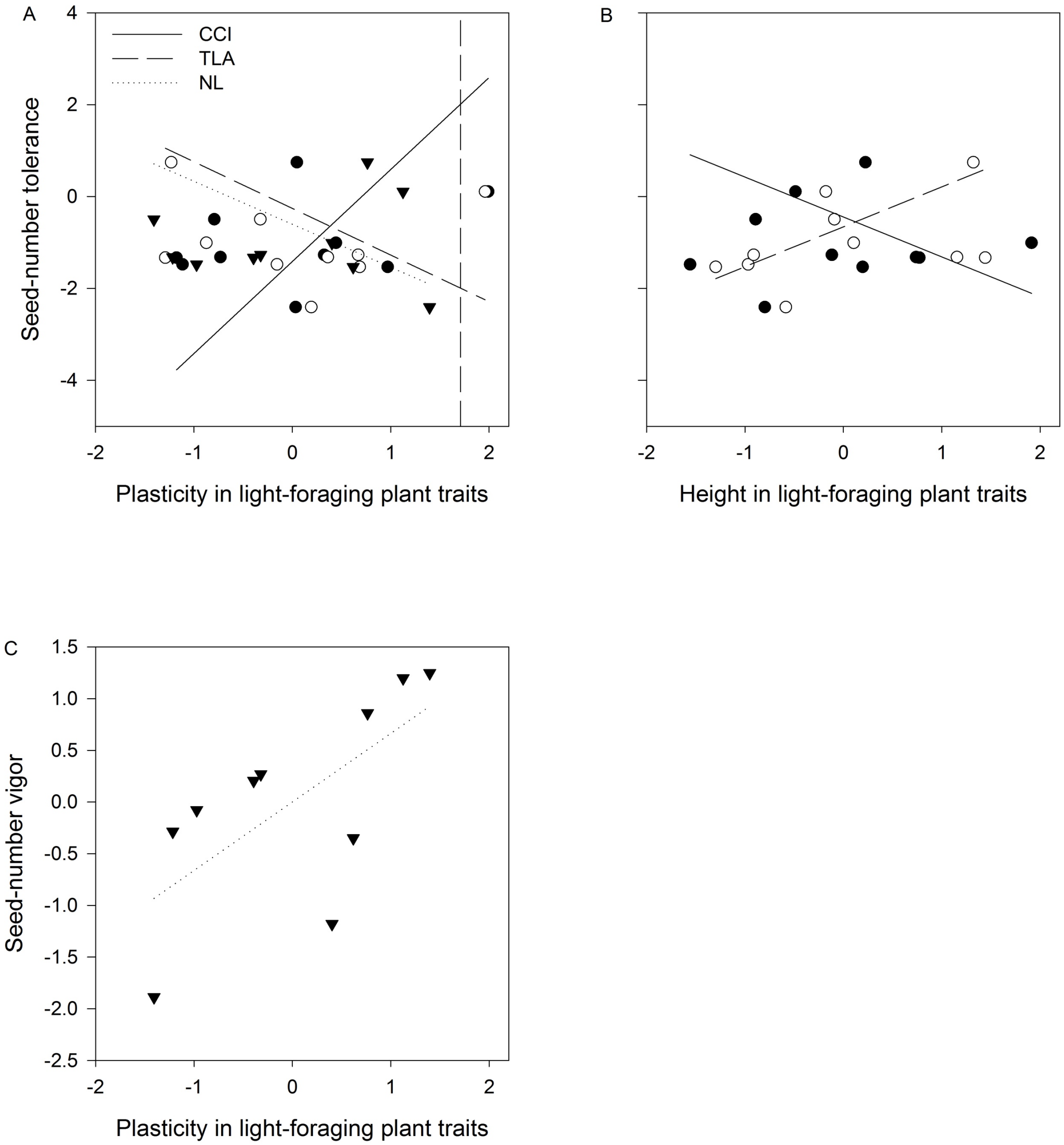

Figure I. Significantly regression coefficients of plasticity and height of light-foraging traits *versus* the seed-number tolerance or vigor in response to defoliation of *Datura stramonium,* growing in a high light environment. The slopes of the regression lines observed were obtained from separate regressions of the numbers of seeds (vigor or tolerance) on light-foraging traits, after controlling for trait correlations. Reference vertical line in the upper-left panel indicates zero plasticity of unstandardized values only for the total leaf area; the other plasticities had positive values in response to defoliation. Plasticity is the difference of defoliated minus control plants; see Methods in Chapter 2). Symbols in all panels as in panel A.

Apéndice II

FLOWER-LEVEL DEVELOPMENTAL PLASTICITY TO NUTRIENT AVAILABILITY IN *DATURA STRAMONIUM*: IMPLICATIONS FOR THE MATING SYSTEM

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Flower-level developmental plasticity to nutrient availability in *Datura stramonium*: implications for the mating system

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- **Background and Aims** Studies of phenotypic plasticity in plants have mainly focused on (1) the effect of environmental variation on whole-plant traits related to the number of modules rather than on (2) the phenotypic consequences of environmental variation in traits of individual modules. Since environmental and developmental factors can produce changes in traits related to the mating system, this study used the second approach to investigate whether within-individual variation in herkogamy-related traits is affected by the environment during plant development in two populations of *Datura stramonium*, an annual herb with a hypothesized persistent mixed mating system, and to determine which morphological traits may promote self-fertilization.
- **Methods** Full-sib families of two Mexican populations of *D. stramonium*, with contrasting ecological histories, were grown under low, mid and high nutrient availability to investigate the effects of genetic, environmental and within-plant flower position on flower size, corolla, stamen and pistil lengths, and herkogamy.
- **Key Results** Populations showed differences in familial variation, plasticity and familial differences in plasticity in most floral traits analysed. In one population (Ticumán), the effect of flower position on trait variation varied among families, whereas in the other (Pedregal) the effect of flower position interacted with the nutrient environment. Flower size varied with the position of flowers, but in the opposite direction between populations in low nutrients; a systematic within-plant trend of reduction in flower size, pistil length and herkogamy with flower position increased the probability of self-fertilization in the Pedregal population.
- **Conclusions** Besides genetic variation in floral traits between and within populations, environmental variation affects phenotypic floral trait values at the whole-plant level, as well as among flower positions. The interaction between flower position and nutrient environment can affect the plant's mating system, and this differs between populations. Thus, reductions in herkogamy with flower positions may be expected in environments with either low pollinator abundance or low nutrients.

Key words: *Datura stramonium*, flower-level developmental reaction norms, nutrient availability, within-individual variation, herkogamy, population variation, probability of self- and cross-fertilized flowers, complete selfing, mating system, reproductive assurance.

INTRODUCTION

The property of a given genotype to produce different phenotypes in response to distinct environmental conditions is a ubiquitous plant character called phenotypic plasticity (Pigliucci, 2001). However, modular organisms, such as plants, whose modules develop sequentially during the plant's ontogeny, face repeated opportunities for small-scale spatial and temporal environmental variation that may affect the module phenotype in a single individual (Winn, 1996). This view assumes that organs in repetitive modules are prone to alter their development in an autonomous way according to programmed developmental responses to changes in the environment during growth (Herrera, 2009). These instances have been referred to as 'developmental organ-level phenotypic plasticity' (Herrera, 2009; 'intra-individual plasticity' *sensu* Kawamura, 2010).

Plasticity can be expressed in whole-plant traits, such as growth, size and fecundity (e.g. Pigliucci and Schlichting, 1995; Pigliucci *et al.*, 1997) as well as in the traits of reiterated structures such as leaves, flowers and fruits (Goodspeed and Clausen, 1915; Schmalhausen, 1949; Sultan, 1987; reviewed in Herrera, 2009). Until recently, phenotypic plasticity studies have mainly focused on the analysis of environmental variation on whole-plant traits related to the number of modules rather than on phenotypic consequences of environmental variation on the traits of individual modules (Herrera, 2009; cf. growth and some reproductive traits, Pigliucci and Schlichting, 1995; Pigliucci, 1997; Pigliucci *et al.*, 1997). This modular nature of plasticity (de Kroon *et al.*, 2005) has striking consequences for plants since it could generate sub-individual variation affecting the evolutionary trajectory of organ traits by setting upper limits in the response to selection (i.e. constraining it), and opens up the opportunity for selection by animals on plant-level variability determining the size of the realized phenotypic space at the individual and population levels (Herrera, 2009).

The role of environmental factors in the expression of vegetative traits in plants is well established (Pigliucci, 2001), in contrast to flowers that have been traditionally considered the least plastic traits at the intra-specific level (Sinnott, 1921), shaped by developmental homeostasis (reviewed by Fenster and Galloway, 1997) and strong stabilizing selection (Armbruster *et al*., 2004). However, some floral traits are subject to marked modifications under stress by both internal and external conditions during development (e.g. flower size; Goodspeed and Clausen, 1915; Schlichting and Levin, 1984; reviewed in Herrera, 2009). In plants that produce flowers sequentially along branches, the order of flower production may have a strong influence on some traits such as herkogamy (Barrett and Harder, 1992; Vallejo-Marin and Barrett, 2009) but not on others (Vogler *et al*., 1999; Bissell and Diggle, 2008; reviewed in Diggle, 2003). Herkogamy, the stigma–anther separation, has been the focus of in-depth research (reviewed in Barrett *et al*., 2009) given its role as a major determinant of mating patterns in plant populations (Darwin, 1862; Webb and Lloyd, 1986). The expression of phenotypic plasticity in herkogamy can modify the frequency of self- and cross-fertilized flowers in self-pollinated species (Vallejo-Marin and Barrett, 2009). This modified rate of self- and cross-fertilization may help to match current environmental conditions. An increase in self-fertilization has been predicted in more stressful environments, where either biotic (e.g. pollinator abundance, Darwin, 1878) or abiotic conditions limit cross-pollination (e.g. dry season and an abundance of ephemeral habitats, Rick *et al*., 1978; Holtsford and Ellstrand, 1992; Elle and Hare, 2002; Moeller and Geber, 2005, reviewed in Levin 2010).

Datura stramonium L. (Solanaceae) is a predominantly self-fertilizing annual herb that has heritable variation in herkogamy that is positively correlated with the outcrossing rate (Motten and Stone, 2000). However, herkogamy can exhibit a remarkable pattern of continuous within-individual variation (cf. *Eichornia paniculata* marked bimodal pattern, Barrett and Harder, 1992) and this has been attributed to family-level inbreeding depression history (Stone and Motten, 2002). The effect of environmental factors on this variation has received less attention, and the search for genotype by environment interactions has been related to among-site variation rather than to specific environmental stressors (e.g. Motten and Stone, 2000). If flowers of *D. stramonium* produce a continuous variation in herkogamy that affects the relationship between selfed vs. outcrossed flowers (Motten and Stone, 2000), it is of interest to determine whether these contrasting floral phenotypes are produced as a systematic within-plant trend of variation. Here, our aim was to determine the effect of specific factors influencing within-plant variation in floral traits of *D. stramonium* and discuss its implications for mating system evolution.

We present results of a study of genetic variation in developmental organ-level reaction norms. A strong increase in plant biomass and total alkaloid content related to soil nitrogen addition has been documented in *D. stramonium* (Weaver and Warwick, 1984). We examined the pattern of developmental trajectories among six sequential flower positions (i.e. successive metamers, Fig. 1), and its plasticity to three different soil nutrient availabilities in two populations that differ in pollinator abundance and soil fertility. Our study addressed three specific questions. (1) How much genetic variation for floral trait within-plant variation and their plasticity exists in two populations of *D. stramonium*? An answer to this question would provide us with some empirical estimates of the potential for the evolution of the shape of organ-level developmental trajectories in heterogeneous environments, (2) Does stress (low nutrient availability) increase the probability of self-pollinated flowers? (3) What floral traits promote an increased self-fertilization?

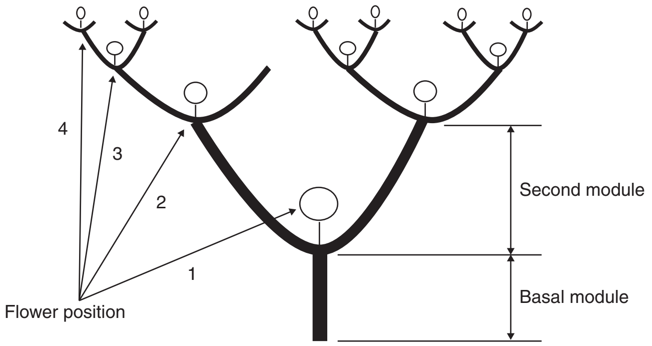

Fig. 1. Leeuwenberg's architectural model of *Datura stramonium*. The model consists of a sympodial succession of equivalent sympodial units (metamers), each of which is orthotropic and determinate in its growth. Then, the apex is expected to transform into one flower in each node. A module refers to a portion of an axis (metamer) made up of a single terminal meristem which corresponds to a sympodial unit. Thus, every flower position represents the traits expressed at each sympodial unit, resulting in the sequence of within-individual floral variation observed; in the figure, there is a sequential decrease in flower size and metamer length for the first four positions.

MATERIALS AND METHODS

Study species and populations

Datura stramonium is a colonizing annual plant widely distributed around the world. Although it is found in all types of soil, it prefers rich soils (Weaver and Warwick, 1984), rapidly assimilating nitrogen in the form of nitrate or ammonium (Lewis and Probyn, 1978; Platt and Rand, 1982). The species produces hermaphroditic self-compatible flowers, with tubular corollas and copious quantities of nectar. The flowers last only one night (Motten and Antonovics, 1992). The stamens and the style are inserted inside the corolla, with the stamen filaments adnate to the corolla tube in the proximal middle part (Motten and Stone, 2000). Herkogamy is positively correlated with the outcrossing rate in *D. stramonium* (Motten and Stone, 2000). Flowers having the stigma at or below the level of the anthers produce only selfed seeds. In flowers with the stigma exserted beyond the level of the anthers (i.e. anther–stigma separation $>3{\cdot}5$ mm), outcrossing increases in proportion to the degree of the exsertion (Motten and Stone, 2000). The average estimated outcrossing rate is low ($t = 1{\cdot}3\,\%$), but variation in t has been observed (range: 0–18 %; Motten and Antonovics, 1992). Narrow-sense heritability ranges from 0·20 to 0·31 in a population derived from a full diallel cross using eight inbred lines (that include a natural occurring polymorphism for hypocotyl and flower colour) in different environments (Motten and Stone, 2000).

Variation in herkogamy attributable to dominance variance appears to be exhibited in the richest environments, with exserted stigmas being recessive (Motten and Stone, 2000). Flower visitors are hawkmoths, honey-bees and bumble-bees (Sharma, 1972; Grant and Grant, 1983; Motten and Antonovics, 1992). The species is considered a weed in some countries, but in México it is a colonizing (ruderal) plant, most commonly encountered in disturbed habitats (Núñez-Farfán and Dirzo, 1994).

In *D. stramonium*, in contrast to many plants where the adult phase begins when sexuality is first apparent, maturity and sexuality are not necessarily synonymous. In this species, the position of flowers may be used to establish an architectural category to which a plant belongs, so that sexuality is also important in the vegetative growth dynamics of the plant. This property is due to its architectural model called Leeuwenberg (Hallé *et al*., 1978), in which branching produces equivalent orthotropic modules, each with deterministic growth culminating in the production of a terminal flower (Fig. 1). An important feature of the model is the decrease in length and primary width of successive modules and leaf size; thus, the first module is the longest, with the largest leaves (Hallé *et al*., 1978). We use the term module to refer to sequential, often semi-autonomous structural and functional sub-units of plants (*sensu* de Kroon *et al*., 2005), which are produced during development (White, 1979; Preston and Ackerly, 2004) but not necessarily at the same time. Then, a module refers to a portion of an axis (metamer) made up of a single terminal meristem which corresponds to a sympodial unit (Bell, 1991). Thus, every flower position represents the traits expressed at each sympodial unit, resulting in the sequence of within-individual floral variation observed (Fig. 1).

Seeds from two populations (natural progeny) of *D. stramonium* from central Mexico were collected: the Ticumán population in the state of Morelos, and the Pedregal de San Angel Ecological Reserve population, south of Mexico City. These populations differ in climate, vegetation and type of soils (Valverde *et al*., 2001). The Ticumán population is pollinated by several hawkmoth species (genus *Sphinx*, A. López, pers. obs.; of 241 marked flowers, 163 flowers were visited in 150 min), and the rate of outcrossing among plants varies between 0 and 80 %, with an average of 18 % (Cuevas, 1996). In contrast, the Pedregal population is incidentally pollinated by honey-bees but in some seasons pollinators are very scarce (J Núñez-Farfán and A. López Velázquez, pers. obs.; from 200 marked flowers, one flower was visited in 120 min in the nearest locality to Pedregal). Previous studies suggest that the Ticumán population possess greater genetic variance in quantitative traits than the Pedregal population (Núñez-Farfán and Dirzo, 1994; Fornoni *et al*., 2003). In contrast, the Pedregal population is highly inbred and self-compatible (91·8 %, $n = 98$ flowers; Núñez-Farfán *et al*., 1996). Moreover, in plants of this population grown in the greenhouse, herkogamy accounted for only 1 % of the variance in seed number per fruit ($r^2 = 0{\cdot}013$; $F = 1{\cdot}4$; $P < 0{\cdot}05$; $n = 476$; Fornoni and Núñez-Farfán, 2000).

Cultivation of plants and experimental treatments

Full-sib families of each field maternal progeny were derived from one generation of selfing in the greenhouse. Seeds were sown in pots (1·5 L) and kept at a 12:12 h (light/dark) photoperiod; the mean temperature was 28/23 °C. Due to low germination in some families, only five and ten families from Ticumán and Pedregal were used, respectively. When cotyledons were fully expanded, seedlings were transplanted to pots, with a mixture of sand, soil and turface (fritted clay).

When plants were 2 weeks old, 12 replicates of each family were randomly assigned to one of three nutrient levels, which were supplied four times at 10 d intervals: ‘low’ (no nutrients added to the soil mixture), ‘medium’ (4 g L^{-1} of water of 20–20–20 NPK solution added), ‘high’ (6 g L^{-1} of water of 20–20–20 NPK solution added). Pots were arranged in a complete randomized design in a common garden at Harvard University, Cambridge, MA, USA. Pots were sunk into the soil to limit fertilizer loss.

Plant traits

Random seedling mortality reduced initial sample sizes from $n = 12$ plants per genotype. For 52 and 112 plants (254 and 563 flowers; not all plants produced flowers in all positions, see below) from Ticumán and Pedregal populations, respectively, three floral traits were measured with a calliper to the nearest 0·1 mm on six sequential flower positions along the plant: (1) corolla length, from the base of the calyx to the top of one lobe; (2) stamen length, from the base of the ovary to the top of the anthers; and (3) pistil length, from the base of the ovary to the top of the stigma. Herkogamy was estimated by subtracting the height of the stamen from the height of the pistil. Positive herkogamy implies that the stigma is exserted above the level of anthers and negative herkogamy implies the opposite (Schoen, 1982). Our aim here was not to compare plants at the same chronological age, which might vary to a great extent in our system because more than one flower is produced at different times in each sequential developmental stage. Hence, we did not use comparisons at a common point in time because they are important in relation to real-time processes such as reproductive output in relation to the length of the growing season but at a common developmental stage (Coleman *et al*., 1994), represented by the metamer number as an index (flower position). This index reveals the inherent growth strategy of the *D. stramonium* architectural model defining both the way the plant elaborates its form and the resulting sequence of activity of the endogenous morphogenetic processes of the organism, resulting in the intra-individual floral variation observed.

Statistical analysis

We assessed within-plant variation in floral characters by means of two analysis of covariance (ANCOVA) models. The first tested for genetic and environmental effects on whole-plant traits related to the number of modules; this analysis was intended to test the differences between populations. The second model tested for genetic and environmental effects, within populations, on the traits of individual modules (i.e. the positional change in the measured traits) or the shape of the developmental trajectories.

After checking for normality and homoscedasticity, only herkogamy did not meet the analysis of variance (ANOVA) assumptions of equal variance. Then, we used the pistil/stamen length ratio instead of length subtraction (see 'Plant traits', above). Results and significance levels were similar using both measures, so descriptive statistics and ANOVA results of subtraction data are presented. Since trait values were standardized (centred on the mean, dividing by the standard deviation) prior to ANOVAs, the mean squares for each trait–factor combination are directly comparable across traits, enabling interpretation of the relative importance of each factor in the analysis for explaining the phenotypic variation (Pigliucci and Kolodynska, 2006). We do not report Bonferroni-like corrections for multiple tests, often applied to maintain the overall probability of committing type I error, because these increase the probability of type II error (Moran, 2003; Banta *et al*., 2010). Instead, we reported the 'native' P-values (e.g. Pigliucci and Kolodinska, 2006). Population pattern reaction norms of floral traits were plotted against the three levels of nutrient availability using the least squares means adjusted for the covariate effect in the full model.

Genetic and environmental effects on whole-plant traits

A full mixed-model ANOVA was conducted to investigate the relative importance of (1) flower position (used as a covariate); (2) population (genetic variation among populations); (3) family, nested within population (genetic variation at the family level); (4) treatment (presence of average phenotypic plasticity regardless of specific populations or families); (5) treatment by population interaction (genetic variation for plasticity among populations); and (6) treatment by family interaction (genetic variation for plasticity among families, within populations). All main effects were considered fixed, except for family and its interactions, which were considered random.

Genetic and environmental effects on the traits of individual modules

We performed a detailed analysis of the reiterated floral traits throughout the six sequential flower positions along the plant, and the interaction of these within-plant trajectories with family and nutrient effects; individual analyses were conducted for each population. An ANCOVA was used according to the model: y = flower position (covariate), family, treatment, family × treatment, flower × family, flower × treatment, where the interaction terms involving flower position were intended to investigate the variation of within-plant trajectories at the family level (i.e. the flower × family term) or at the nutrient level (i.e. plasticity of within-plant trajectories; flower × treatment term). The three-way interaction was never significant and was excluded. The independence of the trait expression in each module of the plant motivated our use of an ANCOVA instead of repeated measures ANOVA (a model more suitable for time-dependent measurements such as for growth traits; e.g. Pigliucci and Schlichting, 1995; Pigliucci *et al*., 1997). Regression analysis of estimates of sequential floral parameters has also been used (e.g. Barrett and Harder, 1992; Vogler *et al*., 1999; Bissell and Diggle, 2008). We also used the genotypic averages of flower positions and measurements of flower traits to plot the architectural developmental trajectories for each genotype in the three environments. We used a 'character state approach' (i.e. using the mean values for each flower position, namely the character state; Pigliucci 2001) to plot architectural developmental trajectories with the aim of inspecting the patterns.

Flower length and phenotypic integration effects across environments

To ascertain whether flower length is a function of flower position, a principal component analysis (PCA) with Varimax rotation was applied to the correlation matrix of four floral measurements for the entire data set. Varimax rotation is a method for orthogonal rotation which results in high loadings for fewer variables; the rest will be near zero (Hair *et al*., 1998). We extracted factors and scores of floral traits on those factors: the first factor explained the most variance, which had high loadings for all floral traits except herkogamy. The scores for each factor reflect the weight and direction of the contribution of each individual plant's combined trait values to that component, and these can be analysed in the same manner as the traits themselves (Pigliucci *et al*., 1997; Kristjánsson, 2002; Engelmann and Schlichting, 2005; Bissell and Diggle, 2008). The scores were all normally distributed and were used as dependent variables in the two ANCOVA models used to evaluate the other floral traits measured (see above).

An additional ANCOVA was used to evaluate differences between populations (main factor) in the slope of size factor on flower position (covariate) in each environment; a significant population by flower position interaction will be interpreted as a change in the slope of the regression line of the size factor on flower position for the two populations (i.e. test of parallelism, e.g. Gianoli, 2004; Gonzalez and Gianoli, 2004).

As a measure of phenotypic integration and to assess the integrated response of floral traits to the effects of nutrient environments, we repeated the PCA for each population/environment matrix. Salient loadings of traits on the first factor at all nutrient environments are interpreted as evidence of strong phenotypic integration (e.g. Bissell and Diggle, 2008). We included traits in the interpretation of a factor if they had a factor of pattern coefficient $\geq 0{\cdot}40$ (i.e. a salient loading, which is the cut-off most often used in PCA, Harman, 1976; Gorsuch, 1983; Bissell and Diggle, 2008). That is, only traits that load consistently across environments on the first factor represent a suite of correlated characters maintained by co-ordinated development (Bissell and Diggle, 2008). Additionally, we performed classical paired comparisons at the phenotypic level for all traits, among correlation environmental matrices for each population and among populations for each environment (Appendix; Supplementary Data Tables S2 and S3).

Modified rate of self- and cross-fertilization

Within-individual variation in contrasting floral phenotypes was evaluated using logistic regression (Barrett and Harder, 1992; Vallejo-Marín and Barrett, 2009). Flowers were classified as contributing to self- (herkogamy $<3{\cdot}5$ mm) or cross-fertilization (herkogamy $\geq 3{\cdot}5$ mm), and coded with one and zero before the analysis, respectively. The threshold of 3·5 mm

Table 1. *ANOVA of individual floral traits of two populations of* Datura stramonium *grown under three levels of nutrient availability*

Trait	Flower (d.f. = 1)		Population (d.f. = 1)		Family (d.f. = 13)		Treatment (d.f. = 2)		Treatment by population (d.f. = 2)		Treatment by family (d.f. = 26)		Error (d.f. = 771)
	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS
Corolla length	0·43	0·42	174·04	**<0·0001**	4·38	**0·0034**	8·23	**0·0037**	2·50	0·14	1·30	**0·0034**	0·67
Stamen length	0·63	0·35	156·52	**<0·0001**	3·28	**0·0118**	4·02	**0·0400**	0·85	0·48	1·19	**0·0206**	0·71
Pistil length	5·56	**0·0037**	76·69	0·0151	10·99	**<0·0001**	16·50	**<0·0001**	1·34	0·28	1·05	**0·0295**	0·66
Herkogamy	6·99	**0·0008**	0·39	0·8750	17·18	**<0·0001**	17·41	**<0·0001**	0·52	0·56	0·92	0·05	0·61
Flower size (factor 1)	0·36	0·47	179·85	**<0·0001**	2·15	0·1152	4·90	0·0263	1·41	0·32	1·27	**0·0080**	0·70

The family term and its interactions are random and nested within populations.
Probability values in bold were considered not significant ($P > 0·05$).
Flower indicates flower position.

was chosen because flowers with stigmas protruding above this level showed continuous variation and a direct influence on the extent of outcrossing in *D. stramonium* (Motten and Stone, 2000; see ‘Study species’ above). The model was fitted using the module GLM with a binomial error, and a logit link function (JMP, Version 7. SAS Institute Inc., Cary, NC, USA) for each population. Statistical significance of the family term is interpreted as evidence of genetic variation in herkogamy, significance of the treatment effect as an indication of environmental effects, and the interaction as evidence of genetic variation for plasticity (Vallejo-Marín and Barrett, 2009). We used flower position as a covariate in the full model, and interaction terms between categorical effects (i.e. family and treatment) and flower position were also included as potential predictors in the logistic regression model (Barrett and Harder, 1992). If any interaction term was significant, we further explored the metameric influence (i.e. position-dependent floral trait expression) on the probability of producing selfed flowers for each nutrient environment using the module Nominal Logistic (JMP, Version 7).

RESULTS

Genetic and environmental effects on whole-plant traits

The first ANOVA model (Table 1; Fig. 2) revealed a significant effect of flower position on pistil length and herkogamy (median values in Supplementary Data Table S1). Populations were significantly different for all traits except herkogamy. Flowers were larger, and had greater corolla, stamen and pistil lengths in the Ticumán population (Fig. 2). Genetic variation among families within populations was significant for all traits except flower size. The experimental increment in nutrient availability had a significant effect for all traits (Fig. 2). Nutrient addition affected flower size (longer lengths for all floral traits). The Ticumán population showed less phenotypic plasticity between the medium and high nutrient availability for all floral traits except herkogamy (Fig. 2). However, genetic differences in plasticity among populations were not significant for any trait (Table 1, treatment by population effect). A significant treatment by family interaction indicates genetic variation for plasticity within populations for all floral traits (Table 1; herkogamy showed marginal significance).

It is interesting to note that for most characters, a large fraction of phenotypic variance is accounted for by genetic differentiation among populations, followed by nutrient availability and family main effects (Table 1). The exception to this pattern was herkogamy, which had little variance associated with population differentiation. These results indicate that genetic differences in plasticity, even when significant, contribute relatively little to phenotypic variation across the nutrient settings examined. From the perspective of whole-plant trait variation examined with this ANOVA model, there is a significant amount of variation explained by flower position for herkogamy and pistil length [Table 1, mean square (MS) of flower effect].

Genetic and environmental effects on the traits of individual modules

Analyses by population reveal that the Ticumán population has significant among-family genetic variation in traits, whereas the Pedregal population does not. In contrast, Pedregal shows both much greater plasticity and environment-dependent genetic variation (i.e. genetic variation for plasticity; Table 2, Fig. 3).

The effect of flower position was significant for herkogamy and pistil length in the Pedregal population, and corolla length in the Ticumán population (Table 2). A significant flower position by family interaction for all traits except stamen length reveals genetic variation for position-dependent floral trait expression in the Ticumán population. Almost all traits except herkogamy showed a significant flower position by treatment interaction (highlighting the modification of developmental trajectories by the nutrient environment) in the Pedregal population (Table 2). In the Ticumán population, both corolla and flower size showed significant flower position by treatment interaction; stamen length showed a marginally significant effect, whereas pistil length and herkogamy did not (Table 2).

The inspection of developmental trajectories showed a more homeostatic development for the Pedregal families for all traits in the low nutrient environment (Fig. 3A–D). Populations showed opposite within-plant trends in this environment, with all traits except herkogamy appearing to increase with flower position for the Ticumán population, but decreasing for the Pedregal population (Fig. 3A–D; see below). Populations did not show opposite trends in mid and high nutrient environments (Fig. 3E–L). In these environments, an increase in phenotypic variance, for almost all traits, was evident in the Pedregal population. However, familial variation and developmental stability for herkogamy is maintained across environments in this population. In the high-nutrient environment, the corolla-dependent

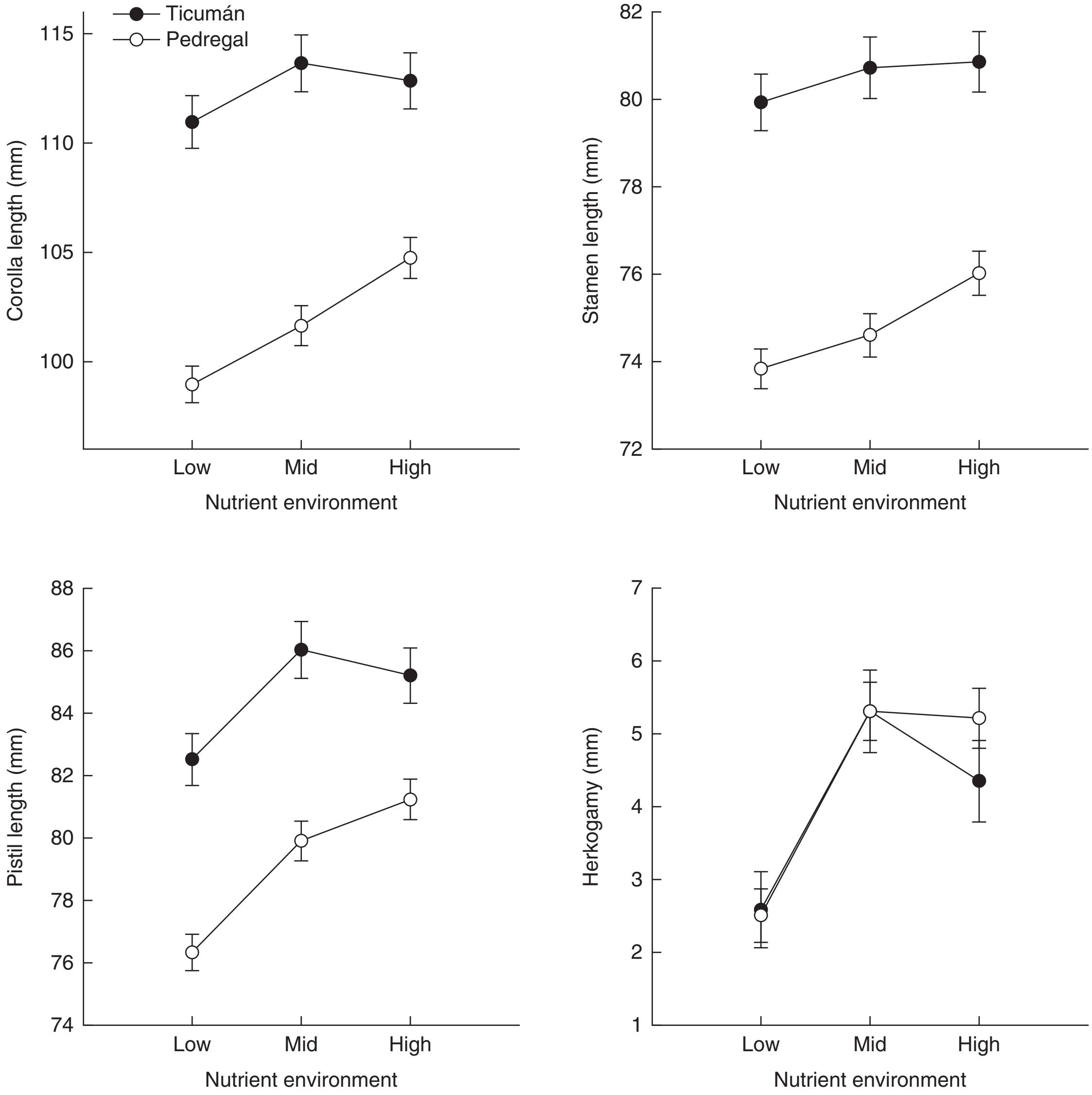

FIG. 2. Reaction norms of floral traits in response to nutrient availability of Ticumán and Pedregal populations of *Datura stramonium*. Mean values (± s.e.) are depicted.

variation in stamen and pistil length was evident for the Pedregal population that showed a very low value for corolla, stamen and pistil length in the fourth flower position (Fig. 3I–K); a similar result was observed for the fifth position in low nutrients (Fig. 3B–D). Notwithstanding, the herkogamy value in these flower positions did not drop as would be expected, highlighting the strong control of herkogamy in this population (Fig. 3L). Conversely, one family in the Ticumán population showed a decrease in the value of corolla length that was associated not with the stamen length but with pistil length (Fig. 3I–K). This resulted in lower herkogamy values in this family for the second flower position (Fig. 3L).

In the Pedregal population, the largest fraction of phenotypic variation was not accounted for by the genetic differentiation among families, but by the main effect of nutrient availability, followed by flower position, flower position by treatment and treatment by family interaction effects; flower position by family interaction explained little phenotypic variance. This implies that environmentally and architecturally phenotypic variation played a more important role than either its interaction or the genetic differentiation for flower position. Conversely, in the Ticumán population, the greatest fraction of phenotypic variation was associated not with the main effect of nutrient treatments but with genetic differentiation among families, followed by flower position by family and flower position by treatment interactions. The main effect of flower position explained little phenotypic variance (Table 2).

Flower size (factor 1) differed between populations and treatments, being smaller in the Pedregal and in the low-nutrient environment for both populations (Table 1; Fig. 4A). The significant flower position × treatment interaction in flower size (both populations) and herkogamy-related traits (corolla, stamen and pistil length in Pedregal) led us to explore metameric variation for these traits in each environment (Table 2).

TABLE 2. *ANCOVA of individual traits within two populations of* Datura stramonium *grown at three levels of nutrients availability*

Population, trait	Flower		Family		Treatment		Treatment by family		Flower by family		Flower by treatment		Error
	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS
Pedregal	(d.f. = 1)		(d.f. = 9)		(d.f. = 2)		(d.f. = 18)		(d.f. = 9)		(d.f. = 2)		(d.f. = 521)
Corolla length	2·36	0·06	1·01	0·74	13·1	**0·0013**	1·58	**0·0015**	0·36	0·85	2·15	**0·0426**	0·68
Stamen length	2·5	0·61	2·04	0·21	6·02	**0·0178**	1·35	**0·0133**	0·37	0·86	3·72	**0·0055**	0·71
Pistil length	5·95	**0·0014**	0·61	0·74	17·77	**<0·0001**	0·94	**0·0463**	0·66	0·33	3·03	**0·0054**	0·57
Herkogamy	3·62	**0·0087**	1·54	0·15	15·84	**<0·0001**	0·89	**0·0350**	0·86	0·10	0·24	0·63	0·52
Flower size (factor 1)	2·29	0·07	1·15	0·64	8·04	**0·0091**	1·51	**0·0042**	0·37	0·86	3·22	**0·0108**	0·63
Ticumán	(d.f. = 1)		(d.f. = 4)		(d.f. = 2)		(d.f. = 8)		(d.f. = 4)		(d.f. = 2)		(d.f. = 232)
Corolla length	2·35	**0·0465**	11·94	**0·0001**	0·96	0·22	0·53	0·51	1·48	**0·0418**	4·33	**0·0008**	0·59
Stamen length	1·68	0·12	5·50	**0·0042**	0·39	0·57	0·65	0·48	0·98	0·22	2·05	0·05	0·68
Pistil length	0·32	0·52	31·38	**<0·0001**	3·72	0·09	1·15	0·16	2·38	**0·0170**	1·33	0·18	0·77
Herkogamy	0·33	0·50	48·26	**<0·0001**	5·11	**0·0301**	0·97	0·23	5·45	**<0·0001**	0·38	0·60	0·73
Flower size (factor 1)	2·22	0·06	4·15	**0·0071**	0·40	0·52	0·57	0·51	1·34	0·08	3·09	0·0082	0·63

The family term is random.
Probability values in bold were considered not significant ($P > 0{\cdot}05$).
Flower indicates flower position.

Opposite directional trends in flower size were observed only in the low-nutrient environment (where flowers were smaller): using mean family values [Ticumán, slope = 0·18 ± 0·07 (mean ± s.e.), $F_{1,\ 28} = 6{\cdot}50$, $P = 0{\cdot}0166$, $R^2 = 0{\cdot}19$; Pedregal, slope = –0·12 ± 0·03, $F_{1,\ 54} = 22{\cdot}61$, $P < 0{\cdot}0001$ $R^2 = 0{\cdot}28$] (Fig. 4B). Differences between populations were significant (ANCOVA, $F_{1,\ 86} = 24{\cdot}26$, $P < 0{\cdot}0001$, $R^2 = 0{\cdot}67$; test of parallelism on family mean values) (Fig. 4B). This highlights the results of developmental trajectory plots observed for corolla, stamen and pistil traits in this environment (see above), the characters with higher loadings for flower size (i.e. factor 1 of the PCA with Varimax rotation; see the Materials and Methods). Corolla showed an opposite within-plant trend of variation only in the low-nutrient environment: using mean family values (Ticuman, slope = 2·01 ± 0·80, $F_{1,28} = 6{\cdot}30$, $P = 0{\cdot}0181$, $R^2 = 0{\cdot}18$; Pedregal, slope = –1·029 ± 0·28, $F_{1,58} = 13{\cdot}25$, $P = 0{\cdot}0006$, $R^2 = 0{\cdot}19$). Differences between populations were significant (ANCOVA, $F_{1,\ 86} = 19{\cdot}44$, $P < 0{\cdot}0001$, $R^2 = 0{\cdot}62$; test of parallelism on family mean values). A within-plant trend of variation was observed in stamen and pistil lengths only in the low-nutrient environment for the Pedregal population: stamen (slope = –0·72 ± 0·15, $F_{1,\ 58} = 23{\cdot}84$, $P < 0{\cdot}0001$, $R^2 = 0{\cdot}29$); pistil (slope = –1·06 ± 0·15, $F_{1,\ 58} = 28{\cdot}56$, $P < 0{\cdot}0001$, $R^2 = 0{\cdot}33$).

Phenotypic integration effects across environments

The integrated response of floral traits to the effects of nutrient environments showed that corolla, stamen and pistil load consistently and with no appreciable differences across environments in both Ticumán and Pedregal populations (i.e. the salient loadings were >0·40 and were constant across environments) (Table 3).

Ratio of self- and cross-fertilization

Flower position in the Pedregal population had a strong influence on the probability of producing self-fertilized flowers [log ratio test (LRT) of flower effect; slope = 0·18 ± 0·06, $\chi^2_{1,\ 521} = 7{\cdot}76$, $P = 0{\cdot}0053$]. Flowers in the low-nutrient environment were smaller and more likely to self (LRT of treatment effect; $\chi^2_{2,\ 521} = 37{\cdot}53$, $P < 0{\cdot}0001$, proportions of selfed flowers = 0·57 ± 0·04, 0·28 ± 0·03 and 0·30 ± 0·04, for low- mid- and high-nutrient environments, respectively), implying an environmentally influenced (i.e. plastic) rate of self- and cross- fertilization. Genetic variation at the family level was significant (LRT of family effect; $\chi^2_{9,\ 521} = 23{\cdot}51$, $P = 0{\cdot}0051$). Interaction effects were not significant in the Pedregal population. In the Ticumán population, only the family effect was significant (LRT of family effect; $\chi^2_{4,\ 232} = 84{\cdot}54$, $P < 0{\cdot}0001$).

We further explored the metameric influence on the probability of producing selfed flowers in the Pedregal population for each environment using the nominal logistic. Results indicated that flower position only affects the production of selfed flowers in the low-nutrient environment: as flower position increases, the probability of producing selfed flowers increases (Fig. 4C; slope = –0·31, $\chi^2_{1,\ 192} = 12{\cdot}44$, $P = 0{\cdot}0004$).

DISCUSSION

The main aim of our study was to find a within-plant trend of variation for floral traits. Irrespective of the causes of variation in these trends, they have striking consequences for the mating system and its evolution in different abiotic environments. The specific mechanisms that govern automatic self-pollination are the most significant aspects of the evolution of selfing (Barrett and Harder, 1992; Barrett *et al*., 2009). One challenging question, regarding the causal nature of within-plant variation, is whether contrasting floral phenotypes of a plant are produced randomly in relation to development, or whether positional effects are involved (Diggle, 2014) and affected by different environmental conditions (Barrett *et al*., 2009). In this sense, we were unable to test for effects attributable to flower position alone, which makes a more complex experimental design necessary. Very often this design implies an additional treatment where flowers are prevented from producing fruits and

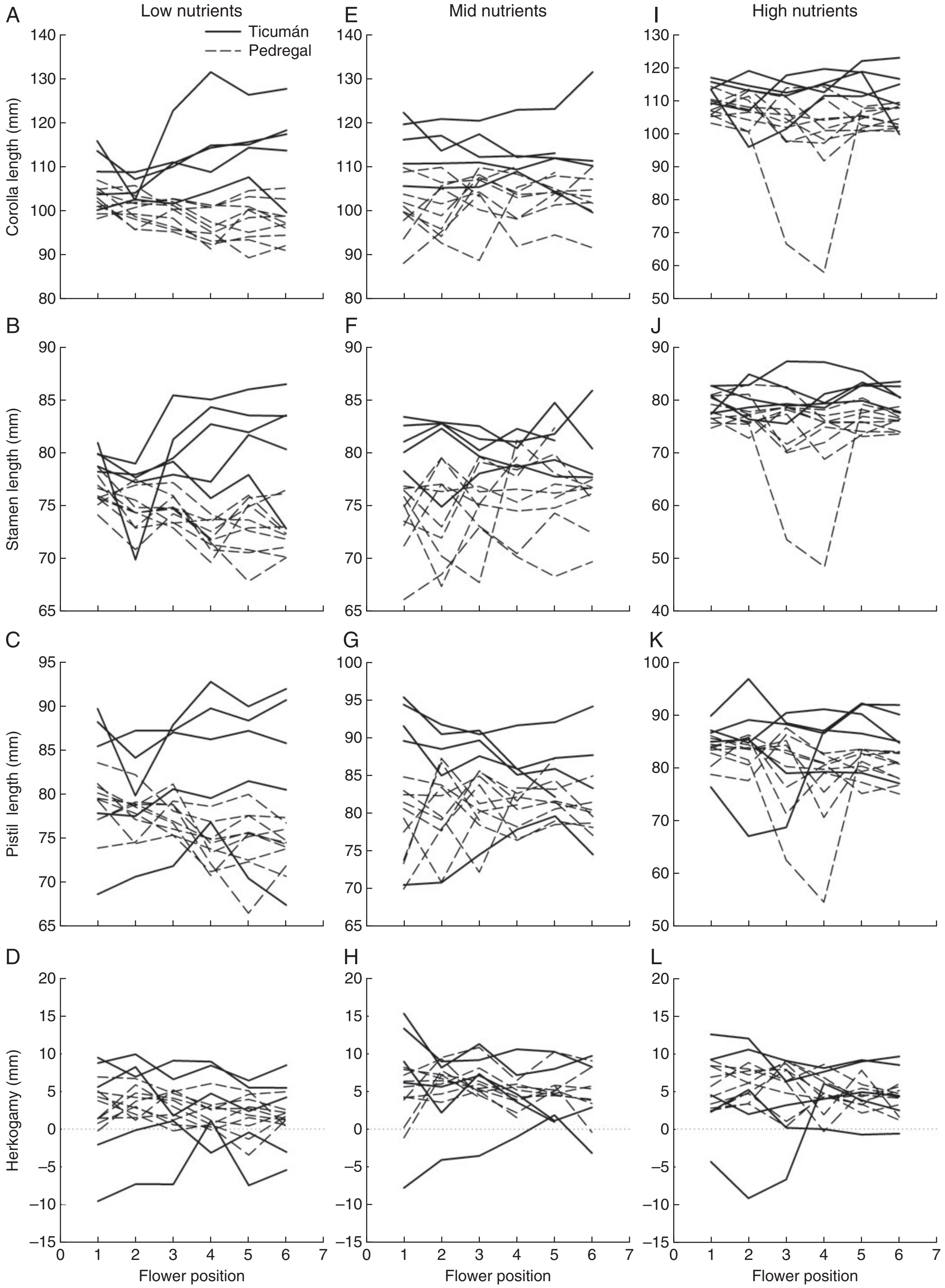

FIG. 3. Flower-level developmental reaction norms for full-sib families of two populations of *Datura stramonium* grown in three nutrient environments. Genotypes from Ticumán and Pedregal populations are plotted. The zero level of herkogamy is indicated by the reference lines in (D), (H) and (L).

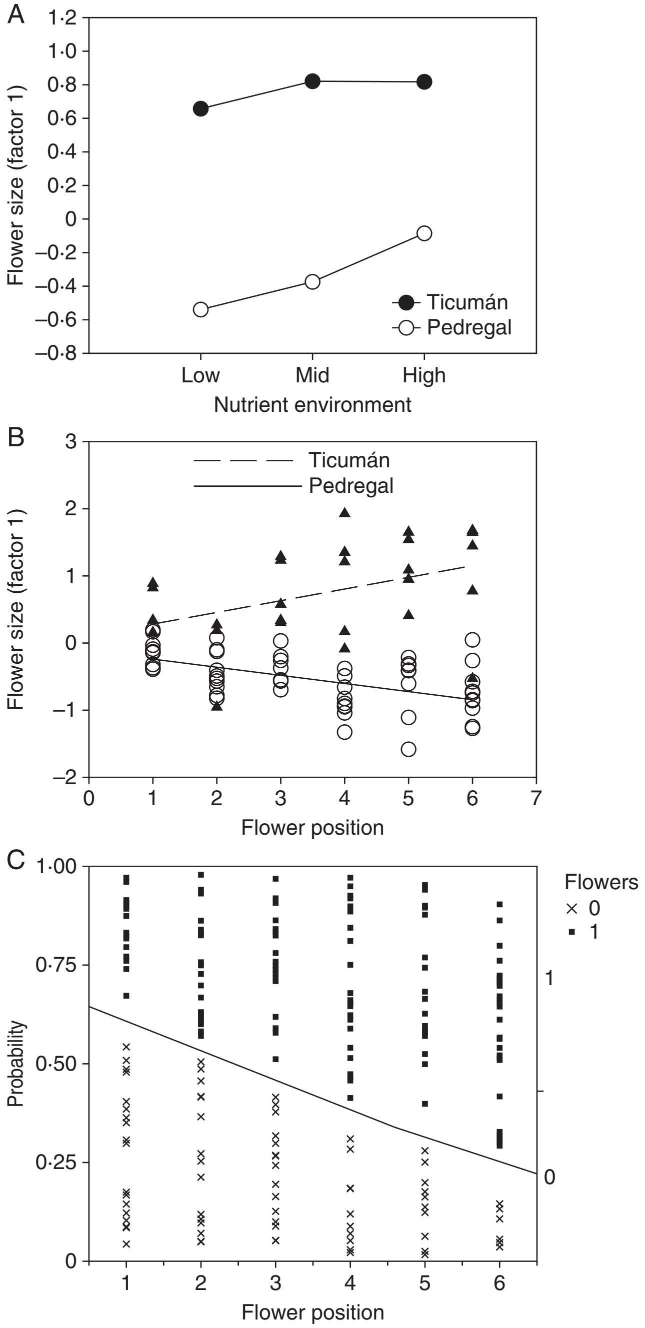

FIG. 4. Environmental and within-plant variation in flower size in two populations of *Datura stramonium*. (A) Population reaction norms to nutrient availability. (B) Among-population ($F_{1,\ 86} = 24{\cdot}26$, $P < 0{\cdot}0001$, $R^2 = 0{\cdot}67$, test of parallelism) differentiation (using mean family values) in systematic trends of within-plant variation in the low-nutrient environment. (C) Probability of producing self-fertilized flowers (squared symbols) in the low-nutrient environment for the Pedregal population using nominal logistic regression ($\chi^2_{1,\ 192} = 12{\cdot}44$, $P = 0{\cdot}0004$, full model). The line represents a sequential decrease in the probability of producing outcross flowers. 1 = selfed flowers, 0 = outcrossing flowers.

compared at equivalent positions of flowers allowed to set fruit (as in our design) (Diggle, 1997, 2003).

In *Datura stramonium*, the effect of flower position on herkogamy variation has not yet been measured, since the analysis of within-plant variation makes an intensive sampling necessary. Also, because not all flowers of a plant display the same phenotype, this might explain an absence of correlation between flower size and herkogamy in this species (e.g. Motten and Antonovics, 1992; Motten and Stone, 2000). However, it is well known that flower size is often affected by changing environmental conditions (Coleman *et al*., 1994; Pigliucci, 2001), resulting in reductions in herkogamy (Fishman *et al*., 2002). In *D. stramonium* we found that the ability to alter flower herkogamy is not independent of environmentally and architecturally induced flower size (cf. *Eichornia paniculata*; Vallejo-Marín and Barrett, 2009). Furthermore, we have shown that populations of *D. stramonium* with different ecological histories may differ in genetic variation for within-plant trajectories, and how the environment alters these. Examination of these trajectories revealed that whole-plant phenotypic plasticity is driven by different within-plant trajectories between populations. In the following, we will discuss our results in terms of changes in nutrient availability, stressing the differences between the two populations examined.

TABLE 3. *Principal component analysis for length factor in two populations of* Datura stramonium *growing in three levels of nutrient availability*

Ticumán				Pedregal		
Trait	Low	Medium	High	Low	Medium	High
Corolla	0·96	0·870	0·89	0·90	0·96	0·94
Stamen	0·98	0·930	0·94	0·97	0·98	0·98
Pistil	0·74	0·620	0·66	0·73	0·80	0·85
Herkogamy	–0·03	0·052	0·12	0·05	–0·01	0·05
% variance	60·85	50·41	53·59	56·91	62·85	64·30

Genetic and environmental effects on flower-level developmental trajectories

Our results show that *D. stramonium* displays a high degree of among-population differentiation. More genetic variation was detected for the Ticumán population, in contrast to the low level of genetic variation in the Pedregal population. Phenotypic plasticity was higher in the Pedregal population and it had significant genetic variation. In contrast, the Ticumán population is less plastic, and genetic variation for plasticity is low, except for herkogamy that showed a marked pattern of plasticity (Table 2; Fig. 2). Flower length was a major determinant of differences between populations; flowers are larger in Ticumán but all other traits followed this pattern. Contrary to the expectations of a consistent correspondence between sexual organs and pollinator positioning (i.e. uniform morphological expression of floral traits) necessary to ensure effective pollen transfer (Lloyd and Web, 1992), floral traits in *D. stramonium* can respond to abiotic environments by means of a variable morphological expression.

The Ticumán population had greater genetic variation both at the whole-plant trait and at the flower position levels; major developmental variability in all environments across flower positions was detected (Fig. 3; see the perturbation of each family line with flower position). Failure to produce organ phenotypes that are closely consistent with those expected from a predetermined developmental and morphogenetic plan may contribute to within-plant variation in the characteristics of reiterated structures (Herrera, 2009). In this population, there was not a systematic within-plant trend of variation in the flower positions for all floral traits in all environments. In contrast to the Ticumán population, in the Pedregal population there was a modification of developmental trajectories by the environment and a directional trend of flower length and herkogamy in the low-nutrient environment.

In this study, the Ticumán population interacts with taxonomically diverse assemblages of mutualists that could differ in their preferences for a given floral trait; this would explain the genetic variation for flower position in this population where mutualists are more heterogeneous in their preferences, and the non-genetic variation for flower position of Pedregal where mutualists are most alike or their abundance is very low. It has been shown that populations that present different pollinator assemblages differ in the average flower phenotype (Johnson, 1997; Johnson and Steiner, 1997; Nattero and Cocucci, 2007). However, in other cases, population differentiation in flower characters could be due to factors related to geographic differences among localities or genetic drift (Dominguez *et al*., 1998; Boyd, 2000; Mascó *et al*., 2004).

Flower length and integration across environments

Pedregal flower length was influenced strongly by nutrient availability: flowers in low-nutrient environments were smaller in contrast to larger flowers in a high-nutrient environment (Table 2, Fig. 4A). This plasticity was not evident in the Ticumán population. Notwithstanding, population differentiation in mean flower length was marked, and the Ticumán population expressed the largest flower length. Developmental trajectories of this character showed a clear trend in the low-nutrient environment (Fig. 4B); the Pedregal population decreased its flower length with flower position as expected for the architectural pattern of *D. stramonium*, where a decrease in the length and primary width of successive modules is correlated with the size of leaves and hence with the magnitude of the carbon contributions to growth of organs placed in those modules (Hallé *et al*., 1978; Herrera, 2009). Reproductive sinks are mainly supplied by their local source leaves (Marshall, 1996). Conversely, the Ticumán population showed a trend of increasing flower size with flower position. These opposite trends exemplify how different developmental trajectories determine mean whole-plant responses. Clearly, an increase in size in the Ticumán population favoured higher developmental variability, whereas a decrease in flower size in the Pedregal population favours lower developmental variability that is correlated with a tendency to reduce herkogamy (slope $= -0{\cdot}34 \pm 0{\cdot}15$, $F_{1,\,58} = 5{\cdot}04$, $P = 0{\cdot}0286$, $R^2 = 0{\cdot}08$), and hence with an increase in the probability of producing selfing flowers (Fig. 4C). Increases in flower size related to greater plant size due to increases in nutrient availability have been reported more often (Kagaya *et al*., 2009).

Phenotypic and genetic correlations among floral traits are expected to occur because of shared developmental pathways (Krizek and Fletcher, 2005) and strong stabilizing selection for floral integration (Armbruster *et al*., 2004). In this study, flower size (length factor) was correlated with all traits except herkogamy; this suite of correlated characters is maintained in the two populations regardless of environmental variation, and they participate in a common function that responds as a whole to flower position (mostly in the low-nutrient environment) (Table 3; Fig. 4B).

We interpret the maintenance of this suite as evidence of common developmental regulation of correlated characters (e.g. Bissell and Diggle, 2008). Since herkogamy was not correlated with length factor, the influence of size is clearly accomplished by means of its correlation with pistil and stamens, which showed a strong association with corolla (Fig. 3). This positive association of pistil and flower size suggests that a reduction in herkogamy is associated with smaller flowers in the Pedregal population. Also, despite the strong correlation of floral parts (i.e. corolla, pistil and stamen lengths), their plastic responses are not equal. In fact, slopes for within-plant trends differ for stamen and pistil lengths, with sequential reduction in pistil length being greater than that for stamens (see Results ‘Genetic and environmental effects on the traits of individual modules’). Thus, pistil height reduction in the low-nutrient environment drives a reduction in herkogamy and increases the probability of self-fertilization (cf. flower and flower by treatment interaction in Table 2). Other studies have shown the opposite for *D. stramonium* (Motten and Antonovics, 1992; Motten and Stone, 2000) and *E. paniculata* (Barrett *et al*., 2009), i.e. no correlation between an estimate of flower size (i.e. corolla or perianth) and herkogamy. However, a more precise estimate of flower size should have the length of all floral organs involved (factor 1 or length factor in Bissell and Diggle, 2008), since, from a morphometric point of view, it is the correlation of these characters that properly measures flower size. Notwithstanding, the latter could not be the case, at least for *E. paniculata*, because during the early stages of the establishment of selfing, the association between perianth and herkogamy does not occur and the stigma–anther separation can be altered independently of changes in flower size (Vallejo-Marín and Barrett, 2009). In fact, the association between herkogamy and perianth size occurs in other groups where reductions in herkogamy are often associated with the evolution of small flowers (e.g. *Mimulus*; Fishman *et al*., 2002). The perianth seems a good estimator of flower size in these groups.

Implications for the mating system

Phenotypic plasticity is a strategy by which individual plants may adjust to environmental heterogeneity (Bradshaw and Hardwick, 1989). Heterogeneity in nutrient availability could be a major regulator of the population dynamics of some herbaceous plant species (Lewis and Probyn, 1978; Thompson, 1994). Depending on the timing of germination and on their proximity to the parent, seedlings of short-lived plant species can be susceptible to the effect of nutrient depletion provided by the litter of the mother plant, and therefore can find themselves in a rich- or in a poor-nutrient environment, which induces major phenotypic changes in terms not only of plant size but of architecture as well (Thompson, 1994). The Pedregal population locality has volcanic thin soils, with a very irregular topography, which creates heterogeneity in the deposition of soil materials as nutrients (Cano-Santana and Meave, 1996). Therefore, phenotypic plasticity could be selected in the Pedregal population to cope with these heterogeneous soil conditions. Variation in the rate of self- and cross-fertilized flowers in this population resulting from plasticity could allow individuals to match current environmental conditions. The increase in self-fertilization in the Pedregal population could be favoured in low-nutrient environments where low abundance of pollinators favours the decrease of cross-pollination. Other studies have reported an increase of selfing in stressful environments (Stebbins, 1957; Elle and Hare, 2002; Elle, 2004; Moeller and Geber, 2005; Vallejo-Marin and Barrett, 2009).

The potential for evolutionary change in the mating system of extensively self-fertilized species such as *D. stramonium* may depend in part on the relative fitness of selfed and outcrossed progeny under different environmental conditions (Motten and Antonovics, 1992). If increased selfing results in mostly homozygous lines in this species, most individuals in a population will thus be largely purged of deleterious recessive alleles and, as a result, the level of inbreeding depression will be decreased (Lande and Schemske, 1985; Yahara, 1992). If this is true for *D. stramonium*, two scenarios could be considered: (1) that outcrossing in a population would eventually be eliminated (Fisher, 1941; Jain, 1976; Wells, 1979; Charlesworth, 1980); or (2) that some level of outcrossing might be favoured if subsequent rounds of outcrossing progeny of homozygous lines generate a very large amount of genetic variability and novelty (Allard *et al*., 1968; Motten and Antonovics, 1992).

Within-individual variation may constitute an adaptive strategy to produce variable progeny, in which individuals produce more than one functional class of reproductive organs (Lloyd, 1984; Herrera, 2009). Given the low levels of outcrossing reported in *D. stramonium*, the species has been hypothesized to represent a persistent mixed mating system rather than a transition to complete selfing (Motten and Antonovics, 1992; Cuevas, 1996; Motten and Stone, 2000). However, the former may occur in populations where long periods of increased pollinator abundance favour the increase of a non-systematic within-plant trend of variation (cf. fig. 6.4b in Herrera, 2009), such as the Ticumán population, which showed genetic variation for almost all traits in relation to flower position. This could lead to adaptive levels of within-plant variance, as it is supported by predictions from genetic models of the adaptive consequences of selection on environmental components of phenotypic variance (Bull, 1987; Zhang, 2005). Conversely, a transition to complete selfing instead of a mixed strategy may be possible in *D. stramonium* if more stressful environments where either biotic (e.g. low pollinator abundance) or abiotic conditions (e.g. nutrient availability causing a reduced flower size) favour the fixation of a systematic within-plant trend of variation that provide reproductive assurance (Darwin, 1878), such as in the Pedregal population. Future field studies are required to investigate in depth whether within-plant variation of herkogamy-related traits in *D. stramonium* has the potential for evolutionary change of the mating system under different ecological conditions and hence if it has any adaptive value.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: median values (first to third quartile) of selected floral traits based on mean family values of six sequential flower positions in 817 flowers of two populations of *Datura stramonium* grown in low, mid and high nutrient availability. Table S2: phenotypic correlation coefficients between floral traits, and flower integration indexes for three nutrient environments in two populations of *Datura stramonium*. Table S3: comparisons of integration pattern among nutrient environments both within and among populations. Each value corresponds to the correlation coefficient between correlation matrices.

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APPENDIX

To determine the level of association of the floral phenotype among environments, we calculated the associations between floral traits in each population with the Pearson product–moment correlation coefficient at the phenotypic level in each environment (Supplementary Data Table S2).

The phenotypic integration level of floral traits for each nutrient environment, in each population, was assessed using the variance of eigenvalues of the corresponding correlation matrix (Wagner, 1984). Because treatments differed in sample size, corrected INT values were estimated for the purpose of comparison among nutrient environments and populations. Standard errors and confidence intervals of each INT were obtained by bootstrapping (Cheverud *et al*., 1989; Herrera *et al*., 2002). To compare the integration patterns among nutrient environments and populations, we performed pairwise comparisons among all correlation matrices using Mantel test (Baker and Wilkinson, 2003; Pérez *et al*., 2007). Standard errors were obtained by bootstrapping (Supplementary Data Table S3).