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RESTRICCIONES GENÉTICAS Y AMBIENTALES SOBRE LA EVOLUCIÓN DE LAS DEFENSAS EN PLANTAS ANTE SUS ENEMIGOS NATURALES.

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Me permito informar a usted, que el Subcomité de Biología Evolutiva y Sistemática, en su sesión ordinaria del día 04 de marzo de 2013, aprobó el jurado para la presentación de su examen para obtener el grado de **DOCTOR EN CIENCIAS**, del Posgrado en Ciencias Biológicas, del alumno **CARMONA MORENO BELLO DIEGO** con número de cuenta **95187228** con la tesis titulada: titulada **"RESTRICCIONES GENÉTICAS Y AMBIENTALES SOBRE LA EVOLUCIÓN DE LAS DEFENSAS EN PLANTAS ANTE SUS ENEMIGOS NATURALES"**, realizada bajo la dirección del **DR. JUAN ENRIQUE FORNONI AGNELLI:**

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

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"[…], es como un gran árbol robusto y sano. La raíz es impermantente y de naturaleza mutable, el tronco es impermanente y de naturaleza mutable, las ramas y las hojas son impermanentes y de naturaleza mutable, la sombra es impermanente y de naturaleza mutable. Si alguien dijera: "la raíz, el tronco, las ramas y las hojas son impermanentes y de naturaleza mutable, pero lo que es la sombrea, ésa es permanente, perpetua, eterna de naturaleza inmutable". ¿Diría bien, quien dijera esto? […]"

Majjhuma Nikaya

Sermón sobre la instrucción de Nandaka (n° 146)

"[…] Así, la mente no está dominada por su tendencia mecánica a aferrarse a conjuntos de categorías fijas y limitadas, ni por reacciones automáticas que son las que en realidad provocan la tendencia a aferrarse inalterablemente a las mencionadas categorías fijas y limitadas. Cada vez que se presente un problema difícil, la mente será capaz, si es necesario, de abandonar las antiguas categorías y crear nuevas formas de revelación racional e imaginativa, útiles para guiar el pensamiento hacia nuevas líneas que puedan ser necesarias para resolver el problema."

David Bohm

Sobre la Creatividad

Esta tesis es dedicada a mi propio Urizen

y, por otro lado, a mi padre.

A ambos con profundo amor.

ÍNDICE

Resumen

La defensa de las plantas ante el daño de los herbívoros es un complejo de atributos, sin embargo, tradicionalmente los estudios la describen como un solo atributo. Paralelamente, la evolución de los caracteres defensivos se ha estudiado principalmente bajo escenarios ecológicos donde las plantas son consumidas por una especie de herbívoro. Ambas aproximaciones no son realistas dada la complejidad del fenotipo defensivo y la importancia de las interacciones multiespecíficas en las comunidades. En esta tesis se propone estudiar de manera explícita el proceso microevolutivo en ambientes más realistas. En el primer capítulo se exploró cómo el incrementar de manera muy sutil la complejidad del ambiente ecológico al manipular dos herbívoros folívoros (***Lema daturaphila*** y ***Epitrix parvula:*** Chrysomelidae), cada uno con su propia fenología de ataque e historia coevolutiva con la planta hospedera, puede explicar la presencia de las dos estrategias defensivas generales: la resistencia y la tolerancia. En particular se exploró si la funcionalidad defensiva (***i.e.*** redundancia/complementariedad) de la expresión simultánea de la resistencia y tolerancia puede ser alterada cuando se manipulan dos de las principales especies de herbívoros que atacan a ***Datura stramonium*** (Solanaceae) en una población particular. A partir de una parcela experimental en donde se condujo un experimento de genética ecológica pudimos detectar cambios en el valor adaptativo de ambas estrategias defensivas y cómo en ciertas circunstancias la selección favorece la expresión simultánea de este par de estrategias defensivas, que en un principio se pensaban redundantes. En el segundo capítulo se mostró que no sólo la manipulación de herbívoros altera el valor adaptativo de las estrategias defensivas, sino que también modifica el valor adaptativo de atributos de historia de vida y la expresión de la estructura genética (la matriz **G**), confirmando la complejidad del fenotipo defensivo. En este caso los análisis revelaron que, a pesar de haberse detectado cambios en los gradientes de selección sobre algunos de estos atributos de manera independiente, el vector de los gradientes de selección (***i.e.*** el vector resultante obtenido de considerar simultáneamente todos los gradientes de selección) no fue alterado por la manipulación del ambiente, sin embargo, el vector de la respuesta a la selección sí. Esta discrepancia es evidencia de que la matriz de varianza-covarianza también es

propensa a cambiar a esta escala microevolutiva. Este resultado deja abierta la pregunta sobre la estabilidad de la matriz **G** y subraya la importancia de considerar ciertas fluctuaciones ambientales a la hora de evaluar el valor predictivo de la ecuación que predice la respuesta a la selección (i.e. la ecuación del criador), cuando es aplicada en un condiciones naturales o semi-naturales, como es el caso de nuestra parcela de experimentación. En el tercer y último capítulo de esta tesis se busca responder qué atributos de las plantas predicen mejor el nivel de resistencia en contra de los herbívoros. Por medio de un metaanálisis (controlando la señal filogenética) basado en 72 estudios (19 familias de plantas) y 499 correlaciones genéticas entre diversos atributos de las plantas y su nivel de resistencia, se pudo mostrar que la susceptibilidad de las plantas al daño por hebívoros no disminuye conforme se incrementa la concentración de los metabolitos secundarios. Por otro lado, esta susceptibilidad sí se ve afectada por atributos de historia de vida (ej. tasa de crecimiento, tiempo a la reproducción), morfología gruesa de la planta (ej. número de ramificaciones, tamaño de la planta) y atributos físicos de resistencia (ej. tricomas, látex). Al final del capítulo se propone la hipótesis de que la selección natural generada por los herbívoros actúa con mayor fuerza sobre las estrategias de historia de vida y atributos físicos de las plantas, que sobre los metabolitos secundarios. No obstante, debido a que las restricciones genéticas de estos últimos pueden ser de menor intensidad que en los atributos de historia de vida o morfología, es posible que la importancia defensiva de los metabolitos secundarios radique no en su gran efecto sobre los herbívoros, sino en la debilidad de sus restricciones genéticas.

Summary

Plant defense against herbivory damage is a complex attribute; however, traditionally studies describe the defense as a single attribute. In parallel, the evolution of defensive traits has been studied mainly in ecological scenarios where plants are consumed by a single herbivore species. Both approaches are not realistic given the complexity of the defensive phenotype and the importance of multispecific interactions in communities. This thesis highlights the importance of explicitly study the micro evolutionary process under more realistic environments. The first chapter explores how a very subtle increase in the complexity of the ecological environment by the manipulation of two herbivores (***Lema daturaphila*** y ***Epitrix parvula:*** Chrysomelidae), each with their own phenology of attack and coevolutionary history with the host plant, may explain the presence of the two general defensive strategies: resistance and tolerance. In particular, we explored whether the defensive functionality (***i.e.*** redundancy / complementarity) of the simultaneous expression of resistance and tolerance can be altered when handling two species of herbivores that, in a particular population, attack ***Datura stramonium*** (Solanaceae). In an experimental plot an experiment was conducted in order to detect ecological changes in the adaptive value of both defensive strategies and ask if such changes favor the simultaneous expression of this pair of defensive strategies, defensive strategies that initially were thought as mutually exclusive. In the second chapter we show that not only manipulation of herbivores alters the adaptive value of defensive strategies but also modify the adaptive value of life history attributes and expression of genetic structure (matrix **G**) confirming the notion of a complex defensive phenotype (chapter I). In this case the analysis revealed that despite the fact of having detected changes in the selection gradients on some traits such alterations were not observed when the vector of selection was analyzed. However, we do detect changes in the vector of the response to selection. This discrepancy is evidence that the variance-covariance matrix is also prone to alterations at a microevolutionary scale. This result address the question about the stability of the matrix **G** and stresses the importance of considering certain environmental fluctuations when assessing the

predictive value of the breeder's equation, especially when applied in a natural or semi-natural conditions. In the last chapter of this thesis we seeks to answer what attributes of plants predict better the level of resistance against herbivores. Through a meta-analysis (controlling the phylogenetic signal) based on 72 studies (19 plant families) and 499 genetic correlations between various plant traits and the level of resistance, we detected that the susceptibility of plants to herbivores damage is not correlated with the amount o presence/absence of secondary metabolites. Furthermore, this susceptibility was affected by life history attributes (eg. growth rate, the reproduction time), gross morphology of the plant (number of branches, size of the plant) and sometimes by physical attributes traditionally related with resistance (eg. trichomes, latex). At the end of the chapter we propose the hypothesis that natural selection generated by herbivores is stronger on life history strategies and physical attributes of plants than on the secondary metabolites. However, because the genetic constraints of the latter may be less intense than in the former attributes it is possible that the defensive importance of secondary metabolites lies not in its great effect on herbivores, but because the constraints on their evolution are the weakest.

INTRODUCCIÓN GENERAL

“La teoría de evolución por selección natural es una teoría ecológica basada en observaciones ecológicas de, quizás, el más grande ecólogo de todos los tiempos [Charles Darwin]”, en 1967, con esta frase, J. L. Harper abría su plática ante la Sociedad Ecológica Británica, señalando la interdependencia que existe entre el estudio de la evolución adaptativa y la ecología. Esta percepción estaba clara entre los ecólogos de inicios del siglo XIX y XX (Futuyma 1986), sin embargo, a lo largo de la historia la cercanía entre ambas disciplinas no siempre fue estrecha. Inicialmente, la ecología se veía como la aproximación necesaria para explicar la evolución adaptativa (McIntosh en Collins 1986). De hecho, en 1900 se vislumbraba que el papel fundamental de la ecología en la biología moderna debía ser el de revelar los misterios de la adaptación (Cowles en Collins 1986). Empero, la ecología comenzó a enfocarse en el funcionamiento de las adaptaciones en lugar de en sus causas últimas (***i.e.*** evolutivas), favoreciendo en muchos casos la concepción de que la ecología era una mera extensión de la fisiología (Cittadino en Collins 1986; Futuyma 1986). Aun así, la sutil relación entre la ecología y evolución se mantuvo gracias al trabajo conjunto de los ecólogos y taxónomos. Por ejemplo, Clements y el botánico Harvey Hall, estaban interesados en los mecanismos evolutivos que actúan en las poblaciones naturales y esperaban que los protocolos de trasplantes diseñados por Gaston Bonnier les permitieran demostrar que las especies trasplantadas evolucionarían, pareciéndose a las especies nativas (Kohler 2002).

A pesar de que en 1924 Brues ya sugería el proceso coevolutivo entre plantas e insectos en su artículo “The specificity of food plants in the evolution of phytophagous insects”, el interés por descubrir las causas últimas de este tipo de patrones (***i.e.*** evolutivas) se vio mermado por un cierto desdén entre los ecólogos por el proceso de selección natural (Futuyma 1986). Mientras tanto, un profundo interés por entender cómo las poblaciones se regulaban, enfocó la atención de los ecólogos en estudiar los procesos denso-dependientes (Orians 1962), dejando en segundo término la importancia del factor genético como determinante en la regulación y estructuración de las poblaciones (Collins 1986). De hecho, aunque la ecología mantuvo la distancia necesaria para no figurar dentro de la síntesis neo-Darwiniana en los años 30 y 40, la evidencia empírica generada por el grupo de Clausen, Keck y Hiesey y otros fue fundamental para aportar bases solidas a esta síntesis (Núñez-Farfán y Schlichting 2001). Sin embargo, fue a finales de los 50 e inicios de los 60 que la ecología evolutiva cobró forma como área de estudio, gracias al trabajo de personajes como Lack, Cain, Sheppard, Kettlewell, Orians, Ford, Lerner, Dobzhansky y el equipo de Clausen y compañia . En conjunto, mostraron cómo cambios genéticos y morfológicos se correlacionaban con cambios en las características del hábitat, características o factores que fueron concebidos como agentes de selección. Este tipo de observaciones enfatizaron la importancia de la heterogeneidad ambiental como mecanismo que puede favorecer la evolución y el mantenimiento de los polimorfismos (Collins 1986).

La heterogeneidad ambiental ocurre tanto a escalas espaciales como temporales, y de hecho puede ser enmarcada dentro de lo que en 1930 Fisher llamó “***degradación ambiental”*** en su libro ***Genetical Theroy of Natural Selection***. Dicho autor

señaló que este constante cambio en el ambiente, tanto biótico como abiótico, impacta negativamente la adecuación poblacional de las especies al alterar la relación que guardan con los picos adaptativos, por lo que lo denominó ***"deterioro ambiental"***. Para Fisher, el cambio en el ambiente biótico es el más significativo ya que es producido por la misma evolución de las especies (Fisher 1930): "[…] probablemente más importante que el cambio en el clima son los cambios evolutivos en progreso entre organismos asociados. Conforme la adecuación de cada organismo se incrementa, la de sus enemigos y sus competidores también aumenta (página 42 de Fisher 1930) [la base de la hipótesis de la reina roja de Van Valen 1973] lo que tendrá un efecto degradante derivado del proceso biótico, un efecto de mayor impacto desde el punto de vista de cada organismo involucrado […]". En esta frase Fisher reconoce la importancia de un ambiente multiespecífico en constante cambio, y asume la complejidad de modelar la dinámica evolutiva de las especies cuando el fenotipo en evolución enfrenta un paisaje adaptativo que no es constante (a diferencia de como lo veía Wright; interpretación de Lewontin 1978 y Pigliucci y Kaplan 2006; pero ver página 362 en Wright 1932), al ser modificado por la continua degradación del ambiente. Tomando en cuenta este importante señalamiento teórico varios investigadores comenzaron a estudiar la evolución de interacciones sencillas (eg. Lerner y Dempster, 1962), pero no fue hasta 1964 (el mismo año en que Ford publicara su libro de Ecological Genetics) que los evolucionistas P. R. Ehrlich y P. H. Raven (1964)-el primero especializado en mariposas y el segundo en plantas- propusieron un mecanismo (ya antes considerado Flor 1955, 1956 en Mode 1957) que permite entender cómo evolucionan las especies ante la degradación ambiental generada por la propia interacción interespecífica entre

organismos: la coevolución. De esta manera el patrón de “especies relacionadas de insectos alimentándose de especies emparentadas de plantas” observado 40 años atrás por Brues, pudo tener una hipótesis sobre las causas últimas hecha a la medida (Spencer 1988).

Desde la aproximación microevolutiva, la hipótesis coevolutiva hace explícita, por primera vez, la necesidad de estudiar la interfase dinámica entre las interacciones ecológicas y el proceso evolutivo bajo selección natural. Sin embargo, dado que las observaciones de Ehlrich y Raven (1964) fueron hechas de manera comparativa entre especies, se instauró la moda de estudiar las relaciones filogenéticas entre grupos de organismos que aparentemente se encontraban en coevolución (Gould 1988). Esto favoreció la generación de una visión macrocoevolutiva del fenómeno, pero descuidó el estudio del proceso coevolutivo a escala microevolutiva (Gould 1988; pero ver Olson ***et al.*** 1974). A pesar de que la hipótesis coevolutiva fue planteada en 1964, no fue hasta 1980 que Janzen la definió formalmente y resaltó dos tipos de procesos coevolutivos: 1) coevolución (que a lo largo de la tesis será referida como coevolución pareada) es “el cambio evolutivo de un atributo fenotípico de una especie en respuesta al cambio en otro atributo fenotípico de otra especie, este segundo atributo de igual manera responderá al cambio evolutivo del primer atributo” y 2) coevolución difusa, cuando “una o ambas poblaciones son representadas por un conjunto de poblaciones que generan presiones selectivas como un grupo”. Es importante resaltar que en esta definición Janzen no describe el tipo de patrón macro evolutivo que debiera observarse bajo un escenario coevolutivo pareado, sino describe, en sí, el proceso coevolutivo (muy escasamente estudiado; ver Garrido ***et al.*** 2012). La primera definición encontró

rápidamente buena aceptación (Kiyosawa 1982; Roughgarden 1983; Kiester ***et al.*** 1984), mientras que el estudio de la coevolución difusa se vio restringido por la falta de criterios metodológicos para definirla y por complicaciones logísticas que hacían difícil su estudio (Gould 1988). Retomando la noción de la degradación ambiental de Fisher (1930), la coevolución pareada asume que la degradación del ambiente de un par de especies interactuantes depende únicamente de ellas. Es decir, asume que las presiones de selección recíprocas entre un par de especies que interactúan ecológicamente son independientes del contexto ambiental biótico donde la interacción tiene lugar. Este supuesto es poco probable biológicamente y, por lo tanto, poco realista (Futuyma y Slatkin 1983), Mientras tanto, la coevolución difusa considera que dicha degradación ambiental es resultado de las interacciones entre múltiples especies. Este tipo de coevolución, también denominada coevolución multiespecífica, fue señalado por varios como un proceso más común (realista) que la coevolución pareada (Fox 1981, 1988; Futuyma y Slatkin 1983; Gould 1988; Levin ***et al.*** 1990) pero con serias complicaciones para ser estudiado (Hougen-Eitzman y Rausher 1994) y algunas más para ser modelado (Levin ***et al.*** 1990; Bergelson ***et al.*** 2001).

Finalmente, Rausher y colaboradores (Hougen-Eitzman y Rausher 1994; Iwao y Rausher 1997; Stinchcombe y Rausher 2001, 2002), en la década de los 90 y principios del nuevo siglo, definieron varios criterios bajo los cuales se podía distinguir la coevolución difusa de la pareada. Estos trabajos, así como subsiguientes investigaciones, se enfocaron principalmente en distinguir el tipo de coevolución que ocurría entre especies interactuantes (revisado en Strauss ***et al.*** 2005). Sin embargo, desde el punto de vista de esta tesis, se busca resaltar que la importancia de estos

criterios va más allá de poder distinguir el tipo de coevolución. Estos criterios permiten iniciar un protocolo de investigación para entender el proceso micro (co)evolutivo en un contexto multiespecífico, es decir cuando la degradación ambiental es producida por el componente biótico (Fisher 1930). Más aún, permite explorar las causas (agentes) de la selección natural, de las cuales aún se tiene un profundo desconocimiento sobre su naturaleza (McCall 2011). Finalmente, si esto deriva en la generación de patrones macro (co)evolutivos pareados o no, debe ser evaluado desde otra aproximación.

En particular, la formulación de estos criterios está dada en términos de la ecuación del criador (presentada por primera vez, la versión univariada, en Lush (1934) pero utilizando la versión multivariada de dicha ecuación: $\mathbf{\Delta z} = \mathbf{G} \times \boldsymbol{\beta}$ (Iwao y Rausher 1997). Donde $\boldsymbol{\beta}$ es el vector de selección (conformado por $\boldsymbol{n}$ número de gradientes de selección), **G** es la matriz de varianza-covarianza genética formada por todos los atributos sujetos a la acción de la selección y $\mathbf{\Delta z}$ es el vector de respuesta a la selección (Iwao y Rausher 1997; Strauss ***et al.*** 2005). Esta ecuación, que inicialmente fue diseñada para predecir la respuesta a la selección bajo esquemas de selección artificial y de mejora de especies domesticadas (Lush 1934), nunca fue utilizada para plantear (hasta ahora) hipótesis que permitieran entender el proceso evolutivo adaptativo de las especies en un ambiente cambiante. En realidad, la inquietud original con respecto al ambiente era encontrar las condiciones óptimas (para luego mantenerlas constantes) que favorecerían la expresión de los rasgos a seleccionar y así, mejorar los fenotipos de interés comercial (Hammond 1947 en Daday ***et al.*** 1973). De esta manera, para demostrar la importancia de las interacciones ecológicas en la evolución de los fenotipos, es necesario manipular experimentalmente la

presencia/ausencia de especies que presuntamente pudieran ser agentes selectivos o alterar la expresión de las varianzas y covarianzas genéticas (Hoffman y Merilä 1999; Stinchcombe ***et al.*** 2012). A grandes rasgos, el empleo combinado de esta aproximación multivariada con el diseño experimental de exclusión de terceras especies, permite evaluar si los componentes de la respuesta a la selección son alterados por cambios en el ambiente (***i.e.*** **G** × **E** y **β** × **E**), promoviendo una evolución difusa o multiespecífica (***i.e***. **Δz** × **E**) y, de ser el caso, determinar las causas ecológicas que promueven tales modificaciones. De hecho, estudiar el proceso micro (co)evolutivo de esta manera, permite determinar experimentalmente (y no a partir de la inferencia de patrones macroevolutivos) uno de los supuestos más importantes de la teoría coevolutiva (Janzen 1980): la ocurrencia de selección recíproca entre especies interactuantes (ver Garrido ***et al.*** 2012).

El diseño experimental básico sobre el cual se aplican los criterios que a continuación se describen, consta de un par de especies focales (ej. A ***vs***. B) y al menos una tercera especie (o especie auxiliar; C). El papel que desempeña cada especie en el análisis puede ser alternado dependiendo del diseño experimental con el que se trabaje (ej: A ***vs***. B y auxiliar C, A ***vs***. C y auxiliar B). De esta manera se pueden evaluar los cambios en los componentes a la respuesta a la selección de las especies focales cuando la presencia/ausencia de la tercera es manipulada experimentalmente. El primer criterio se basa en determinar la presencia de correlaciones genéticas entre atributos fenotípicos de una de las especies focales, cada uno asociado a la interacción con una diferente especie de interactuante (Hougen-Eitzman y Rausher 1994; Wise y Rausher 2013). La falta de dicha correlación entre estos atributos daría como resultado

la evolución independiente de cada rasgo, favoreciendo una (co)evolución pareada entre la especie focal y cada una de las especies interactuantes. El segundo criterio evalúa la correlación entre la expresión de un atributo en diferentes ambientes (presencia/ausencia de una tercera especie; Stinchcombe y Rausher 2001). Una correlación total (***i.e. r*** = 1) indicaría la ausencia de plasticidad fenotípica y por lo tanto, la respuesta a la selección se mantendría constante, favoreciendo un escenario de evolución pareada, ya que ni las varianzas ni covarianzas genéticas que conforman la matriz G se verían afectadas cuando se manipula la presencia/ausencia de la tercera especie. Por otro lado, correlaciones parciales (***r*** < 1) o la falta total de correlación (***r*** = 0) pueden favorecer patrones de (co)evolución difusa. De manera interesante, este criterio reta la concepción de que la matriz **G** es estable (Steppan ***et al.*** 2002; tema que se discute en el segundo capítulo). Estos dos primeros criterios claramente están vinculados con la matriz **G**, el primero considera que la estructura de correlaciones de **G** promueve una evolución difusa, mientras que el segundo reconoce que **G** puede ser alterada por el ambiente (***i.e.*** **G** × **E**). El último criterio analiza lo que ocurre con la selección natural en ambientes cambiantes, es decir, busca determinar la existencia de una interacción entre el ambiente y los patrones selectivos (**β** × **E**). Si los patrones de selección actuando sobre uno o varios atributos de la especie focal son alterados por la manipulación de una tercera especie, dos conclusiones se pueden hacer: a) la tercera especie es un agente selectivo y b) la selección es difusa y probablemente generará un cambio en la respuesta a la selección, favoreciendo un patrón de evolución difusa (Stinchombe y Rausher 2001).

Esta tesis en particular saca provecho de la aplicación del protocolo ya mencionado para explorar la importancia de incorporar ambientes ecológicamente más realistas para poder generar hipótesis sobre la evolución de los fenotipos. En esta tesis se usa como sistema de estudio la evolución de las estrategias defensivas y rasgos de historia de vida que las plantas usan en contra de sus herbívoros.

En el primer capítulo se evalúa si la comunidad de herbívoros puede seleccionar la presencia de estrategias mixtas defensivas (Herbivores can select for mixed defensive strategies in plants; Carmona and Fornoni 2013). Modelos teóricos basados en la teoría de defensa óptima (van der Meijden ***et al.*** 1988; Mauricio ***et al.*** 1997) señalan que ambas estrategias defensivas deberían de funcionar de manera redundante (***i.e.*** no complementarias) y, por lo tanto, no ser expresadas de manera simultánea por los fenotipos, no obstante, este patrón no se observa en la naturaleza. Individuos de poblaciones naturales expresan simultáneamente la resistencia y tolerancia en las poblaciones naturales, contradiciendo las predicciones teóricas. Aquí se propone la necesidad de manipular el ambiente selectivo (presencia/ausencia de dos especies de herbívoros) para determinar la importancia que tiene este factor en la evolución simultánea de las estrategias defensivas. De esta manera buscamos generar una hipótesis alternativa sobre el por qué no observamos lo que predicen los modelos que, dicho sea de paso, fueron construidos bajo la idea de un ambiente selectivo constante y no multiespecífico. En particular este capítulo se centra en el cambio de la superficie del paisaje adaptativo debido a la interacción $\boldsymbol{\beta} \times \mathbf{E}$ mejor conocida como: selección difusa o multiespecífica.

En el segundo capítulo (Understanding the causes of adaptive evolutionary changes in ecologically variable environments: the predictive power of the breeder's equation) se explora la respuesta defensiva de las plantas desde una perspectiva más integrada, tanto por la inclusión de atributos de historia de vida en el estudio de la defensa como por la aproximación estadística. En particular, el efecto de un ambiente multiespecífico sobre la evolución de la resistencia fue examinado en conjunto con la evolución de la tasa de crecimiento y de inicio de la reproducción, explorando los límites de la respuesta defensiva de los fenotipos. En cuanto a la aproximación estadística se propone “la prueba del trinche de asado” (carving fork analysis), que permite evaluar al mismo tiempo la importancia evolutiva que tiene el ambiente multiespecífico sobre **β** y **G** para determinar la evolución del fenotipo (**Δz**), en este caso del fenotipo defensivo. De esta manera, este método no sólo reconoce el protocolo de Rausher y colaboradores, sino que permite evaluarlo de manera simultánea, haciendo más accesible el probar hipótesis microevolutivas basadas en un contexto comunitario multiespecífico.

El último capítulo (Plant traits that predict resistance to herbivores; Carmona ***et al.*** 2011), inspirado en la idea de que el fenotipo defensivo es complejo, explora la pregunta sobre cuál es el atributo que predice mejor la resistencia de las plantas ante sus enemigos (Plant traits that predict resistance to herbivores). En particular este capítulo permite discutir, en el contexto de la tesis, si las hipótesis de estudio sobre cómo evoluciona una adaptación no sólo deben considerar un ambiente multiespecífico y un fenotipo multivariado, sino también el cómo delimitamos lo que es una adaptación.

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Research

Herbivores can select for mixed defensive strategies in plants

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Summary

- Resistance and tolerance are the most important defense mechanisms against herbivores. Initial theoretical studies considered both mechanisms functionally redundant, but more recent empirical studies suggest that these mechanisms may complement each other, favoring the presence of mixed defense patterns. However, the expectation of redundancy between tolerance and resistance remains unsupported.
- In this study, we tested this assumption following an ecological genetics field experiment in which the presence/absence of two herbivores (*Lema daturaphila* and *Epitrix parvula*) of *Datura stramonium* were manipulated. In each of three treatments, genotypic selection analyses were performed and selection patterns compared.
- Our results indicated that selection on resistance and tolerance was significantly different between the two folivores. Tolerance and resistance are not redundant defense strategies in *D. stramonium* but instead functioned as complementary defenses against both beetle species, favoring the evolution of a mixed defense strategy. Although each herbivore was selected for different defense strategies, the observed average tolerance and resistance were closer to the adaptive peak predicted against *E. parvula* and both beetles together.
- In our experimental population, natural selection imposed by herbivores can favor the evolution of mixed defense strategies in plants, accounting for the presence of intermediate levels of tolerance and resistance.

Introduction

Plants simultaneously allocate resources to two general defense mechanisms against herbivores and pathogens: resistance (the ability to avoid or reduce the probability of being eaten or infected) and tolerance (the ability to reduce the negative fitness effect of damage or infection once it occurs; Rosenthal & Kotanen, 1994; Strauss & Agrawal, 1999). Initial studies conceived both strategies as functionally redundant alternative defense mechanisms (van der Meijden *et al.*, 1988; Fineblum & Rausher, 1995), because the same fitness benefits can theoretically be obtained by each defense strategy alone. In turn, if both strategies are associated with fitness costs, a genotype should be either highly tolerant or resistant but not both (Abrahamson & Weis, 1997; Mauricio *et al.*, 1997). This would result in an adaptive surface with two alternative fitness peaks, one of maximum tolerance and minimum resistance and the other of maximum resistance and minimum tolerance (Fig. 1a). Although theoretical work has been shaped by researchers assuming defense strategies are redundant, redundancy has only rarely been supported in previous studies (Mauricio *et al.*, 1997; Tiffin & Rausher, 1999; Mauricio, 2000; Pilson, 2000).

Empirical evidence indicated that individual plants usually allocate resources to both strategies, expressing a range of combined patterns of defense allocation (mixed defense strategy; reviewed in Núñez-Farfán *et al.*, 2007; Fig. 1c,d). More recent theoretical efforts examined the conditions for varied mixed defense strategies within host populations (Fornoni *et al.*, 2004a; Restif & Koella, 2004). When the benefits of expressing both strategies simultaneously are higher than those gained when resistance and tolerance are expressed alone, these strategies represent complementary defense mechanisms (Fornoni *et al.*, 2004a). Hence, if both strategies are complementary rather than redundant, an adaptive landscape with a peak selecting for a mixed allocation pattern of defense will represent an adaptive strategy (Fig. 1c,d). Nevertheless, whether this pattern is truly favored by selection under natural conditions or represents transient states to complete allocation to either tolerance or resistance (Fig. 1a,b) remains unresolved.

The presence of intermediate levels of tolerance and resistance can also result from fluctuating selection pressures related to variations in the amount, types and/or identity of the species imposing damage (reviewed in Núñez-Farfán *et al.*, 2007). In particular, when the pattern of plant defenses selected against an enemy is altered by the presence/absence of an additional consumer, diffuse selection has been invoked as the underlying mechanism (Hougen-Eitzman & Rausher, 1994; Stinchcombe & Rausher, 2002; Strauss *et al.*, 2005). Fluctuating selection acting on the tolerance–resistance adaptive landscape could reflect changes in the extent of redundancy or complementarity between the two strategies and could explain the presence of mixed patterns of defense allocation (Fig. 1). Even when the adaptive

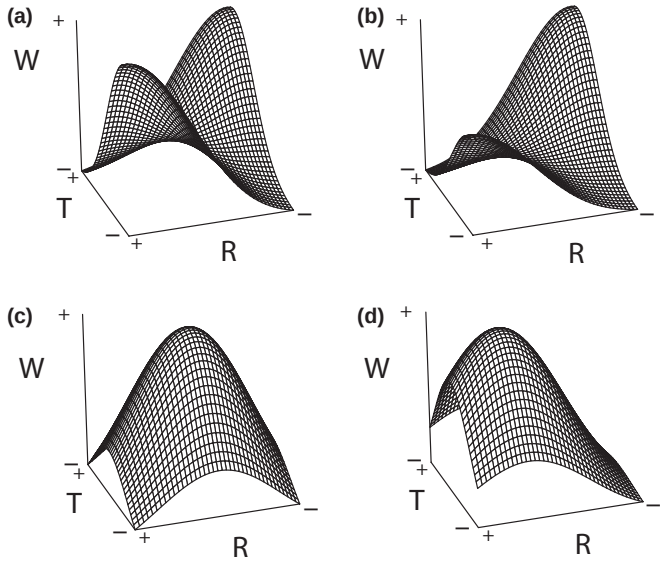

Fig. 1 Four hypothetical fitness (W denotes relative fitness) surfaces for the allocation to resistance (R) and tolerance (T). (a, b) Surfaces with two adaptive peaks and a valley of minimum fitness corresponding to intermediate levels of tolerance and resistance. (a) This surface corresponds to a scenario in which tolerance and resistance function as mutually exclusive alternatives, as maximum allocation to tolerance or resistance provides similar fitness benefits, but there are subadditive benefits of having intermediate levels of both strategies. (b) This surface corresponds to the situation in which the extent of the redundancy between tolerance and resistance is reduced because in this hypothetical example maximal allocation to tolerance provides more fitness benefits than maximum allocation to resistance. (c, d) Surfaces with one adaptive peak corresponding to a mixed pattern of allocation to tolerance and resistance (mixed defense strategy). These two panels differ in the position of the fitness peak and illustrate the range of hypothetical combinations of tolerance and resistance that correspond to a complementary defense strategy. When the presence/absence of different species of consumers accounts for qualitative or quantitative changes in the adaptive surface, diffuse selection can be invoked as a mechanism that can maintain the variation in plant defenses (Strauss *et al.*, 2005).

surface presents an intermediate peak, its position within the surface may be affected by changes in the selection pattern (Fig. 1c,d). Although diffuse selection has been demonstrated to act independently on tolerance (Stinchcombe & Rausher, 2002) and resistance (Pilson, 1996; Juenger & Bergelson, 1998; Stinchcombe & Rausher, 2001; Lankau, 2007), studies quantifying simultaneous selection on both strategies did not account for this source of variation (Tiffin & Rausher, 1999; Pilson, 2000; Fornoni *et al.*, 2004b). Thus, determining whether tolerance and resistance function as redundant or complementary defenses and whether they depend on the ecological context constitutes a major step in explaining observed mixed patterns of defense allocation. To understand how the effect of several natural enemies conditions the fitness consequences of the simultaneous expression of tolerance and resistance, we experimentally manipulated the presence/absence of different species of natural herbivores to gain a clear picture of the adaptive value of mixed defense strategies.

In a natural habitat, plants are likely to be eaten by diverse species, each with a particular eco-evolutionary dynamic with the host (Johnson & Stinchcombe, 2007). Generalist herbivores are usually more susceptible than specialists to plant secondary metabolites (Ali & Agrawal, 2012), a pattern consistent with the observed adaption of several specialized herbivores to specific toxic compounds (Metcalf, 1986; Shonle & Bergelson, 2000; Agrawal *et al.*, 2012). Whenever the benefits of resistance are reduced by the herbivores' adaptation, tolerance, as a more general response, could play a major role in the plant's defense strategy, as tolerance can provide fitness benefits in the presence of damage (Agrawal & Fishbein, 2008; Garrido *et al.*, 2012). Hence, when a given host is consumed by several natural enemies with different magnitudes of specialization, tolerance and resistance may function as complementary rather than redundant defense mechanisms. In this study, we performed an ecological genetic field experiment using controlled crosses in the annual herb *Datura stramonium* and two natural herbivores (*Lema daturaphila* and *Epitrix parvula*) to determine (1) the presence of genetic variation in tolerance and resistance against leaf damage; and (2) the pattern and intensity of genotypic selection in tolerance and resistance under combinations (presence/absence) of two natural enemies.

Materials and Methods

Study system

Datura stramonium L. (Solanaceae; jimsonweed) is an annual weed that frequently inhabits disturbed areas throughout North America (Valverde *et al.*, 2001). Within central Mexico, mean herbivore damage can fluctuate between 10% and 50% among populations (Valverde *et al.*, 2001), and 100% of leaf damage to individual plants can occur within populations (Núñez-Farfán & Dirzo, 1994). Plants from this species simultaneously express resistance by producing foliar trichomes and secondary compounds (tropane alkaloids; Shonle & Bergelson, 2000; Valverde *et al.*, 2001) and exhibiting tolerance (Fornoni *et al.*, 2004b). Both defense strategies are heritable, and selection in breeding values has been recorded in two natural populations (Fornoni *et al.*, 2003, 2004b).

In central Mexico, *D. stramonium* is attacked by two folivore beetles: *E. parvula* and *L. daturaphila* (Chrysomelidae) (Fig. 2). *E. parvula* can consume a broader spectrum of Solanaceous species, while *L. daturaphila* is mainly a specialized enemy of the genus *Datura* (Kogan & Goeden, 1970). *E. parvula* begins to consume the foliage tissue at the seedling stage leading to, in some populations, up to 23% leaf area loss at the end of the plant's life cycle (Núñez-Farfán & Dirzo, 1994; Valverde *et al.*, 2001; Fornoni *et al.*, 2003). By contrast, *L. daturaphila* tends to oviposit on plants near the onset of reproduction, removing up to 100% of the leaf area of individual plants during the larval stage (Núñez-Farfán & Dirzo, 1994). The study site was located in Teotihuacan, Mexico (19°47′27″N; 98°51′4″W, 2294 masl; mean annual precipitation: 559.6 mm; mean annual temperature: 14.8°C; Valverde *et al.*, 2001) within a xerophytic shrub community. At this site, an experimental plot (2048 m^2) was established and plowed to remove natural vegetation before the seedlings were transplanted.

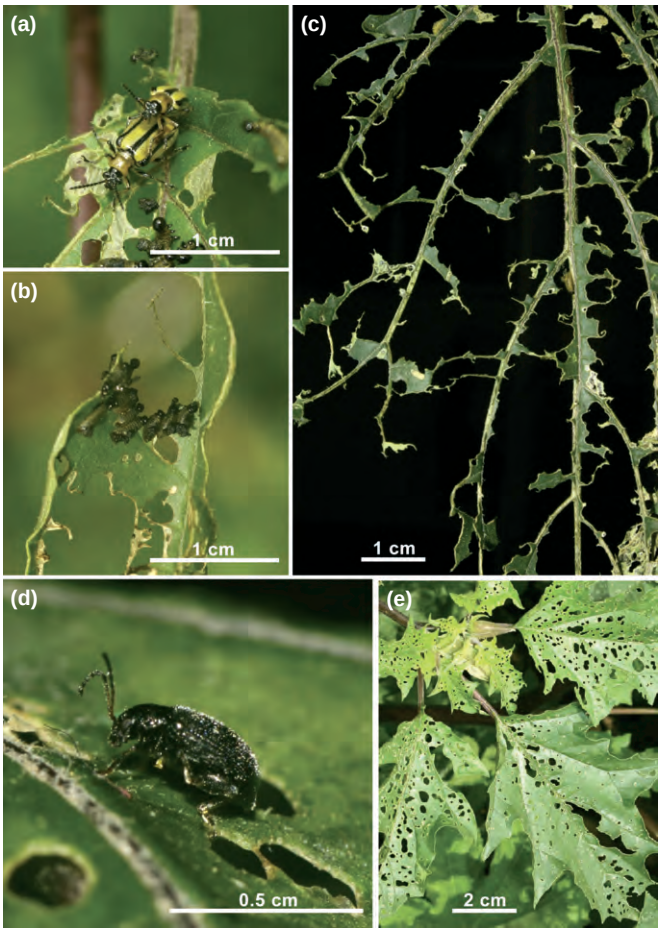

Fig. 2 Photographs of two beetles and types of damage imposed on the host plant *Datura stramonium* (Solanaceae). (a) *Lema daturaphila* adults. (b) *Lema daturaphila* third-instar larvae. (c) Leaf damaged by *L. daturaphila* larvae. (d) *Epitrix parvula* adult. (e) Leaf damaged by *E. parvula* adults.

Genetic material

During the summer of 2004, seeds from 115 plants were collected from a natural population of *D. stramonium*. Thirty seeds from one fruit on each plant were sown in plastic pots (2 l) and germinated in a glasshouse at the Instituto de Ecología of the Universidad Nacional Autónoma de México. One randomly chosen seedling from each family (i.e. seeds from the same fruit) were transplanted to a plastic pot (2 l) and manually self-pollinated to produce full-sibs. In 2005, these selfed full-sibling families were self-pollinated and grown again in the glasshouse to reduce maternal effects. After germination, in the first week of July 2006 a total of 64 full-sib maternal families (1536 plants) were transplanted to the experimental plot.

Experimental design

An ecological genetic field experiment with an incomplete factorial design was performed to manipulate the presence/absence of the most important herbivores of *D. stramonium*, *L. daturaphila* and *E. parvula*. We used a randomized complete block design in which eight siblings per family were assigned to each treatment combination (64 families × 4 blocks × 3 treatments × 2 replicates/family/block). Blocks were used to control for environmental variation. Plants were spaced 1 m apart in the experimental plot.

In the *E. parvula* treatment (E), beetles were allowed to naturally colonize plants while *L. daturaphila* were excluded. *Lema daturaphila* eggs and larvae were removed manually every 5 d, reducing the chances that laid eggs would hatch. Based on previous laboratory surveys, larvae emerge 5–7 d after oviposition (E. Garrido, pers. comm.); thus, eliminating eggs every 5 d ensured that no larvae emerged and ate foliar tissue. The *L. daturaphila* treatment (L) was established by spraying each plant with SevinXP® (Bayer de México, Ecatepec, México) at a concentration of 25 mg l^{-1} every week during the plants' prereproductive period, as *E. parvula* mainly consume the plant during the seedling and juvenile stages (D. Carmona and J. Fornoni, pers. obs.). SevinXP® is a contact carbamyl insecticide reported to have little effect on plants and was used previously in the same study system (Shonle & Bergelson, 2000). An additional set of 50 plants was sprayed with the insecticide and the amount of damage compared with that of the combined herbivore treatment when both herbivores were present (i.e. the natural condition in this population). The mean leaf damage on the sprayed plants was 3.6% (± 0.0012% SE), while the mean leaf damage in the LE treatment was 13 ± 0.004% (Table 1). This indicated that the insecticide was highly effective in deterring herbivores in our experimental plot. We adjusted the frequency of the insecticide applications during the first 3 wk to determine the number of days after which the insecticide was no longer effective and did not affect the preference of *L. daturaphila*. As after 7 d of insecticide application *L. daturaphila* adults started laying eggs again, subsequent applications were performed once a week. Results indicated that levels of damage in treatment L were higher (14.6 ± 0.0038%; Table 1) than those observed in the LE treatment, indicating that applying the insecticide did not negatively affect the preference of *L. daturaphila*. However, we cannot rule out possible side effects that may be associated with our experimental manipulation of the presence of *E. parvula*. Thus, any inference derived from the selection regime in which plants are eaten only by *L. daturaphila* should be treated with caution. Plants in the LE treatment were those with natural levels of herbivore damage caused by the folivore species *L. daturaphila* and *E. parvula* (LE). Even though the type of leaf damage produced by both herbivores can be distinguished (Fig. 2c,e), damage inflicted by *E. parvula* is usually masked by the extensive damage produced by *L. daturaphila* (Fig. 2c). Thus, estimating the amount of damage imposed by each herbivore in the LE treatment is impossible. Plants that were not sprayed with insecticide were sprayed with water as a control. We recorded the following variables for each individual plant: transplant date; number of leaves after 26 d (initial size); percentage of foliar damage; and total fruit production. The percentage of foliar damage was recorded when fruits matured and was estimated using a Color Windows Image Analysis System (WinDIAS-Basic; Delta-T Devices Ltd, Cambridge, UK). Thirty leaves were sampled

Table 1 Mean (SE) herbivory damage and fruit production under three treatment combinations: presence of *Lema daturaphila* and *Epitrix parvula* (LE), presence of *L. daturaphila* (L), and presence of *E. parvula* (E)

	Treatment				
	LE	L	E	*F* (df)	*P*
Damage	13.0 (0.005)[b]	14.6 (0.004)[a]	7.4 (0.004)[c]	122.06 (2,1164)	< 0.0001
				χ^2(df)	*P*
Fruit production	3.72 (0.528)[b]	4.96 (0.511)[a]	4.76 (0.525)[a]	87.141 (2,1771)	0.0375

Differences in damage and fruit production among treatments (denoted by different superscript letters) were detected using Tukey's and Wald's test respectively.

randomly per plant, unless plants had < 30 leaves, in which case all leaves were collected.

Data analyses

Variation in damage and fitness among treatments A one-way ANOVA and subsequent Tukey tests were performed to determine the effects of the amount of damage and plant fitness. Because the total seed number is highly correlated with the number of fruits (Fornoni *et al.*, 2003), it was used as a proxy for maternal fitness. In addition, because *D. stramonium* is a highly selfing species, maternal fitness probably reflects the majority of seeds sired by a given genotype (Motten & Antonovics, 1992; Núñez-Farfán *et al.*, 1996). Differences among treatments in the number of fruits were determined with the package *glm* of the program *R* (R version 2.11.1, 2010) which allows the generalized linear model to be fitted with a quasi-Poisson error distribution. Because we detected overdispersion, we corrected the standard error using a quasi-generalized linear model in which the variance was given by $\varphi \times \mu$, where μ is the mean and φ the estimated dispersion parameter (Zuur *et al.*, 2009).

Operational definition of resistance and tolerance Resistance for each individual plant was estimated as 1 – the proportion of damaged leaf area (Simms & Rausher, 1987), assuming that all plants were readily available for the herbivore population in the experimental plot. As all plants suffered from herbivore damage within the experimental plot, this assumption is probably validated. Given that we used multiple replicates per family, estimates of resistance at the genetic level are likely to converge to the true resistance value per family.

Tolerance was estimated as the relationship between *fitness* and the proportion of damaged leaf area (Mauricio *et al.*, 1997; Strauss & Agrawal, 1999; Tiffin & Rausher, 1999; Stinchcombe & Rausher, 2002; Fornoni *et al.*, 2003, 2004b). Before estimating tolerance, we examined whether the relationship between fitness and damage was nonlinear as this can bias the tolerance estimation (Tiffin & Inouye, 2000). Based on preliminary analyses indicating the absence of a general nonlinear effect of damage on fitness ($\chi^2_1 = 0.04$; $P = 0.8275$) and the Akaike information criterion (AIC), we excluded the quadratic term in subsequent analyses. Hence, to obtain family estimates of tolerance, we performed regression analysis on fitness to obtain partial regression coefficients for each family (tolerance estimates; more details in the following section).

Genetic variation for fitness, tolerance, and resistance As our experimental design was unbalanced and included random effects, we used mixed model methodology to test for significant random effects (Littell *et al.*, 2002). We searched for genetic variations in fitness, tolerance, and resistance to herbivores using mixed models and the restricted maximum-likelihood (REML) iterative algorithm in the statistical module *lmer* of the program *R* (R version 2.11.1, 2010). A generalized linear mixed model (GLIMM) was used to detect genetic variation in fitness and tolerance because the fitness estimator (number of fruits) followed a Poisson distribution. In this case, the function *glmer* from the *lme4* package of the program *R* (R version 2.11.1, 2010) was used with the quasi-Poisson error distribution and a *log* link function, given that preliminary analyses revealed important levels of overdispersion. The final model for fitness included the fixed effect treatment, and the random effects of block, family, and the interaction term family × treatment. Day of transplant and initial plant size (estimated as the number of leaves) were included as covariables to reduce the error mean square. AIC scores were used to select the best statistical model. Significant effects of family and its interactions with other factors were tested with the likelihood-ratio χ^2 test (Zuur *et al.*, 2009). A significant interaction between damage and family indicates the presence of genetic variation in tolerance. This interaction evaluates the presence of differences in the relationship between fruit number and damage among full-sib families. Because fruit number and proportion of damage were regressed using a log link function, our approach for estimating tolerance avoided the kind of bias that arises when the model that estimates tolerance combines additive and multiplicative scales and uses an identity link function (Wise & Carr, 2008). In our model, the interaction damage × family × treatment indicated that the expression of genetic variation in tolerance is conditioned by the herbivore species that cause damage. Following the same mixed model used for fitness, the genetic variation in resistance was analyzed using the GLIMM with a normal error structure.

Natural selection Natural selection acting on resistance and tolerance was examined at the genotype level (Rausher, 1992) within each treatment following multiple regression analyses. For

these analyses, full-sib family estimates for fitness, resistance, and tolerance from previous mixed models (best linear unbiased predictors (BLUP)) were obtained using the *ranef* function of package *lme4* in *R*. Given that our experimental design was analyzed using mixed model theory because of unbalance, BLUPs are much better estimators of genotypic family values than standard least square means (Littell *et al.*, 2002). Before the analyses were conducted, BLUPs for untransformed fitness values were relativized as (fitness + absolute value of the fitness of the family with lowest fitness)/(mean fitness + absolute value of the fitness of the family with the lowest fitness); thus, the mean relative fitness equals 1 (Pilson, 2000). BLUPs for tolerance and resistance were standardized to a mean of zero and a standard deviation of 1.

Because damage was used to estimate resistance and tolerance, we tested for a genetic correlation between the two defensive traits before conducting the selection analyses. Our results found no evidence of an association between tolerance and resistance, eliminating this source of collinearity in the analyses (see the Results section). Another possible artifact that can also affect the estimation of selection gradients in tolerance occurs when the mean and variance in fitness among genetic families are highly positively correlated. In this case, families with low variance in fitness are likely to express high levels of tolerance because of low statistical power to detect significant shallow slopes resulting in negative directional selection acting on tolerance (Agrawal *et al.*, 2004). Previous studies in which this form of bias has been detected found negative directional selection acting on tolerance and a strong positive association between fitness means and variances ($r \geq 0.81$; Agrawal *et al.*, 2004; Baucom & Mauricio, 2008). In the present study, we found a similar association between fitness means and variances ($r = 0.79$; $P < 0.0001$) but no evidence of negative directional selection (see the Results section), suggesting that families with high levels of tolerance (shallow slope between fitness and damage) were not necessarily those with lower mean fitness. In addition, a bias affecting selection gradients on tolerance could arise because fitness is used in the predictor (tolerance) and the response variable in subsequent selection analyses. However, in an analysis of covariance on fitness in which the effects of family and family × damage are included, the partial regression coefficients for each family (tolerance estimates) are statistically independent from the family mean fitness values in the absence of multicollinearity. As a consequence of the lack of an algorithm for detecting such nonindependence between random terms, we estimated the variance inflation factor (VIF) from a model in which both effects (family and damage × family) were considered fixed. Results for each treatment indicated that these values were < 1.28, suggesting low chances of multicollinearity between family mean fitness and tolerance (Zuur *et al.*, 2009). Finally, although BLUPs are better estimators of family means than least squares when family replicates are unbalanced, the use of BLUPs in selection analyses has an undesired statistical behavior (Hadfield *et al.*, 2010); thus, any inference about future responses to selection should be made with caution.

To detect directional selection (β) acting on resistance and tolerance, only linear terms were included in the partial regression analyses. A second model was performed that included linear and quadratic effects to test for the presence of stabilizing/disruptive (γ_{ii}), and correlational (γ_{ij}) selection (Lande & Arnold, 1983). The estimation of the quadratic selection gradient (γ_{ii}) was multiplied by 2 to obtain the correct selection strength (Stinchcombe *et al.*, 2008). Differences among treatments in the pattern and the intensity of selection in plant defenses were examined using an ANCOVA on relative fitness. Further analyses between pairs of treatments were performed to test for the presence of differences in selection patterns (LE vs E and LE vs L). Significant differences between treatments E and L indicate that the simulated damage environments imposed different selection regimes.

Visualization of the resistance–tolerance fitness surfaces

Although Lande & Arnold (1983) observed that selection gradients can be used to detect general differences in the pattern of simultaneous selection on two traits, a better picture of selection patterns can be obtained through visualizing fitness surfaces. Detailed portrayals of the resistance–tolerance fitness surfaces in each treatment combination were obtained using a nonparametric spline procedure (Schluter & Nychka, 1994). This graphical analysis represents a useful complement to nonlinear selection gradients as it can detect more complex selection patterns. Selective surfaces were plotted using thin-plate spline fit, a three-dimensional analog of cubic spline (Zuur *et al.*, 2009), using the *Tps* function from the *fields* package in *R*. The smoothing parameters for each spline were chosen based on generalized cross-validation.

Results

Variation in damage and fitness among treatments

Plants growing in different treatments differed in the amount of foliar damage ($F_{2,1164} = 122.06$; $P < 0.0001$; Tukey HSD at 95% confidence; Table 1). The mean estimated damage in the LE treatment (i.e. where *L. daturaphila* and *E. parvula* were present) was 13%, and when *E. parvula* was excluded, the foliar damage imposed by *L. daturaphila* alone increased to 14.6%. However, when *L. daturaphila* was excluded, the foliar damage imposed by *E. parvula* was 7.4%, indicating that the amount of damage caused by the two herbivore species interacted negatively in a nonadditive way. These results showed that the main consumer of foliar tissue in the natural environment was *L. daturaphila*. Significant differences among treatments in the fruit production were detected (Table 1). Plants in the LE treatment produced a lower number of fruits per plant than in the other two treatments (Table 1). The numbers of fruits produced in the presence of *L. daturaphila* and *E. parvula* were similar, but significant differences in the amount of damage were detected (Table 1).

Genetic variation

Significant genetic variation among full-sib families was detected for fitness (fam; $\chi^2_1 = 778.05$; $P < 0.0001$), resistance

($\chi^2_1 = 23.988$; $P < 0.001$), and tolerance (damage × fam; $\chi^2_1 = 22.201$; $P < 0.0001$; Supporting Information Table S1). In addition, the expression of genetic variation in these traits was conditioned by the presence/absence of particular herbivore species (Table S1). Genetic differences among families were observed for all traits in all treatment combinations, except for resistance in treatment E (Table S1). We did not detect the presence of a genetic correlation between resistance and tolerance in any treatment combination (LE: $r = 0.123$; $P = 0.403$; L: $r = 0.032$; $P = 0.824$; E: $r = 0.059$; $P = 0.686$).

Natural selection

Multiple regression analyses detected significant genotype selection acting upon tolerance and resistance. Manipulation of the presence/absence of folivore species altered the selection pattern in resistance and tolerance (Tables 2, S2, S4). In the presence of *E. parvula* alone (treatment E), analyses detected stabilizing selection favoring intermediate levels of resistance (*F* value, degrees of freedom, and probability for the full model including linear and quadratic effects: $F_{5,42} = 3.145$; $P = 0.0168$; Table 2). Differences in the nonlinear component of selection on resistance were detected between treatments E and LE, indicating the presence of diffuse selection (i.e. differences in the pattern of selection against a focal species when another interacting species is present; Table S4). By contrast, in the presence of the other beetle (treatment L), only marginally directional selection on tolerance was detected (*F* value, degrees of freedom and probability for the linear model: $F_{2,45} = 2.811$; $P = 0.0707$; Table 2). Although directional selection on tolerance was favored only in the presence of *L. daturaphila* (Table 2), we did not detect differences in the selection pattern in this strategy among treatments (Table S3). Between treatments L and E, significant differences were detected in the nonlinear component of selection on resistance, indicating that each simulated environment exerted a different selective regime on plant defenses in the experimental population (Table S2). In the treatment LE, the analysis detected a significant negative correlational selection gradient acting on the simultaneous expression of tolerance and resistance (*F* value, degrees of freedom, and probability for the full model including linear and quadratic effects: $F_{5,42} = 3.141$; $P = 0.0169$; Table 2).

Graphical inspection of the selective surfaces in all treatments supported the general result of the multiple regression analyses indicating the presence of one dominant fitness peak in the resistance–tolerance adaptive landscape (Fig. 3). Differences were also evident in the shape and position of the adaptive peaks depending on the presence/absence of the two herbivore species (Fig. 3). When both herbivores were present (treatment LE), the fitness surface indicated the presence of two partially alternative fitness peaks, one represented by high tolerance and intermediate levels of resistance, and the other by high resistance and low tolerance (Fig. 3; LE). Although tolerance and resistance interacted negatively, the adaptive surface indicated that the higher fitness peak corresponded to a mixed pattern of defense allocation (high tolerance and intermediate levels of resistance). This scenario partially matched the hypothetical scenario depicted in Fig. 1(a) as in our experimental population the two fitness peaks should not be considered true equivalent alternatives (the high tolerance–intermediate resistance peak provided higher fitness benefits than the other peak). This pattern contrasts with those observed in the presence of each beetle alone as only one fitness peak is favored in both cases. In the presence of *L. daturaphila* (treatment L), the fitness peak corresponded to high tolerance and low resistance (Fig. 3L), mimicking the hypothetical scenario depicted in Fig. 1(b) where only one defense mechanism is favored. In the presence of *E. parvula* (treatment E), the adaptive surface indicated the presence of an intermediate peak for resistance running parallel to the tolerance axis (Fig. 3E). This pattern partially matched the hypothetical surface simulated in Fig. 1(d), as only an intermediate optimum level of one strategy was detected. The observed changes between treatment LE and treatments E and L corresponded to a qualitative difference in the shape of the fitness landscape (i.e. transition between Figs 1a,b and c). In treatment E, the mean observed level of resistance and tolerance (circled cross, Fig. 3) was closer to the adaptive peak than in treatments LE and L (Fig. 3).

Table 2 Selection gradients (standard errors within parentheses) (directional (β_i), stabilizing/disruptive (γ_{ii}), and correlational (γ_{ij})) on tolerance and resistance obtained from independent multiple regression analyses in three treatments corresponding to the manipulation of the presence/absence of two folivore beetles: (LE) presence of *Lema daturaphila* and *Epitrix parvula*, (L) presence of *L. daturaphila*, and (E) presence of *E. parvula*

	Treatment		
Defensive trait	LE	L	E
Directional selection (β_i)			
Resistance	0.048 (0.054)	−0.072 (0.051)	0.035 (0.061)
Tolerance	0.063 (0.054)	**0.097** (0.050)†	−0.007 (0.065)
Stabilizing/disruptive selection (γ_{ii})			
Resistance^2	−0.128 (0.106)^{b}	−0.068 (0.095)^{b}	−**0.264** (0.079)^{a}**
Tolerance^2	0.065 (0.066)	−0.137 (0.073)	0.150 (0.090)
Correlational selection (γ_{ij})			
Resistance × tolerance	−**0.181** (0.006)^{a}**	−0.137(0.073)^{a}	−0.027 (0.072)^{a}

Significant effects are indicated in bold, and different letters correspond to statistically significant differences between pairs of treatments (critical $\alpha = 0.016$ after a Bonferroni correction) in the pattern of selection obtained from an ANCOVA (further details are presented in Supporting Information Tables S2–S4). Each quadratic selection gradient (i.e., Resistance^2 and Tolerance^2) was corrected following Stinchcombe *et al.* (2008) (see the Materials and Methods section).
**, $P < 0.01$; †, $P = 0.057$.

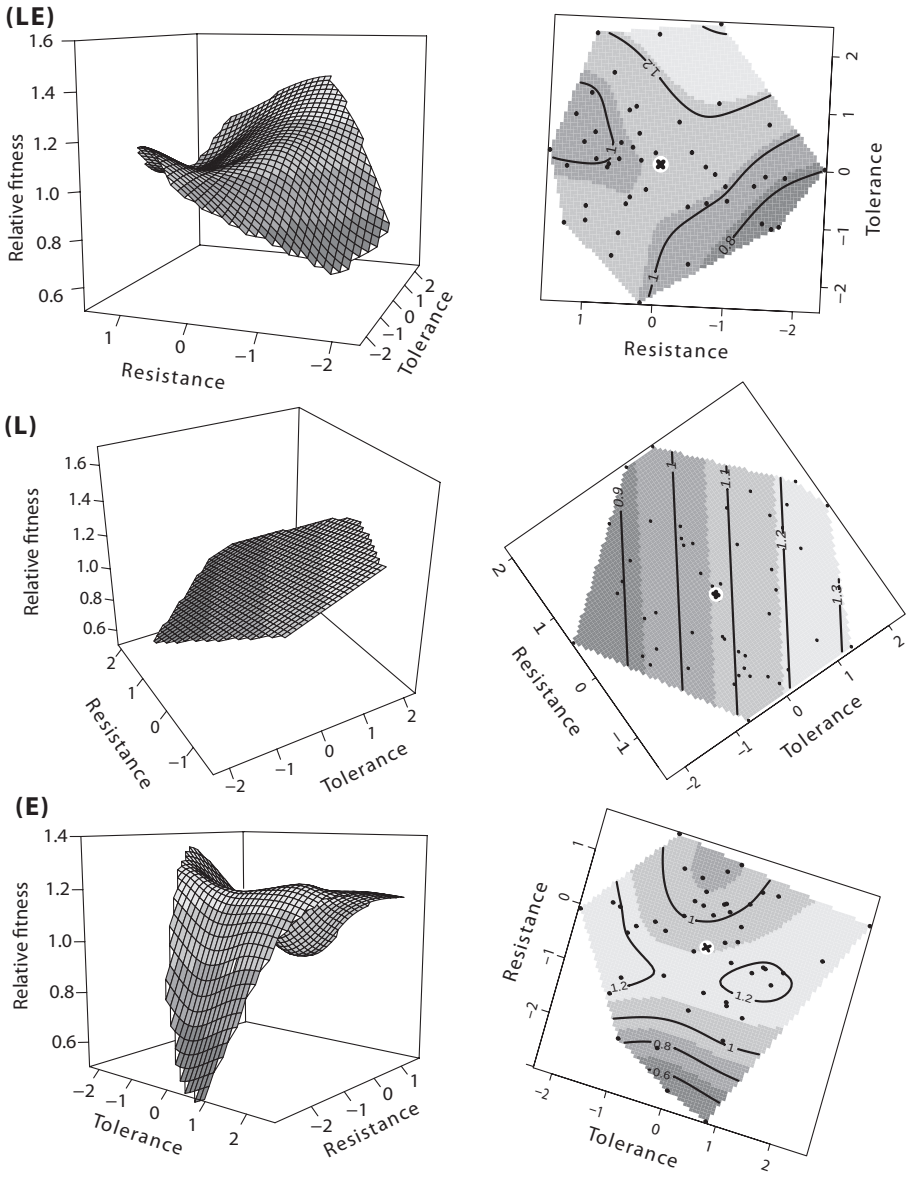

Fig. 3 Resistance–tolerance adaptive surfaces (left) and contour plots (right) describing the bivariate fitness function in each treatment. For each contour plot, the circled crosses indicate the mean level of tolerance and resistance observed in the experimental population. Treatments: LE, treatment with both herbivores present, *L. daturaphila* and *E. parvula*; L, only *L. daturaphila*; E, only *E. parvula*.

Discussion

Rather than being redundant, tolerance and resistance in *D. stramonium* functioned as complementary defenses in the presence of only *E. parvula* or both herbivores, favoring the evolution of a mixed defense strategy. By contrast, *L. daturaphila* selected for increased tolerance and diminished resistance, suggesting that resistance is useless against this more specialized herbivore (Garrido *et al.*, 2012). Although the idea that resistance and tolerance could function as complementary defenses against natural enemies has been theoretically examined (Tiffin, 2000; Fornoni *et al.*, 2004a; Restif & Koella, 2004), we showed for the first time the validity of this expectation in a natural plant–herbivore system. Our results demonstrated that different experimental methods for altering herbivore damage resulted in different selection modes on resistance and tolerance, affecting the simultaneous evolution of tolerance and resistance and the extent of their complementarity. Although this may account for the presence of intermediate levels of both strategies in our population (circled crosses in Fig. 3), the observed mean level of tolerance and resistance was closer to the adaptive peak predicted when the two herbivores and *E. parvula* alone were present. Thus, these two scenarios favoring a mixed defense strategy are likely to be the most common selective environments and/or historical selection pressures exerted by *E. parvula* have been more intense than that imposed by *L. daturaphila*. Personal observations in the region confirmed that the presence of *E. parvula* and the two herbivores together are the most frequent combinations in central Mexico (J. Hernández-Cumplido & J. Fornoni, pers. obs.).

An important source of variation that can account for the presence of intermediate levels of tolerance and resistance is diffuse selection when traits are exposed to temporal and/or spatial

fluctuation in the selection patterns derived from the presence of different consumer species (i.e. changes in the selective pattern acting on a particular trait involved in an interaction due to the presence/absence of a third species; reviewed in Strauss *et al.*, 2005). Previous studies addressing diffuse selection focused on single traits (Pilson, 1996; Juenger & Bergelson, 1998; Stinchcombe & Rausher, 2002; Lankau, 2007; Sahli & Conner, 2011), although our approach using tolerance and resistance allowed us to test whether diffuse selection acted on the combined expression of the two defense strategies. Analyses detected changes in selection acting on resistance and not on tolerance, although this strategy was selected only in one treatment combination (i.e. treatment L). Further inspection of the adaptive surfaces indicated that, although the presence/absence of *L. daturaphila* changed the position of a combined fitness peak, the manipulation of *E. parvula* altered the extent of the complementarity, as when only *L. daturaphila* was present the combined fitness peak observed in the LE treatment changed to one in which maximum tolerance and minimum resistance were favored. Thus, our results indicated that changes in the amount of damage after our experimental manipulation of two herbivore species can affect the position of the tolerance–resistance fitness peak, and suggest that it may also affect the complementarity between the two defenses.

Selection on tolerance imposed by the beetle *L. daturaphila*, a common response to specialist herbivores, suggests that this herbivore has a long history of interaction with the plant and is more likely to be locally adapted. A recent cross-infection experiment involving *D. stramonium* and *L. daturaphila* in the same study site indicated weak local adaptation by *L. daturaphila* (Garrido *et al.*, 2012) that may be enough to reduce the benefits of resistance. By contrast, resistance appears to be more effective against *E. parvula*, a consumer of several genera within the Solanaceae. The closer position of mean resistance and tolerance to the expected fitness peak in the presence of *E. parvula* and the two beetles together suggests that this herbivore has exerted more frequent and/or intense selection on resistance than *L. daturaphila*. Although both folivores belong to the Chrysomelidae and to the same functional guild, the narrower diet specialization of *L. daturaphila* suggests that the adaptive value of tolerance depends to some extent on the coevolutionary state of the interaction (reviewed in Fornoni, 2011).

Our results are consistent with Roy & Kirchner's (2000) theoretical expectation that, whenever tolerance provides a fitness benefit (when *L. daturaphila* eats the plants), natural selection should fix tolerance at maximum levels, whereas resistance is more likely to be maintained at intermediate levels (in the presence of *E. parvula*) because of the negative feedback with fitness. Although correlational selection of the form necessary to favor the evolution of a negative genetic correlation between the two strategies was detected, further analyses revealed no evidence that resistance and tolerance are being constrained by a negative genetic correlation between them. Despite genetic variation in tolerance and resistance still being available, we cannot rule out the possibility that further genetic constraints will prevent the evolution of optimum levels of plant defenses (Strauss & Agrawal, 1999; Tiffin & Rausher, 1999; Lankau, 2007; Johnson *et al.*, 2009; Fornoni, 2011; Sahli & Conner, 2011).

Theoretically, a mixed defense strategy would be favored when the nonlinear cost and benefit functions of the two strategies interact to generate an adaptive surface with maximum fitness at intermediate levels (Fornoni *et al.*, 2004a; Restif & Koella, 2004). The shape of the adaptive surface and the mean level of tolerance and resistance provided strong support for the hypothesis that the two strategies functioned as complementary defenses providing more than additive fitness benefits. The absence of redundancy and mutual exclusivity could occur because resistance does not reduce damage completely and tolerance does not fully compensate for the effect of damage (Mauricio, 2000). Our results suggest that functional complementarity could be favored in a scenario in which resistance is partially effective against herbivores and tolerance could provide additional benefits reducing the negative effect of damage on fitness. In this sense, the coevolutionary response of natural enemies could break down the functional redundancy as natural enemies can rapidly adapt to their host resistance (Garrido *et al.*, 2012). This explanation is also consistent with evidence supporting the expectation of mutual exclusivity between tolerance and resistance against two abiotic selective agents (frost and glyphosate damage; Agrawal *et al.*, 2004; Baucom & Mauricio, 2008). Hence, if the presence of a coevolutionary process can break down functional redundancy between tolerance and resistance to natural enemies, future models of plant defense should reconsider the expectation of redundancy.

Concluding remarks

Our results indicated that the average tolerance and resistance values are closer to the adaptive peak predicted in the presence of *E. parvula* and both herbivores together, suggesting that these scenarios are probably the most common under natural conditions in the studied population. Differences in the selection pressure imposed by two folivore beetles may result in the evolution of markedly different defense strategies. This suggests that, when a plant is eaten by several herbivore species, each with a particular history of interaction with the plant, the probability of finding functional redundancy between resistance and tolerance to the whole community of consumers will decrease. Thus, if different herbivores sharing the same host plant generate selection on resistance and tolerance, or a single enemy generates selection on both, both mechanisms may be maintained, resulting in the expression of a combined defense pattern. Although diffuse selection may actually affect the evolution of defensive traits, the most common ecological setting favors the evolution of a mixed defense strategy. Hence, the simultaneous expression of tolerance and resistance in our experimental population is more consistent with the predicted selective surfaces than with the presence of environmental or genetic constraints. Finally, if conditions for mutual exclusivity against natural enemies are rarely encountered in nature, our results could represent a general pattern.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Results from ANOVAs to detect genetic variance in fitness, resistance and tolerance

Tables S2–S4 ANCOVAs testing differences in selection gradients between treatments

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Supporting Information Tables S1–S4

Table S1 Results from ANOVAs on fitness (fruit production), resistance and tolerance to detect overall, within and among treatment differences in genetic variance. Values correspond to χ^2 (degrees of freedom are indicated in parenthesis) after likelihood ratio tests. ANOVAs for fitness and tolerance corresponded to generalized linear models with the quasi-Poisson error distribution. LE: Presence of *Lema daturaphila* and *Epitrix parvula*, L: Presence of *L. daturaphila*, E: Presence of *E. parvula*. Significant effects are indicated in bold.

Plant traits	**Overall Family effect**	**Family effect within treatments**			**Differences among Family effect across treatments**
		LE	L	E	
Fitness	**778.05***** (1)	**304.05***** (1)	**581.0***** (1)	**452.69***** (1)	**589.28** *** (10)
Resistance	**23.988***** (1)	**12.931***** (1)	**4.115*** (1)	0.357 (1)	**18.01**** (6)
Tolerance	**22.201** *** (1)	**183.95***** (1)	**162.58***** (1)	**168.94***** (1)	**789.76***** (9)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table S2 Results from generalized linear (A) and non-linear (B) models on relative fitness using Poisson error distribution to contrast selection patterns acting on tolerance and resistance between Treatment E and L. After a Bonferroni correction, significant effects ($P < 0.016$) are indicated in bold. † $0.016 < P < 0.05$.

A.

Source	df	Type III SS	*F-values*	*P*
Intercept	**1**	**51.897**	**431.0223**	**< 0.0001**
Treatment	1	0.024	0.1954	0.6595
Resistance	1	0.042	0.3473	0.5571
Tolerance	1	0.002	0.0139	0.9062
Treatment × Resistance	1	0.104	0.8649	0.3549
Treatment × Tolerance	1	0.317	2.6360	0.1080
Error	90	10.836		

B.

Source of variation	d.f.	Type III SS	*F-values*	*P*
Intercept	**1**	**26.1330**	**247.6823**	**< 0.0001**
Treatment	1	0.0271	0.2565	0.613838
Resistance	1	0.1920	1.8194	0.181010
Tolerance	1	0.0019	0.0177	0.894436
Resistance^2	**1**	**1.0624**	**10.0693**	**0.002107**
Tolerance^2	1	0.2678	2.5378	0.114908
Resistance × Tolerance	1	0.0136	0.1289	0.720450
Treatment × Resistance	1	0.1169	1.1080	0.295532

Treatment × Tolerance	1	0.4526	4.2896	0.041416[†]
Treatment × Resistance^2	**1**	**1.0147**	**9.6175**	**0.002624**
Treatment × Tolerance^2	1	0.3070	2.9097	0.091742
Treatment × Resistance × Tolerance	1	0.0147	0.1390	0.710227
Error	84	8.8628		

Table S3 Results from generalized linear (A) and non-linear (B) models on relative fitness using Poisson error distribution to determine whether the presence/absence of *Epitrix parvula* altered the pattern of selection acting on tolerance and resistance (contrast between Treatment L vs LE. A significant Treatment × trait interaction indicates that *E. parvula* imposes diffuse selection on plant defenses. After a Bonferroni correction, significant effects ($P < 0.016$) are indicated in bold.

A.

Source	df	Type III SS	*F-values*	*P*
Intercept	**1**	**55.018**	**456.0294**	**< 0.0001**
Treatment	1	0.047	0.3910	0.53337
Resistance	1	0.234	1.9412	0.16697
Tolerance	1	0.448	3.7127	0.05716
Treatment × Resistance	1	0.327	2.7087	0.47503
Treatment × Tolerance	1	0.062	0.5146	0.10329
Error	90	10.858		

B.

Source of variation	d.f.	Type III SS	*F-values*	*P*
Intercept	**1**	**21.4266**	**198.5644**	**< 0.0001**
Treatment	1	0.0450	0.4174	0.51999
Resistance	1	0.1415	1.3116	0.25535
Tolerance	**1**	**0.7334**	**6.7967**	**0.01080**
Resistance^2	1	0.0586	0.5430	0.46325
Tolerance^2	1	0.1261	1.1690	0.28270

Resistance × Tolerance	1	0.3974	3.6828	0.05837
Treatment × Resistance	1	0.0909	0.8427	0.36127
Treatment × Tolerance	1	0.1654	1.5327	0.21916
Treatment × Resistance^2	1	0.0157	0.1450	0.70428
Treatment × Tolerance^2	1	0.2160	2.0022	0.16077
Treatment × Resistance × Tolerance	1	0.0762	0.7064	0.40304
Error	84	9.0642		

Table S4 Results from generalized linear (A) and non-linear (B) models on relative fitness using poisson error distribution to determine whether the presence of *Lema daturaphila* altered the pattern of selection acting on tolerance and resistance (contrast between Treatment E vs. LE. A significant Treatment × trait interaction indicates that *L. daturaphila* imposes diffuse selection on plant defenses. After a Bonferroni correction, significant effects ($P < 0.016$) are indicated in bold.

A.

Source	df	Type III SS	*F-values*	*P*
Intercept	**1**	**52.211**	**376.8143**	**< 0.0001**
Treatment	1	0.005	0.0354	0.8513
Resistance	1	0.047	0.3390	0.9074
Tolerance	1	0.002	0.0136	0.5619
Treatment × Resistance	1	0.029	0.2115	0.3446
Treatment × Tolerance	1	0.125	0.9026	0.6467
Error	90	12.470		

B.

Source of variation	d.f.	Type III SS	*F-values*	*P*
Intercept	**1**	**26.4379**	**237.4186**	**< 0.0001**
Treatment	1	0.0044	0.0399	0.842105
Resistance	1	0.2157	1.9374	0.167620
Tolerance	1	0.0022	0.0196	0.888893
Resistance^2	**1**	**1.1932**	**10.7155**	**0.001544**
Tolerance^2	1	0.3007	2.7006	0.104048

Resistance × Tolerance	1	0.0153	0.1372	0.712015
Treatment × Resistance	1	0.2045	1.8363	0.179023
Treatment × Tolerance	1	0.0881	0.7912	0.376289
Treatment × Resistance^2	**1**	**1.1713**	**10.5185**	**0.001696**
Treatment × Tolerance^2	1	0.0135	0.1215	0.728261
Treatment × Resistance × Tolerance	1	0.0002	0.0022	0.963004
Error	84	9.3539		

UNDERSTANDING THE CAUSES OF ADAPTIVE EVOLUTIONARY CHANGES IN ECOLOGICALLY VARIABLE ENVIRONMENTS: THE PREDICTIVE POWER OF THE BREEDER'S EQUATION

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ABSTRACT

In a changing multispecific environment, alterations in selection gradients (diffuse selection) have been inferred as a principal cause of modifications in the response to selection (diffuse (co)evolution). Meanwhile, the other component of the response to selection, the **G** matrix, has been conceived as a stable source of genetic constrains. To assess the role of diffuse selection and the **G** matrix on the response to selection under a changing multispecific environment we conducted an ecological genetic field experiment where the presence of the two most important herbivores of *Datura stramonium* were manipulated. In this study we provide evidence showing a marginal effect of diffuse selection but a significant effect of $\mathbf{G} \times \mathbf{E}$ interaction causing diffuse evolutionary pattern. We discuss that the stability of **G** matrix must not be taken for granted at the micro evolutionary level. We also point out that misleading conclusions about the importance of diffuse selection can be generated if not evaluated using a multivariate approach. Finally, we conclude that the criteria to distinguish between pairwise and diffuse coevolution can be view as a basal eco-evolutionary protocol to examine a more general situation, a multivariate and dynamic phenotype evolving within a multispecific and dynamic selection regime.

Keywords: diffuse selection, diffuse evolution, diffuse coevolution, fluctuating selection, fluctuating evolution, variance-covariance matrix, G-matrix, plant-herbivore interaction, natural selection, life history traits, resistance, damage, biotic interactions.

INTRODUCTION

Adaptive evolution of quantitative traits is represented by the Breeder's equation ($\mathbf{\Delta z} = \boldsymbol{\beta} \times \mathbf{G}$; Fig 1) where a multivariate phenotypic change ($\mathbf{\Delta z}$) is determined by the product of a vector of selection gradients ($\boldsymbol{\beta}$) and the variance-covariance matrix ($\mathbf{G}$) underlying the expression of correlated traits (Lande 1979). This formalization of the response to selection remains as the central hypothesis to understand and predict evolutionary changes (Lush 1934; Lynch and Walsh 1998). During decades, the scope of the Breeder's equation have relied upon the assumption that $\mathbf{G}$ can be assumed as a relatively constant component while $\boldsymbol{\beta}$ (vector of selection gradients) captures how environmental changes affect the adaptive value of quantitative traits. Under this conception, $\mathbf{G}$ has been visualized as a potential "invariant" constrain that condition the direction of short-term evolutionary changes when the direction of the vector of selection is not aligned with the variance-covariance matrix (Steppan et al. 2002; Walsh and Blows 2009). The temporal stability of $\mathbf{G}$ received considerable theoretical analyses (Arnold et al. 2008), but these mainly focused on the strength of $\mathbf{G}$ to counteract the effect of other evolutionary processes like mutation, drift and gene flow (Roff 2000; Walsh and Blows 2009). However, both $\boldsymbol{\beta}$ and $\mathbf{G}$ are known to react against environmental changes induced by alterations in the ecological context (Strauss et al. 2005). The existence and ultimate consequences of $\mathbf{G} \times \mathbf{E}$ interactions affecting $\mathbf{G}$ remains almost unexplored. Asking about the temporal and spatial scale at which $\mathbf{G}$ change is of central importance to predict short-term adaptive responses to selection.

The concept of diffuse selection (changes in the adaptive value of a focal trait involved in the interaction between two species when a third species is present), provides a straightforward framework to understand how ecological variable environmental conditions affect the pattern of selection (Iwao and Rausher 1998; Stinchcombe and Rausher 2001; Sahli and Conner 2011).

This approach however, has not been empirically extended to a multivariate scenario were several correlated traits are simultaneously evaluated (hereafter diffuse multivariate selection; but see Wise and Rausher 2013). Given that selection is expected to affect the evolution of correlated traits, the univariate approach to test for diffuse selection (evolution) can lead to misleading conclusions about the real direction of evolutionary changes when only one isolated trait is considered. Empirical evidence indicated that neither the multivariate consequences of changes in **β** nor the sensibility of **G** to ecologically variable environments (**G** × **E**) have been jointly examined until now (Pigliucci and Kaplan 2006).

Quantitative genetic parameters are population specific estimators since depend on the allelic frequencies of genes underlying quantitative traits (Lush 1934; Falconer and MacKay 1996). However, other sources of variation like **G** × **E** interactions can also affect the major components of the **G** matrix (Schlichting and Pigliucci 1997). An increasingly amount of studies confirm that changes in heritabilities and additive genetic (co)variances occurs when genotypes of a given provenance face different environmental conditions (Daday et al. 1973; Mazer and Schick 1991; Ebert et al. 1993; Simons and Roff 1994; Sgrò and Hoffmann 1998; Bégin and Roff 2001; Stinchcombe and Rausher 2001, 2002; Conner et al. 2003; Fornoni et al. 2003; Cano et al 2004; Husby et al. 2011). Although this support the fact that **G** × **E** interactions can affect the expression of the G matrix (Hoffmaan and Merila 1999), direct implications on the stability of **G** and the overall response to selection has been seldom stressed (Mitchell-Olds and Rutledge 1985; Mazer & Schick 1991; Conner et al. 2003; Pigliucci and Kaplan 2006; Walsh and Blows 2009). Most studies addressing this issue focused on changes in the abiotic conditions of the environment comparing stressful versus less stressful scenarios like those imposed by differences in temperature (Sgrò and Hoffmann 1998), daylight length, moist (Daday et al. 1973), diet

composition (Falconer and Latyszewski 1952), desiccation regimes (Cano *et al.* 2004), greenhouse and field environments (Conner et al. 2003). To our knowledge only Mazer and Schick (1991) and Stinchcombe and Rausher (2001, 2002) have considered intraspecific and interspecific biotic interactions respectively as a source of environmental dependent expression of **β** and **G**. Thus, whether ecologically variable environmental conditions can affect the response to selection through changes in **β** and/or **G** still remains empirically unexplored.

In this study we put together two fundamental pieces of evidence; 1) the occurrence of environment dependent expression of **β** and **G**, and 2) its ultimate consequences on the response to selection following a multivariate approach (Walsh and Blows 2009). Using a cross-breeding design, we specifically tested if under common garden conditions the artificial manipulation of the ecological environment (presence/absence of two herbivores, *Lema daturaphila* and *Epitrix parvula*) affects the vector of selection (**β**) involving resistance, flowering date and growth rate and/or the **G** matrix in the annual herb *Datura stramonium*. In addition we explored whether the potential response to selection is sensitive to fluctuations in the composition of the natural enemies assemblage. Because alterations in either the pattern of selection (**β**) and/or the **G** matrix can produce a pattern of diffuse evolution, we finally evaluated the relative importance of changes in the ecological environment on the potential response to selection vector (**Δz**).

METHODS

Study System

The annual weed *Datura stramonium* L. (Solanaceae) is usually consumed by two folivorous insects that can impose variable levels of damage (10 - 50%) among populations in Central Mexico (Valverde et al. 2001). Individual plants can even suffer as much as 100% of defoliation

(Núñez-Farfán and Dirzo 1994). In Central Mexico *D. stramonium* face the attack of two folivore beetles of the family Chrysomelidae. The flea beetle *Epitrix parvula* is a specialized feeder of the Solanaceae family, while *Lema daturaphila* is a specialist consumer of the genus *Datura* (Kogan and Goeden 1970). Field observations demonstrate that *E. parvula* begins the consumption of foliar tissue at the seedling stage, while *L. daturaphila* tends to oviposit on plants near the onset of flowering (Carmona and Fornoni 2013).

Genetic material

During the summer of 2004, seeds from 115 plants were collected from a natural population of *D. stramonium* located in Teotihuacan, State of Mexico (19° 47´27´´ N; 98° 51´4´´W, 2294). Thirty seeds from a single fruit of each plant (half-sibs) were sowed in plastic pots (2 L) and germinated in a greenhouse at the Institute of Ecology (Universidad Nacional Autónoma de México). One randomly chosen seedling from each half-sib family was transplanted to a plastic pot (2 L) and manually self-pollinated to produce full-sibs. In 2005, these genetic families were grown again in the greenhouse to reduce maternal effects. After germination, the first week of July 2006 a total of 64 full-sibs maternal families (1536 plants) were transplanted to the experimental plot located within a xerophytic shrub community in the area of the natural population. The experimental plot (2048 m^2) was exposed to an average mean annual precipitation of 559.6 mm and a mean annual temperature of 14.8°C (Valverde et al. 2001).

Experimental design

A partial factorial design was performed to manipulate the presence/absence of the two most important herbivores of *D. stramonium, L. daturaphila and E. parvula.* We followed a randomized complete block design where eight siblings per family were assigned to each of the

three treatment combinations (64 families × 4 blocks × 3 treatments 2 × replicates/family/block). Blocks were used to control for micro-environmental variation within the plot. Plants were spaced 1 m apart from each other in the experimental plot. Plants in the LE treatment were those with natural levels of herbivore damage caused by both herbivores: *L. daturaphila* and *E. parvula.* Even though the type of leaf damage produced by both herbivores can be distinguished, the damage inflicted by *E. parvula* is usually masked by the extensive damage produced by *L. daturaphila.* Thus, estimating the amount of damage imposed by each herbivore in the LE treatment is impossible. In the *E. parvula* treatment (E), beetles were allowed to naturally colonize plants while *L. daturaphila* were manually removed every five days (see Carmona and Fornoni 2013). Based on previous laboratory surveys, larvae emerge five to seven days after oviposition (E. Garrido, personal communication); thus, eliminating eggs every five days ensured that no larvae emerged and ate foliar tissue. The *L. daturaphila* treatment (L) was established by spraying each plant with SevinXP® at a concentration of 25 mg/l every week during the plants' pre-reproductive period since *E. parvula* mainly consume the plant during the seedling and juvenile stage (D. Carmona and J. Fornoni, *personal observation*). SevinXP® is a contact carbamyl insecticide reported to have little effect on plants and was used previously in the same study system (Shonle and Bergelson, 2000; see Carmona and Fornoni 2013).

Characters measured

For each plant we estimated growth rate as [(ln number of leaves$_{t1}$ – ln number of leaves$_{t0}$) / (t_1-t_0)] (Pearcy et al. 1989), flowering day as the number of days before the first flower was produced and resistance to herbivory as 1-relative damage obtained for the whole sample of leaves of each plant. In the case of growth rate the first record of number of leaves (t_1) was taken 30 days after transplant corresponding to the plant juvenile stage while the second record was

taken 80 after transplant during the plant's reproductive stage. Finally, maternal plant fitness was estimated as the total number of fruits per plant since this has a strong correlation with total number of seeds ($r^2 = 0.984$, $P < 0.0001$, n= 35; Fornoni et al. 2003). Given that *D. stramonium* is a highly selfing species (Núñez-Farfán et al. 1996), total seed production is a good estimator of both maternal and paternal fitness.

Data Analyses

Genetic variances and heritabilities

Before constructing the genetic variance-covariance matrices (**G**), we first determined the existence of significant genetic variation for all measured traits. Broad-sense heritabilities (h_b^2) and coefficients of variation (CV) of family variance component were estimated performing mixed lineal models using the package lme (R.2.13). In these analyses the family term was included as a random factor. Because our experimental design was unbalanced, we used restricted maximum likelihood (REML) to estimate the family and individual (i.e. residual) components of variance (Shaw et al. 1995). A significant family effect was interpreted as evidence of the presence of genetic variation including additive and non-additive effects. Even though we tested the significance of the genetic component (i.e. family) using a likelihood-ratio test, we also implemented a jackknife resampling procedure in order to assess the statistical significance of h_b^2 and CV through the estimation of 95% confidence intervals (CI). In particular, the estimation of CI's allowed to test explicitly if h_b^2 and CV were different from zero.

Genetic variance-covariance matrices

A genetic variance-covariance matrix was estimated for each treatment. The estimation at the genetic level was performed directly from the covariances among-family means (Arnold and

194 Phillips 1999; Ashman 2003). Despite the fact that these matrices are biased estimates from the
195 matrices calculated via the variance components using mixed models, they are a better option
196 when parametric tests (Arnold and Phillips 1999) or extensive iterations procedures are going to
197 be implemented.

198 Univariate selection gradients

199 Natural selection gradients at the phenotypic level were estimated within each treatment
200 following the Lande and Arnold´s (1983) multiple regression approach (e.g. Wise and Rausher
201 2013). Mixed regression modeling was implemented since the term family was included as a
202 random factor. Including this term controlled for the lack of independence of observations
203 generated by the genetic relatedness among plants within families, when phenotypic estimations
204 were performed. In all cases, selection gradients were estimated assuming that plant fitness has a
205 normal distribution. However, because fitness (i.e. fruit production) was originated by a Poisson
206 process we confirmed the statistical significance of each term using a quasi-GLMM model
207 (glmmPQL function from the package MASS; R.13.0) (Zuur 2009). Growth rate, flowering date
208 and resistance to herbivory were standardized and plant fitness relativized. Finally, an ANCOVA
209 including the factor Treatment was performed to test for the presence of univariate diffuse
210 selection (see Sahli and Conner 2011).

211 Environmental effects on the multivariate response to selection

212 **G** and **β** were multiplied for each treatment combination to obtain the response to selection
213 vector **Δz**. Changes in the response to selection vector were tested through the analysis of
214 differences in the direction (angle θ) and magnitude (length: $|\mathbf{\Delta z_A}|/|\mathbf{\Delta z_B}|$) of **Δz** (Fig 1). To this
215 end we propose an extension of the skewer selection analysis named the carving fork analysis.

This test follows a similar rationale as that proposed by the random skewer selection analysis (Cheverud 1996). The skewer selection analysis tests for differences between variance-covariance matrices (**G**) through the comparison of the response to selection vectors that were obtained after multiplying the two observed **G** matrices by the same randomly generated vector of selection. If the average angle or length ratio between pairs of response to selection vectors is significantly larger than that expected by chance, the skewer selection analysis indicates a significant difference in the variance-covariance matrices between environments (treatments). Instead, our approach tested for differences between treatments in $\mathbf{\Delta z}$ with the aim of disentangling the importance of the observed vectors of selection and observed variance-covariance matrices.

The carving fork analysis (script is available in supporting information) is based on a randomization process to generate a null hypothesis of no differences between pairs of treatments. Genetic families were randomly reshuffled between two treatments. All the simulated treatments contain the full list of genetic families and individuals, without considering their place in the original treatments. This restriction during the construction of the null model was needed to avoid pseudo-treatments without genetic variability. After each randomization, we calculated for each treatment the vector of selection ($\boldsymbol{\beta}$), the variance-covariance matrix (**G**), which were used to obtain the vector of response to selection ($\mathbf{\Delta z}$), the angle between vectors (θ), and the ratio of the length between vectors ($|\ _{A}|/|\ _{B}|$). After 9,999 iterations we obtained an expected frequency distribution under the null hypothesis of no differences between treatments for each parameter. We rejected the null hypothesis if the observed value was higher than 95% of the pseudo-values. The total number of pseudo-values was estimated including the observed value

because if the null hypothesis is true the observed value is just another value from the randomization distribution (Manly 1997).

Based on this randomization procedure we tested the following hypotheses. Hereafter subscripts $_A$ and $_B$ correspond to any pair of treatments. The first hypothesis examined whether the direction (angle) and magnitude (length) of the vectors of selection ($\boldsymbol{\beta}_A$ and $\boldsymbol{\beta}_B$) are equal (*i.e.*, if $\theta_{AB} = 0$, and if $|\boldsymbol{\beta}_A|/|\boldsymbol{\beta}_B| = 1$). Significant differences in any of the properties of the vectors of selection gradients will indicate the existence of multivariate diffuse selection. Differences in the angle between vectors indicate that the relative strength (*i.e.*, direction in the multivariate space) of each selection gradients differ between treatments. Differences in the length between vectors indicate that these differ in the intensity of multivariate selection.

The second hypothesis tested whether the vectors of response to selection ($\boldsymbol{\Delta z}$) are equal in terms of direction and magnitude following the same rationale described above for the vector of selection. In other words, this test examined if there is evidence of differences in the multivariate response to selection ($\boldsymbol{\Delta z}$). Because **G** matrices may affect the response to selection either constraining (*i.e.* when response to selection vectors are equal despite of detected differences in the multivariate vector of selection) or enhancing it (*i.e.* when the response to selection vectors are different despite of undetected differences in the multivariate vector of selection), it is necessary to disentangle the relative role of $\boldsymbol{\beta}$ and **G** on changes in $\boldsymbol{\Delta z}$**.** To solve this issue we used the same randomization process while fixing, at the observed values, either $\boldsymbol{\beta}$ or **G** before estimating the differences in direction and length of $\boldsymbol{\Delta z}$'s. These procedures correspond to the null model with no differences between treatments either in the multivariate selection vectors or in the **G** matrices. In the first case (randomized $\boldsymbol{\beta}$**'**s, fixed **G'**s), we tested whether differences in $\boldsymbol{\beta}$ vectors between treatments account for differences between $\boldsymbol{\Delta z}_A$ and

261 $\mathbf{\Delta z_B}$. In the second scenario (randomized **G**'s, fixed **β'**s), we examined whether differences in **G**
262 between treatments accounted for differences in **Δz**'s. Because in both procedures the observed
263 values of the angle and length ratio between **Δz**'s may either be smaller than expected (for
264 example if **G** constrain the response to selection, see above) or larger than expected under the
265 null hypothesis, we considered a significant value if they were either smaller than 2.5% or larger
266 than 97.5% of the simulated values.

267 A Monte Carlo procedure was conducted in order to determine the number of
268 observations (genetic families in our case) needed to detect a significant angle between the
269 responses to selection vectors, considering **G** matrices with different number of traits. Results
270 presented as supplementary information indicates that the minimum observed angle needed to be
271 detected as statistically significant increases with the number of traits in **G**, but decreases with
272 the number of observations (Fig S1 A and B). For 50 observations in each treatment and three
273 characters (roughly our case), angles between responses to selection vectors greater that 0.8
274 radians (transforming as: radian × 180 / π, is equal to 45° degrees) could be detected as
275 significantly different from zero.

276 **RESULTS**

277 **Genetic variances and heritabilities**

278 For all plant traits significant genetic components of variance and heritabilities were detected
279 (Table S1). Life history traits (flowering date and growth rate) had higher coefficient of genetic
280 variation than resistance, and heritabilities ranged between 0.04 for flowering date in treatment **L**
281 to 0.12 for resistance in treatment **LE** (Table S1). The expression of heritabilities and CV of
282 genetic variation was altered by the experimental treatments. Significant genetic variation was

283 detected for resistance and flowering date but not for growth rate in the presence of *L.*
284 *daturaphila* (Treatments **LE** and **L**) (Table S1). In the presence *E. parvula* alone (Treatment **E**)
285 no variation was detected for resistance while significant variation was detected for growth rate
286 and flowering date (Table S1). For those treatments where *L. daturaphila* was present (treatment
287 **LE** than **L**), higher genetic variation for resistance and flowering date was detected (Table S1).
288 In general, the analyses detected a significant treatment effect on the diagonal component of the
289 **G**-matrix.

290 **Environmental effects on the selection gradients**

291 ANCOVA indicated that selection gradients differed among treatments as showed by the
292 significant Treatment × trait interaction (Table 1). In particular, this analysis detected that the
293 adaptive value of resistance was altered by the presence/absence of herbivores (i.e., Treatment ×
294 resistance effect). While no selection on resistance was observed in treatments **LE** and **E**,
295 directional selection favoring a reduction in resistance was observed in treatment **L** (Table S2).
296 The adaptive value of the combination of growth rate and flowering date also expressed a
297 significant treatment effect (i.e., Treatment × growth rate × flowering date) (Table 1). Only the
298 pattern of selection recorded in treatment **L** indicates the presence of correlational selection
299 favoring either higher growth rate and early flowering or slow growth rate and late flowering. In
300 all treatments higher growth rate was favored, and early flowering was selected in treatments **LE**
301 and **E** (Table S2). Overall, significant differences were detected in the individual components of
302 the vector of selection **β** among all pairs of treatments when the presence of two herbivore
303 species were independently manipulated (Table S3 to S5).

304 **Environmental effects on the multivariate response to selection**

Results indicated that the experimental manipulation of the ecological environment (presence/absence of two insect herbivores) altered the potential multivariate response to selection in the host plant. The direction of the response to selection vectors differed between treatments **LE** and **L** as well as between treatments **L** and **E** (Table 2; Fig 2). Marginal differences between treatment **L** and **E** were also detected for the vector of selection (Table 2; Fig 2). Despite having found variation in the individual selection gradients among treatments (see results above) we found fewer differences among properties of the vectors of selection (direction and length). In all contrasts, no differences were detected in the length of the vectors (Table 2; Fig 2).

Using the carving fork analyses we explored the causes of the environmental effects on the multivariate response to selection. The first series of analyses (randomizing **G´s** and fixing **β**), indicated treatment effect on **G** affecting (Fig 3) changes in **Δz.** Results indicated marginally significant differences between treatments **LE** and **L**, and significant differences between **L** and **E** in the direction of the response to selection vector (Table 3A; Fig 2). Also, marginal differences were detected between treatment **L** and **E** in the length of the response to selection vector (Table 3A; Fig 2). In particular we detected that environmental effects on the expression of **G** clearly changed the direction between **Δz** observed in treatment **L** and **E**.

The second series of analyses (randomizing **β** and fixing **G´s**), indicated treatment effects on **β** affecting changes in **Δz.** Because only the contrast **L** vs. **E** showed significant differences in the response to selection vector and marginal differences in the vector of selection gradients (Table 2; Fig 2), the second series of analyses focused mainly on this pair of treatments. Again a difference in the direction of the response to selection was detected for the contrast between treatments **L** and **E** (Table 3B; Fig 2). These results also indicated a significant effect of **β** on

changes in **Δz**. Significant differences were also detected for the length of the response to selection vector between treatments **LE** and **L** (Table 3B) but the absence of differences in the previous analyses precluded further interpretations. Qualitative differences between **G** matrices of treatments **L** and **E** indicated that the most important differences observed were changes in the intensity of the negative genetic correlation between growth rate and resistance and on the positive genetic correlation between growth rate and flowering date (Table 4).

DISCUSSION

Despite the presence of **G** × **E** interactions in nature is amply recognized (Schlichting & Pigliucci 1997; DeWitt and Scheiner 2004) its ultimate consequences on our ability to predict evolutionary changes have been little examined. In this study we provide experimental evidence showing the presence of significant **G** × **E** interactions affecting the expression of the **G** matrix and the response to selection favoring a fluctuating evolutionary pattern (i.e. changes in the evolutionary fate of the phenotype). Also environmentally induced changes in the vector of selection gradients affected the response to selection. The presence of **G** × **E** adds an additional component of variation promoting the instability of **G**, reducing the predictive power of the Breeder's equation. Thus the stability of **G** against local ecologically variable environments should not be taken for granted (Schlichting and Pigliucci 1997; Hoffman and Merilä 1999; Stinchcombe and Rausher 2001; Conner et al. 2003; DeWitt and Scheiner 2004). Our results from multivariate selection analyses suggest that future studies addressing evolutionary changes in plant defensive traits should included other correlated life history traits as these can be part of the plant defensive phenotype (Carmona et al. 2010). Although this suggestion applies in general to any adaptive trait and question the limits of an adaptation, studies of plant defense evolution usually does not include other (non-defensive) correlated traits.

A central assumption behind the use of the quantitative genetic approach as a long-term predicting tool, linking microevolutionary processes with macroevolutionary patterns, is the stability of the **G** matrix and the adaptive landscape (Lande 1979; Pigliucci and Kaplan 2006; Arnold et al. 2001; Steppan et al. 2002; Begin and Roff 2003, but see Lande 1979 and Schluter 1996). However, the occurrence of **G** × **E** at the population level can reduce the stability of **G**. Such alterations on the **G** matrix components (i.e. variance and covariances) has been previously considered (Stearns 1989; Pigliucci 2006) and empirically demonstrated (Falconer and Latyszewski 1952; Daday et al. 1973; Mazer and Schick 1991; Ebert et al. 1993; Simons and Roff 1994; Sgrò and Hoffmann 1998; Conner et al. 2003; Husby et al. 2011; Bégin and Roff 2001; Stinchcombe and Rausher 2001 and 2002; Fornoni et al. 2003; Cano et al, 2004). However, our results are the first to show that such alterations on **G** promoted by changes in the biotic environment can condition the response to selection. This empirical evidence indicates the importance of **G** × **E** as a source of variation affecting the predictive power of the Breeder´s equation (Mitchell-Olds and Rutledge 1985; Mazer and Schick 1991; Conner et al. 2003; Pigliucci and Kaplan 2006; Walsh and Blows 2009).

The instability of **G** is strongly dependent upon the number and kind of traits considered as well as on the particular responsiveness of each trait to changes in environmental conditions (Pigliucci 2006). Jones et al. (2003) theoretically demonstrated that **G** instability is common in matrices with low covariance structure (result of low pleiotropic mutation and low correlational selection after several generations) particularly in small populations. Hence, alteration in the correlational selection pattern due to diffuse selection (i.e. diffuse selection on correlational selection gradients as observed on Table 1) and alteration of the covariance structure due to direct environmental alteration (Stearns 1989) can condition the stability of **G**. Here, we found

such changes in the covariance structure when the correlation between growth rate and flowering date and negative genetic correlation between growth rate and resistance were estimated in different treatments.

The concept of diffuse selection (evolution) was proposed to determine whether predicting evolutionary changes require an explicit consideration of multispecific community contexts (Hougen-Eitzman and Rausher 1994; Strauss et al. 2005). Although initially applied to traits involved in the interaction between plants and herbivores (Hougen-Eitzman and Rausher 1994), the same rationale can be extended into many other ecological situations (e.g. Sahli and Conner 2011). If the evolution of two interacting species is affected by the presence of a third species, fluctuation in the presence/absence of this third species can affect the (co)evolution of focal interacting species. Empirical evidence using this protocol evaluated the occurrence of diffuse selection as changes in selection gradients of traits following Lande and Arnold's multiple regression approach (e.g. Juenger and Bergelson 1998; Stinchcombe and Rausher 2001; Gomez 2003; Cariveau et al 2004; Irwin et al 2004; Sahli and Conner 2011; Carmona and Fornoni 2013). Such approach allows understanding changes in the pattern of selection on each trait by examining separately their correspondent selection gradient (i.e. following a univariate perspective). Instead, we followed a multivariate approach examining changes in the vector of selection rather than on individual selection gradients (Conner 2012). In this study we found that whereas univariate diffuse selection was detected acting on resistance and on the correlation between growth rate and flowering date (Table 1 and Table S3-S5), the overall pattern from a multivariate perspective contrast with these results. Only marginal changes were detected in the direction of the vector of selection, when comparing the most extreme ecological scenarios (L vs. E). These results indicated that the conclusion about the necessity of including more species

(agents of selection) to understand the evolution of a trait can be misleading when we considered that evolution of traits occurs simultaneously and under complex phenotypes. Furthermore, extending our rationale to the response to selection vector allowed us to ask whether multivariate diffuse selection translates to multivariate diffuse evolution or not. In our study, the carving fork analysis revealed that multivariate diffuse selection is playing only a marginal role on the response to selection despite significant diffuse univariate selection gradients were detected. Since traits involved in adaptation can be integrated within functional modules a multivariate approach to predict evolutionary changes is desirable.

As natural selection erodes genetic constraints and the pattern of selection remains relatively constant, the **G** matrix is expected to align with the vector of selection (Schluter 1996). In this study, the shape of **G** matrix and the vector of selection observed when *L. daturaphila* was removed (i.e. **E** treatment) was statistically identical to the one observed in the treatment **LE** (Fig 2). As expected, we also found no differences in the response to selection between these treatments. Hence, we can infer that the generalist *E. parvula* is the main factor leading the evolutionary change in natural conditions. Under this scenario it is easy to imagine a similar evolutionary fate of the defensive phenotype under LE and E scenarios. Meanwhile, when the specialist *L. daturaphila* was the only herbivore present, the vectors of selection was marginally affected (as described above) when compared with the E treatment but the shape of **G** and consequently the response to selection was clearly affected when contrasted with either the natural environment (i.e. LE treatment) or the environment were *E. parvula* was only present. These results suggest that the presence/absence of the generalist *E. parvula* can make a strong difference in the defensive response to selection of *D. stramonium*. This can be due to two non exclusive arguments: a) the early damage produced by *E. parvula* can induce a change in the

growth rate and time to reproduction of the plant. Such effect cannot be produced by *L. daturaphila* since the phenology of damage starts when plants are in their reproductive stage; and b) because *E. parvula* is a generalist, life history traits alterations can be seen as a plant general response to a broad stressful environment (Hoffman and Merilä 1999) that when damage (i.e. stress) is produced by the specialist *L. daturaphila*. Finally, contrary to what it was traditionally expected, our results indicate that diffuse selection (i.e. $\boldsymbol{\beta} \times \mathbf{E}$) played a marginal role relative to the effect of $\mathbf{G} \times \mathbf{E}$ on **G** when promoting diffuse evolution.

Concluding remarks

Several studies have demonstrate that **G** matrix is stable at macroevolutionary scales (Lande 1979; Steppan et al. 2002; but see Pigliucci and Kaplan 2006), however, we highlight in this study the importance of addressing the stability of **G** matrix against fluctuating ecological environmental conditions promoted by other members of the community. Since it is at this level where quantitative genetic theory works with fewer assumptions (Pigliucci and Kaplan 2006), and more importantly, where the constant deterioration of the environment (multispecific context) occurs due changes in biotic interactions (Fisher 1930). In this way, we took the criteria built by Rausher and coworkers to distinguish between pairwise and diffuse coevolution as a protocol to examine a more general situation, a multivariate and dynamic phenotype evolving within a multispecific and dynamic selection regimes. We believe this can be considered as a fundamental protocol in the study of eco-evolutionary dynamics.

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562

563 Supporting information:

564 Table S1: Broad sense heritabilities and genetic variation for resistance.

565 Table S2: Phenotypic selection analyses for each treatment

566 Table S3-S5: Contrast between pair of treatments using an ANCOVA

Figure S1. Surface that describes how the minimum significant angle in radian above which the carving fork analysis become statistical sensible changes with the number of traits included in the analysis and the number of observations.

S6: R script for running a carving fork analysis.

Table 1. Contrast among treatments (LE, L and E) using an ANCOVA on fitness (total number of fruits). Procedure based on a GLMM (generalized linear mixed model) with poisson error distribution using the glmmPQL function to correct standard errors given the presence of overdispersion. Pairwise contrasts between treatments are reported in Table S3-S5.

Source of variation	Numerator d.f.	Denominator d.f.	F-value	p-value
Treatment (T)	**2**	**737**	**7.9507**	**0.0004**
Growth rate (G)	**1**	**737**	**100.9639**	**<.0001**
Resistance (R)	1	**737**	0.2784	0.5979
Flowering date (F)	**1**	**737**	**27.8661**	**<.0001**
T × G	2	**737**	1.7732	0.1705
T × R	**2**	**737**	**4.3534**	**0.0132**
T × F	2	**737**	2.1134	0.1216
T × G × R	3	**737**	0.5644	0.6386
T × G × F	**3**	**737**	**3.7325**	**0.0111**
T × R × F	3	**737**	2.1497	0.0927

Table 2. Results from the randomization tests to determine the existence of significant differences between pairs of treatments in the vector of selection (**β**), and the vector of the response to selection (**Δz**). These analyses were performed examining whether the direction (the angles $\boldsymbol{\theta}_{\boldsymbol{\beta}}$ and $\boldsymbol{\theta}_{\boldsymbol{\Delta Z}}$; above the diagonal) and the length (magnitude) between two vectors (|**β**|/|**β**| and |**Δz** |/|**Δz**|; below the diagonal) are significantly different. Angles are reported in radians. † marginally significant p-value. See figure 2 to have a depicted reference of these results.

Multivariate fluctuating selection (evolution)			
	LE	**L**	**E**
LE		$\boldsymbol{\theta}_{\beta}$ = 0.37, *P* = 0.2	$\boldsymbol{\theta}_{\beta}$ = 0.31, *P* = 0.30
		$\boldsymbol{\theta}_{\Delta z}$ = 0.77, *P* = 0.036	$\boldsymbol{\theta}_{\Delta z}$ = 0.33, *P* = 0.41
L	**|β|/|β|** = 0.80, *P* = 0.16		$\boldsymbol{\theta}_{\beta}$ = *0.51, P = 0.06*†
	|Δz|/|Δz| = 0.93, *P* = 0.17		**$\boldsymbol{\theta}_{\Delta z}$ = 0.91, *P* = 0.008**
E	**|β|/|β|** = 1.44, *P* =0.27	**|β|/|β|** = 1.79, *P* = 0.16	
	|Δz|/|Δz| = 1.23, *P* = 0.31	**|Δz|/|Δz|** = 1.31, *P* = 0.17	

Table 3. Results from the randomized carving fork analysis to test whether treatment effects on **G** and/or **β** account for differences in **Δz** between treatments. **A**) Results of the analyses searching for the differences between treatments in the direction (angle) and magnitude (length) of the response to selection vector (**Δz**) using randomized values of **G** and fixed (observed) values of **β**. A significant effect indicate that differences in **Δz** correspond to treatment effects on **G**. **B**) Results of the analyses searching for the differences between treatments in the direction (angle, θ) and magnitude (length, $|\ _a| / |\ _b|$ or $|\mathbf{\Delta z_a}| / |\mathbf{\Delta z_b}|$) of the response to selection vector (**Δz**) using randomized values of **β** and fixed (observed) values of **G**. A significant effect indicates that differences in **Δz** correspond to treatment effects on **β**. Significant p-values are indicated in bold. † marginally significant p-value.

A

Effect of G on Δz

Angle		Length	
Contrast	p-value	Contrast	p-value
$\boldsymbol{\theta}_{\mathbf{\Delta z}[\text{LE vs. L}]}$	$0.087^{\dagger}$	$\lvert\mathbf{\Delta z}_{[\text{LE}]}\rvert / \lvert\mathbf{\Delta z}_{[\text{L}]}\rvert$	0.909
$\boldsymbol{\theta}_{\mathbf{\Delta z}[\text{LE vs. E}]}$	0.365	$\lvert\mathbf{\Delta z}_{[\text{LE}]}\rvert / \lvert\mathbf{\Delta z}_{[\text{E}]}\rvert$	0.352
$\boldsymbol{\theta}_{\mathbf{\Delta z}[\text{L vs. E}]}$	**0.021**	$\lvert\mathbf{\Delta z}_{[\text{L}]}\rvert / \lvert\mathbf{\Delta z}_{[\text{E}]}\rvert$	$0.064^{\dagger}$

B

Effect of β on Δz

Angle		Length	
Contrast	p-value	Constrast	p-value
$\boldsymbol{\theta}_{\mathbf{\Delta z}[\text{LE vs. L}]}$	$0.050^{\dagger}$	$\lvert\mathbf{\Delta z}_{[\text{LE}]}\rvert / \lvert\mathbf{\Delta z}_{[\text{L}]}\rvert$	**0.028**
$\boldsymbol{\theta}_{\mathbf{\Delta z}[\text{LE vs. E}]}$	0.348	$\lvert\mathbf{\Delta z}_{[\text{LE}]}\rvert / \lvert\mathbf{\Delta z}_{[\text{E}]}\rvert$	0.132
$\boldsymbol{\theta}_{\mathbf{\Delta z}[\text{L vs. E}]}$	**0.035**	$\lvert\mathbf{\Delta z}_{[\text{L}]}\rvert / \lvert\mathbf{\Delta z}_{[\text{E}]}\rvert$	0.896

606 Table 4. For each treatment, the composition of vectors of selection (**β**; lineal selection gradients
607 reported in Table S2), variance-covariance genetic matrices (**G**) and vectors of response to
608 selection (**Δz**) are reported. LE: presence of both herbivores (*L. daturaphila* and *E. parvula*), L:
609 presence of *L. daturaphila*, E: presence of *E. parvula*.

Treatments	**β**	**G**			**Δz**
		Growth rate	Resistance	Flowering date	
LE					
Growth rate	**0.374**	1			0.362
Resistance	-0.022	-0.1359	1		-0.007
Flowering date	**-0.282**	0.0535	-0.2300	1	-0.257
L					
Growth rate	**0.360**	1			0.331
Resistance	**-0.140**	-0.1788	1		-0.184
Flowering date	-0.138	0.3889	-0.1449	1	0.022
E					
Growth rate	**0.482**	1			0.485
Resistance	0.093	0.0057	1		0.152
Flowering date	**-0.209**	-0.0136	-0.2700	1	-0.241

610

Figure 1. Graphical representation of the multivariate breeder equation ($\mathbf{\Delta z} = \mathbf{G} \times \boldsymbol{\beta}$) considering a pair of hypothetical traits. The vector of selection is constructed using the selection gradients estimated following the multiple regression approach between these two traits and relative fitness (e.g. $w = z_1 + z_2$). The ellipse describing the **G** matrix is produced by the pattern of genetic variance and covariance of two hypothetical traits traits (z_1 and z_2). Here we present the simplest case: a pair of traits with equal variances and no covariance between them. A perfect circle indicates equal variances among traits, while, the crux within the circle indicates the orientation of the eigenvectors. When covariance between variables is equal to 0, the eigenvectors (i.e. the crux) become parallel to its corresponded axis (as in this case). **Δz** is the product of the vector of selection (**β**) and the **G** matrix. | **β** | and | **Δz** | denotes the length that can be interpreted in the former case as the magnitude of selection and in the latter case as the magnitude of evolutionary change. θ_{β} and $\theta_{\Delta z}$ denotes an angle and can be interpreted as the direction of selection and the direction of evolutionary change, respectively. Finally, alterations in the environment can cause changes in **G** or **β** and consequently modifications in **Δz**, in other words, in the evolutionary dynamic of the phenotype.

Figure 2. Graphical representation of diffuse multivariate selection and evolution. Here we only represent alterations on the direction of the vectors because no alteration on the vector´s length was observed. Angles in radians from Table 2 were transformed (radians × 180 / π) into degrees for the sake of the visualization.

Figure 3. Graphical representation of the contrast between **G** matrices estimated under three experimental conditions: when the damage is produced by *L. daturaphila* and *E. parvula* (LE), only *E. parvula* (E) and only *L. daturaphila* (L).

Figure 1.

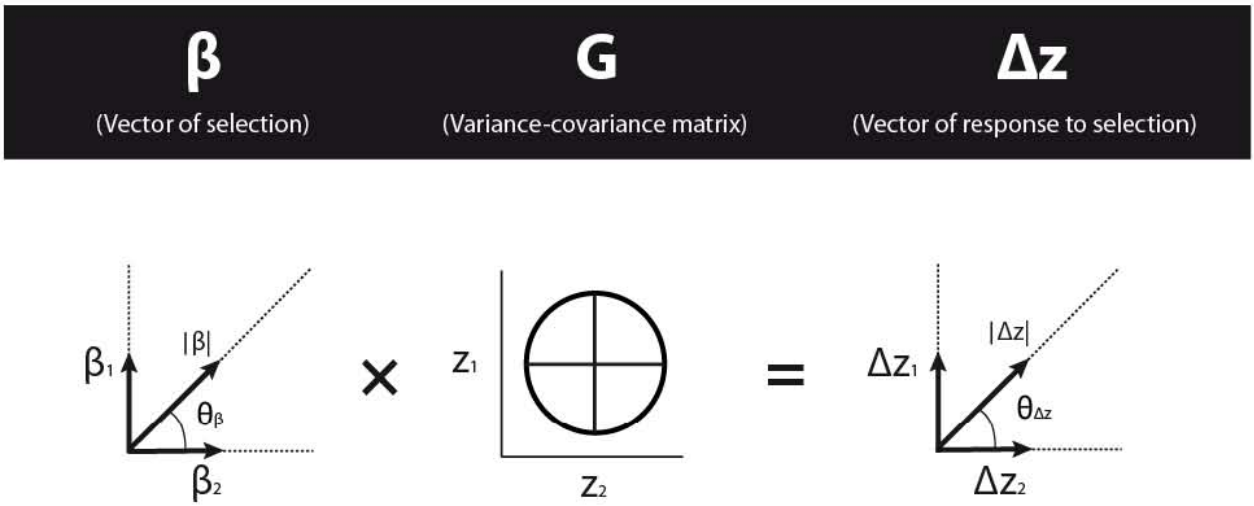

β
(Vector of selection)
G
(Variance-covariance matrix)
Δz
(Vector of response to selection)
β1
|β|
θβ
β2
×
z1
z2
=
Δz1
|Δz|
θΔz
Δz2

Figure 2.

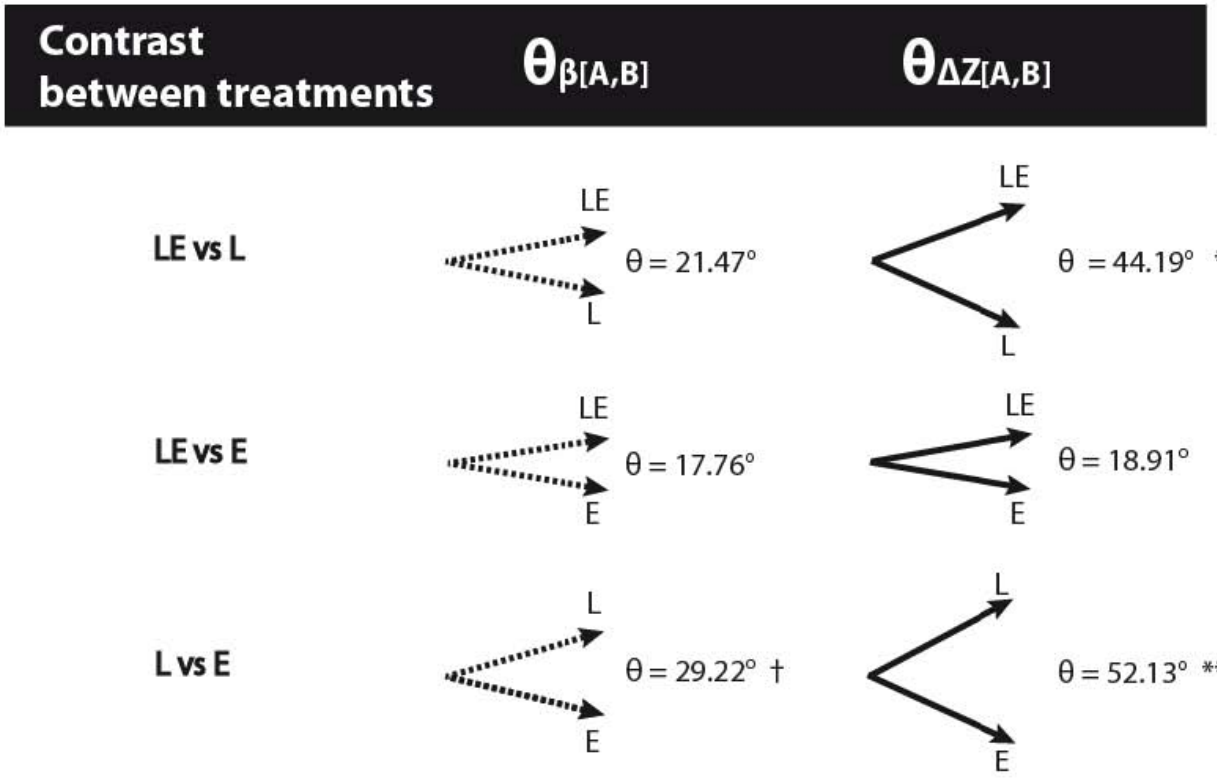
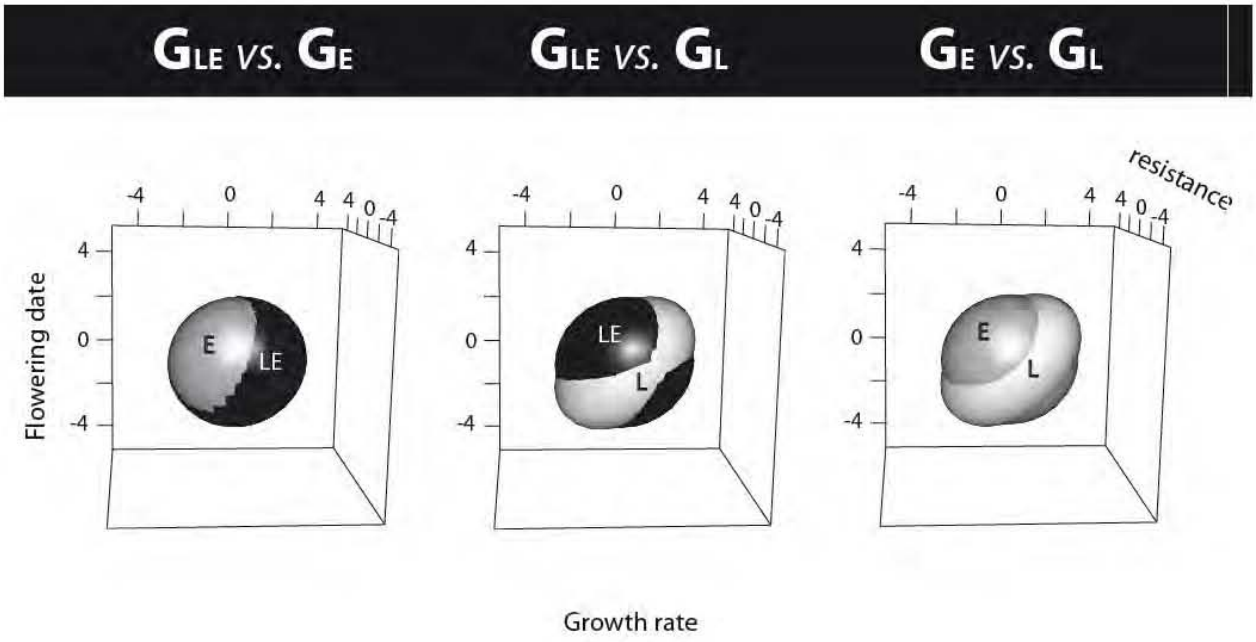

Contrast between treatments
θβ[A,B]
θΔZ[A,B]
LE vs L
LE
L
θ = 21.47°
LE
L
θ = 44.19° *
LE vs E
LE
E
θ = 17.76°
LE
E
θ = 18.91°
L vs E
L
E
θ = 29.22° †
L
E
θ = 52.13° **

Figure 3.


GLE VS. GE
GLE VS. GL
GE VS. GL
resistance
Flowering date
Growth rate
E
LE
L

Supporting information

Table S1. Broad sense heritabilities and coefficient of genetic variation for resistance to herbivory, growth rate and flowering date. Within parentheses confidence intervals (CI) obtained after jackknife is reported. Letters summarized the differences among treatments detected when CIs do not overlap between them. CIs not including 0 are also denoted as statistically significant (*).

Parameter estimated	**Mean across treatments**	**Within treatments**		
		LE	L	E
		Resistance		
h^2	0.041*	[a] 0.119*	[b] 0.068*	[c] 0.003
	(0.038-0.044)	(0.111-0.128)	(0.060-0.075)	(-0.000-0.006)
CV	1.947*	[a] 3.471*	[b] 2.371*	[c] 0.320
	(1.876-2.0179)	(3.336-3.606)	(2.238-2.2503)	(0.197-0.531)
		Growth rate		
h^2	0.025*	0	0	[a] 0.062*
	(0.022-0.028)			(0.054-0.071)
CV	4.518*	0	0	[a] 6.836*
	(4.247-4.789)			(6.366-7.307)
		Flowering date		
h^2	0.059*	[a] 0.075*	[b] 0.040*	[bc] 0.051*
	(0.055-0.064)	(0.065-0.086)	(0.033-0.047)	(0.046-0.056)
CV	4.445*	[a] 4.900*	[b] 3.663*	[bc] 3.906*
	(4.273-4.617)	(4.537-5.264)	(3.319-4.007)	(3.704-4.108)

Table S2. Phenotypic directional and correlative selection gradients (β) acting on growth rate, resistance and flowering day in three different environments. The estimation of selection (β and γ) were obtained from a multiple regression model with normal errors, while the statistical significance was examined using glmmPQL with quasipoisson error distribution. The factor *family* was considered random in order to control for the covariance among replicates within genetic families (see methods).

				glmmPQL	
LE)			d.f	F	P-value
	β	s.e.			
Growth (G)	**0.374**	**0.0831**	**1, 217**	**24.43102**	**<.0001**
Resistance (R)	-0.022	0.0831	**1, 217**	0.15925	0.6902
Flowering day (F)	**-0.282**	**0.0825**	**1, 217**	**16.75674**	**0.0001**
G × R	0.018	0.076	1, 211	0.0587	0.9057
G × F	-0.190	0.069	1, 211	2.5156	0.1610
R × F	0.119	0.100	1, 211	1.0781	0.3003
L)					
	β	s.e.			
G	**0.360**	**0.089**	**1, 213**	**20.1575**	**<0.0001**
R	**-0.140**	**0.088**	1, 213	**3.9271**	**0.0488**
F	-0.138	0.088	1, 213	2.8842	0.0909
G × R	-0.102	0.099	1, 207	0.2788	0.5981
G × F	**-0.224**	**0.090**	**1, 207**	**5.2538**	**0.0229**
R × F	-0.054	0.104	1, 207	0.8194	0.3664
E)					
	β	s.e.			
G	**0.482**	**0.093**	**1, 198**	**38.1658**	**<0.0001**
R	0.093	0.093	1, 198	2.4921	0.1151
F	**-0.209**	**0.093**	**1, 198**	**9.0349**	**0.0030**
G × R	-0.016	0.087	1,192	1.3081	0.2531
G × F	-0.140	0.088	1,192	0.2557	0.6194
R × F	-0.035	0.108	1,192	0.0387	0.8442

Table S3. Contrast between treatments LE *vs.* L using an ANCOVA on fitness (total number of fruits). Procedure based on a GLMM (generalized linear mixed model) with poisson error distribution using the glmmPQL function to correct standard errors given the presence of overdispersion.

Source of variation	Numerator d.f.	Denominator d.f.	F-value	p-value
Treatment (T)	**1**	**487**	**14.5482**	**0.0002**
Growth rate (G)	**1**	**487**	**60.8552**	**<.0001**
Resistance (R)	1	**487**	3.7117	0.0546†
Flowering date (F)	**1**	**487**	**15.9230**	**0.0001**
T × G	1	**487**	0.4164	0.5191
T × R	1	487	0.5884	0.4434
T × F	**1**	**487**	**4.4621**	**0.0352**
T × G × R	2	**487**	0.6461	0.5245
T × G × F	**2**	**487**	**4.6486**	**0.0100**
T × R × F	**2**	**487**	**2.9286**	**0.0544**

Table S4. Contrast between treatments LE *vs.* E using an ANCOVA on fitness (total number of fruits). Procedure based on a GLMM (generalized linear mixed model) with poisson error distribution using the glmmPQL function to correct standard errors given the presence of overdispersion.

Source of variation	Numerator d.f.	Denominator d.f.	F-value	p-value
Treatment (T)	**1**	**471**	**13.8961**	**0.0002**
Growth rate (G)	**1**	**471**	**77.6795**	**<.0001**
Resistance (R)	1	471	0.9406	0.3326
Flowering date (F)	**1**	**471**	**30.9507**	**<.0001**
T × G	1	471	1.5457	0.2144
T × R	**1**	**471**	**5.8023**	**0.0164**
T × F	1	471	0.9592	0.3279
T × G × R	2	471	0.4759	0.6216
T × G × F	2	471	2.3941	0.0924
T × R × F	2	471	1.2992	0.2737

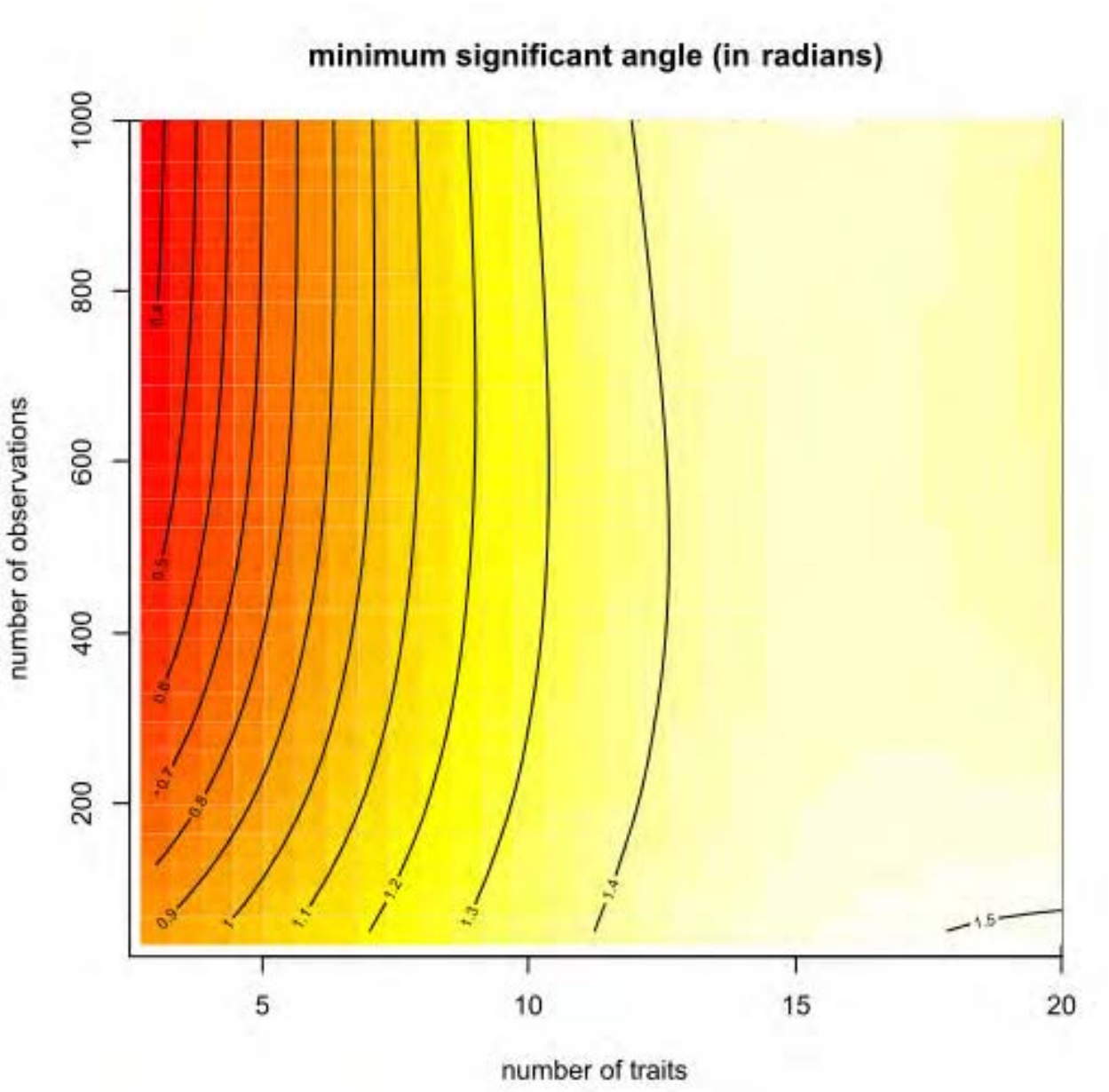
Table S5. Contrast between treatments L *vs.* E using an ANCOVA on fitness (total number of fruits). Procedure based on a GLMM (generalized linear mixed model) with poisson error distribution using the glmmPQL function to correct standard errors given the presence of overdispersion.

Source of variation	**Numerator d.f.**	**Denominator d.f.**	**F-value**	**p-value**
Treatment (T)	1	465	0.0018	0.9661
Growth rate (G)	**1**	**465**	**67.2224**	**<.0001**
Resistance (R)	1	465	0.0030	0.9563
Flowering date (F)	**1**	**465**	**11.7503**	**0.0007**
T × G	1	465	2.3045	0.1297
T × R	**1**	**465**	**7.9046**	**0.0051**
T × F	1	465	1.1692	0.2801
T × G × R	2	465	0.3813	0.6832
T × G × F	**2**	**465**	**3.9850**	**0.0192**
T × R × F	2	465	1.8395	0.1600

Figure S1. Monte Carlo procedure to determine the number of observations (genetic families in our case) needed to detect a significant angle given in radians between the responses to selection vectors, considering **G** matrices with different number of traits. A) When 20 traits are considered, B) a zoom in considering from three to six traits. Number overlapping the isocline denotes the minimum significant angle in radian above which the carving fork analysis become statistical sensible. It is clear that as the number of traits increase the number of observations must also be increased in order to evaluate significant angles.

780 Figure S1.

781 A)

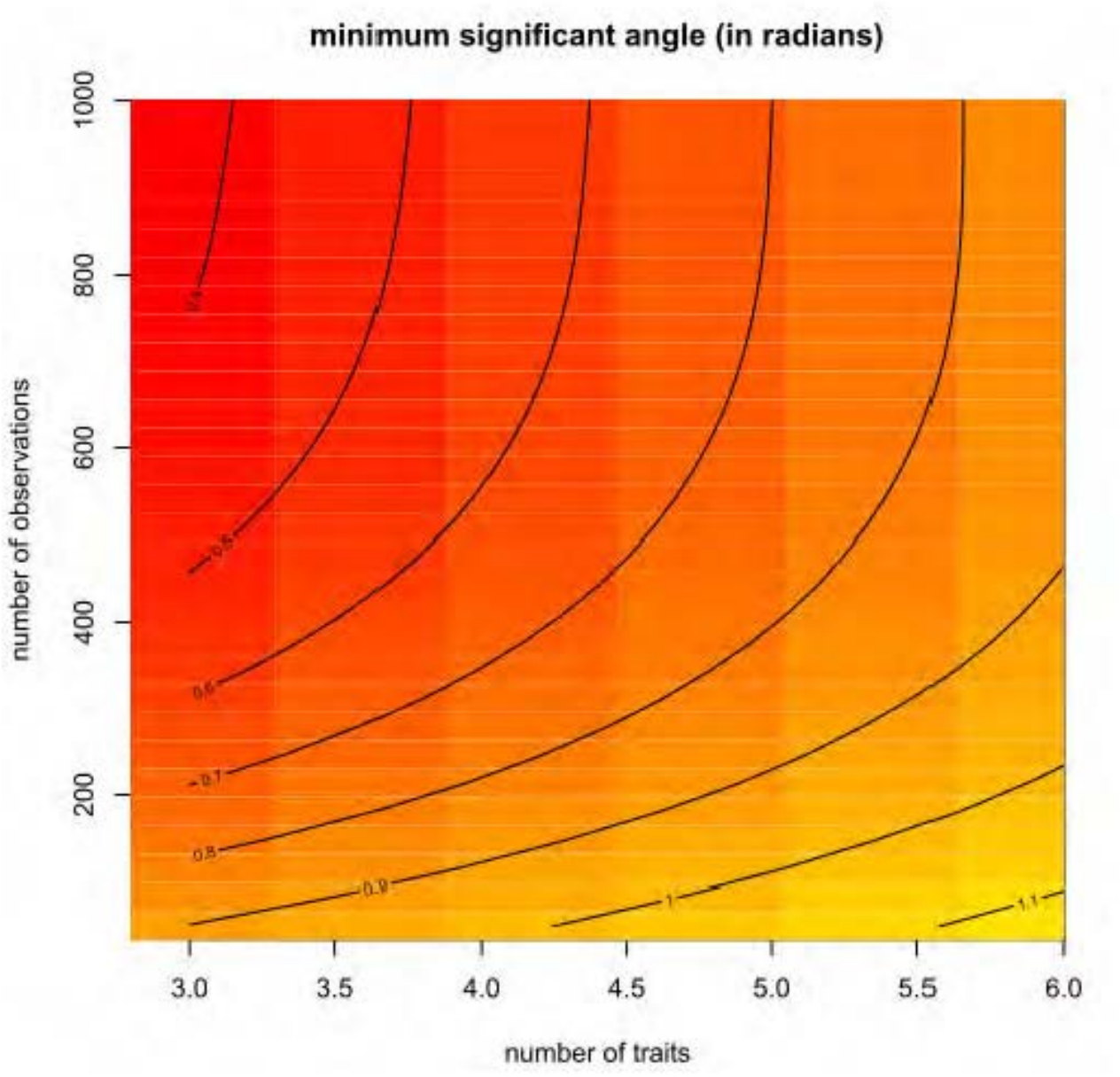


782

783

784

B)


minimum significant angle (in radians)
number of observations
1000
800
600
400
200
0.5
0.6
0.7
0.8
0.9
1
1.1
3.0
3.5
4.0
4.5
5.0
5.5
6.0
number of traits

```
# S6

##############################################################################################
# R script for the carving fork selection analysis that allows to estimate the
diffuse evolutionary patterns
# and detect the importance of diffuse selection and G matrix simultaneously.
#
##############################################################################################
#                PROGRAM NOTES
                 #
##############################################################################################
# 1. R available from http://www.r-project.org/
# 2. This program uses the "lme4" package for R, therefore such package needs to be
installed prior
# to  using the script. To install, type the followin at the command prompt upon
opening R.
# bla bla bla
##############################################################################################
#                DESCRIPTION OF INPUT FILE FORMATS
                 #
##############################################################################################
# The script expect just 1 data base in which at least two treatments (or
environments) must be included.
# Because the carving fork is a pairwise test, if more than two treatments are
included in de data base they
# can be subseted as showed below. The data base most includ fitness, focal traits
and possible covariates
# factors to remove undesirable effects (as showed below).
#
# Note that each file should contain a header row containing names for the
# appropriate fitness component, trait, or covariate.
#
#

# Loading package

library (lme4)



##############################################################################
#                            BEGIN USER INPUT SECTION                        #
#           (Note the direction of slashes in specifying file paths)         #
##############################################################################

#
###---------------------------------------###
### 0- Loading database
###---------------------------------------###

# using linux

basep<-read.table("/.../database.txt", header = TRUE)
basep$bloque<-as.factor(basep$bloque)
basep$fam<-as.factor (basep$fam)
```

```
# using windows
basep<-read.table("C:/.../database.txt", header = TRUE)
basep$bloque<-as.factor(basep$bloque)
basep$fam<-as.factor (basep$fam)


###---------------------------------------###
### 0- Subseting principal data base
###---------------------------------------###

Base.LE<-subset (basep,trat=="LE")
Base.L<-subset (basep,trat=="L")
Base.E<-subset (basep,trat=="E")
Base.S<-subset (basep,trat=="S")

###---------------------------------------###
### Selecting the treatments to work, the number
### of iterations, and the columns to be considered
### in the analysis
###---------------------------------------###

a<-Base.LE # Enter the name of the subseted data base that must be used in the
analysis
b<-Base.L  # Enter the name of the subseted data base that must be used in the
analysis
num_iter<-9999  # enter the number of iterations

A<-na.omit(a[,c(2,3,6,7,8,9,11)]) # Select the columns to be considered
B<-na.omit(b[,c(2,3,6,7,8,9,11)]) # select the columns to be considered


###---------------------------------------###
### 0- Removing undesirable effects with covariates
###---------------------------------------###

# A

afitc <-lm(crece ~ dtrans + bloque, data=A)
A$rcrece<-scale(afitc$res)
afitr <-lm (R~dtrans+bloque, data=A)
A$rR <-scale(afitr$res)
afitf <-lm (flor~dtrans+bloque, data=A)
A$rf <-scale(afitf$res)

# B
bfitc<-lm (crece~dtrans+bloque, data=B)
B$rcrece<-scale(bfitc$res)
bfitr<-lm (R~dtrans+bloque, data=B)
B$rR<-scale(bfitr$res)
bfitf<-lm (flor~dtrans+bloque, data=B)
B$rf<-scale(bfitf$res)

###---------------------------------------###
### 0- Estimating relative fitness
###---------------------------------------###

A$relw<-A$frutos/mean(A$frutos)
B$relw<-B$frutos/mean(B$frutos)



#########################################################
#####   Needed Functions
```

```
#########################################################

###-------------------------------------------------####
### 1- estimate G based on family means
###-------------------------------------------------####

### ARGUMENTS
# base: data.frame object including the phenotypic variables to be used.
# vars: a vector indicating the phenotypic variables to be used.
# type: Use "G" to estimate G based on family means. Use "P" to estimate P matrix
based on individual values.
# scale: if TRUE, fucntion estimates correlation matrix, otherwise covariance matrix
is estimated.
# fam: Only if Type = "G". Factor indicating families. Same length as base

### OUTPUT
# a correlation  or covariance matrix

estG <- function(base, vars, fam, type = "G", scale = TRUE){
  if (type == "G"){
    if (scale == TRUE) {
  m1 <- aggregate(base[,vars], by=list(base[, fam]), mean, na.rm=TRUE)
  cor(m1[,-1])
    }
    else {
      m1 <- aggregate(base[,vars], by=list(base[, fam]), mean, na.rm=TRUE)
      cov(m1[,-1])
    }
  }
  else {
    if (scale == TRUE) {
      cor(base[,vars])
    }
    else {
      cov(base[,vars])
    }
  }
}

###EXAMPLE
Ga <- estG(base= A, vars= c("rcrece", "rR", "rf"), fam = "fam", type ="G",
scale=TRUE)
Ga

###-----------------------------------------###
### 2- estimate linear selection gradients
###-----------------------------------------###

### ARGUMENTS
# formula: a formula object for lm or lmer, used to estimate linear selection
gradients
# base: a data.frame object containing fitness values (scaled to mean=1) and
# (standardized) phenotypic variables. Optionally, it may contain information about
families.
# Type: use "lmer" (the deffault) to estimate linear gradients using mixed models
through the lmer function
# from lme4 package. Otherwise gradiets are estimated using a common linear model
through the lm function
# from stats package

### OUTPUT
# a numeric vector od standardized linear selection gradients. Intercept is ommited
```

```
estB <- function(formula, base, type = "lmer"){
  if (type == "lmer") {
    afitbeta<-lmer(formula=as.formula(formula),data=base)
    abeta.obs  <-fixef(afitbeta)
    abeta.obs[-1]
  }
  else {
    afitbeta<-lm(formula=as.formula(formula),data=base)
    coef(afitbeta)[-1]
  }
}

###EXAMPLE
Ba <- estB(relw~rcrece+rR+rf + (rcrece|fam) + (rR|fam) + (rf|fam), base = A, type =
"lmer")
Ba
Ba <- estB(relw~rcrece+rR+rf, base = A, type = "lm")
Ba

###-----------------------------------###
### 3- scaling vectors to unit length
###-----------------------------------###

### ARGUMENTS
# vec: a numeric vector

### OUTPUT
# a (scaled) numeric vector

scaleV <- function(vec){
  escL <- vec/sqrt((t(vec)%*%vec))
  escL
}

### EXAMPLE
scaleV(Ba)

###-----------------------------------###
### 4- estimate angle between vectors
###-----------------------------------###

# ARGUMENTS:
# vec1, vec2: two numeric vectors of the same length

### OUTPUT
# an angle (in radians)

estA <- function(vec1, vec2){
  ang <- c(acos((t(vec1)%*%vec2)/ sqrt( (t(vec1)%*%vec1)*(t(vec2)%*%vec2))))
  ang
}

###EXAMPLE
Ba <- estB(relw~rcrece+rR+rf + (1|fam), base=A, type="lmer")
Bb <- estB(relw~rcrece+rR+rf + (1|fam), base=B, type="lmer")
estA(Ba, Bb)

###-----------------------------------------###
### 5- estimate ratio between vector lengths
###-----------------------------------------###

# ARGUMENTS:
# vec1, vec2: two numeric vectors of the same length
```

```
### OUTPUT
# the ratio between vector lengths

estL <- function(vec1, vec2){
  lon<-sum(vec1^2)/sum(vec2^2)
  lon
}

###EXAMPLE
Ba <- estB(relw~rcrece+rR+rf + (1|fam), base=A, type="lmer")
Bb <- estB(relw~rcrece+rR+rf + (1|fam), base=B, type="lmer")
estL(Ba, Bb)


#############################################################################
### estimate observed parameters for carving fork analysis
#############################################################################

### ARGUMENTS

# formula: argument to be passed to estB function.
# A formula object for lm or lmer, used to estimate linear selection gradients.

# vars: argument to be passed to estG fucntion. A vector indicating the phenotypic
variables to be used.

# fam1, fam2: name of the column with information about family identity in base1 or
base2 data frames.

# base1, base2: data frames containing standardized phenotypic traits, relative
fitness values,
# and family information

# Btype: argument to be passed to estB function. Use "lmer" (the deffault) to
estimate linear gradients
# using mixed models through the lmer function from lme4 package. Otherwise gradiets
are estimated using
# a common linear model through the lm function from stats package.

# Gtype: argument to be passed to estG function. Use "G" to estimate G based on
family means.
# Use "P" to estimate P matrix based on individual values.

# scale: argument to be passed to estG function. If TRUE (the deffault), G is
estimated as a correlation
# matrix. Otherwise G is estimated as a covariance matrix.

### OUTPUT
# A named vector containing the observed values needed for carving fork analysis.
Include the angle between
# selection gradient vectors (ang.b), the angle between response to selection
vectors (ang.z),
# the ratio between selection gradient vector lengths (lr.b), the ratio between
response to selection
# vector lengths (lr.z), the absolute differences between each selection gradient
(dif.b.1, dif.b.2, etc.),
# the absolute differences between each response to selection (dif.z.1, dif.z.2,
etc.) and the absolute
# differences between corresponding cells in G matrices (for example dif.G.1.1
indicate differences between
# the variances of trait 1; dif.G.3.4 indicate differences between the covariances
of traits 3 and 4).
```

```
carving.obs<-function(formula, vars, fam1, fam2, base1, base2, Btype="lmer",
Gtype="G", scale = TRUE){
  Ba <- estB(as.formula(formula), base = base1, type = Btype)
  Bb <- estB(as.formula(formula), base = base2, type = Btype)
  Ga <- estG(base1, vars, fam = fam1, type = Gtype, scale = scale)
  Gb <- estG(base2, vars, fam = fam2, type = Gtype, scale = scale)
  Za <- Ga%*%scaleV(Ba)
  Zb <- Gb%*%scaleV(Bb)
  angB <- c(acos((t(Ba)%*%Bb)/ sqrt( (t(Ba)%*%Ba)*(t(Bb)%*%Bb))))
  angZ <- c(acos((t(Za)%*%Zb)/ sqrt( (t(Za)%*%Za)*(t(Zb)%*%Zb))))
  LB <- estL(Ba,Bb)
  LZ <- estL(Za,Zb)
  res <- c(angB, angZ, LB, LZ, abs(Ba-Bb), abs(Za-Zb), abs(c(Ga)-c(Gb)))
  #nombrador
  Bdif<-paste("dif.b", 1:length(Ba), sep=".")
  Zdif<-paste("dif.z", 1:length(Ba), sep=".")
  g1<-paste(rep(c(1:length(Ba)), each= length(Ba)), rep(c(1:length(Ba)),
length(Ba)), sep=".")
  g2<-paste("dif.G", g1, sep=".")
  nom<-c("ang.b", "ang.z", "lr.b", "lr.z", Bdif, Zdif, g2)
  #salida
  names(res)<-nom
  res
}

### EXAMPLE
OBS<-carving.obs(formula= relw~rcrece+rR+rf + (1|fam), vars = c("rcrece","rR",
"rf"),
                 fam1= "fam", fam2 = "fam", base1=A, base2=B, Btype="lmer",
Gtype="G", scale = TRUE)
OBS


#################################################################
###CARVING FORK ANALYSIS
#################################################################
###ARGUMENTS
# N: Number of randomizations to be used. Deffault is 1000.

# formula: argument to be passed to estB function.
# A formula object for lm or lmer, used to estimate linear selection gradients.

# vars: argument to be passed to estG fucntion.
# A vector indicating the phenotypic variables to be used.

# fam1, fam2: name of the column with information about family identity in base1 or
base2 data frames.

# base1, base2: data frames containing standardized phenotypic traits, relative
fitness values,
# and family information

# Btype: argument to be passed to estB function. Use "lmer" (the deffault) to
estimate linear gradients
# using mixed models through the lmer function from lme4 package. Otherwise gradiets
are estimated using
# a common linear model through the lm function from stats package.

# Gtype: argument to be passed to estG function. Use "G" to estimate G based on
family means.
# Use "P" to estimate P matrix based on individual values.
```

```
# scale: argument to be passed to estG function. If TRUE (the deffault), G is
estimated as a correlation
# matrix. Otherwise G is estimated as a covariance matrix.

# H0: Hypothesis to be tested. 1 (the deffault) test for diffuse selection and
diffuse evolution.
# Indicates that linear gradients and G matrices are estimated in each randomization
# 2 test for the importance of G matrix in diffuse evolution. Indicates that G
matrices are
# estimated in each randomization, but observed gradients values are used to build
the response to selection.
# 3 (or any character) test for the importance of phenotypic selection in diffuse
evolution.
# Indicates that linear selection gradients are estimated en each randomization, but
observed G matrices
# are used to build the response to selection.

### OUTPUT
# A named data.frame containing columns with the pseudo-values needed for carving
fork analysis, and one
# row for each randomization. Include the angle between selection gradient vectors
(ang.b), the angle
# between response to selection vectors (ang.z), the ratio between selection
gradient vector lengths
# (lr.b), the ratio between response to selection vector lengths (lr.z), the
absolute differences between
# each selection gradient (dif.b.1, dif.b.2, etc.), the absolute differences between
each response to
# selection (dif.z.1, dif.z.2, etc.) and the absolute differences between
corresponding cells in G matrices
# (for example dif.G.1.1 indicate differences between the variances of trait 1;
dif.G.3.4 indicate
# differences between the covariances of traits 3 and 4).

carving.rand<-function (N=1000, formula, vars, fam1, fam2, base1, base2,
                        Btype="lmer", Gtype="G", scale = TRUE, H0 = 1){
  carving.int<-function(formula, vars, fam1, fam2, base1, base2, Btype, Gtype,
scale, H0){
    #warning
    Lf1<-levels(base1[,fam1])
    Lf2<-levels(base2[,fam2])
    if(length(Lf1) != length(Lf1[Lf1 %in% Lf2])) {
      warning ("different number of families")
    } else {
      F<-levels(base1[,fam1])
    }
    #asignador
    T<-c("t1","t2")
    Z1<- numeric(length(F))
    for (i in 1:length(F)) Z1[i]<-sample(T, size=1)
    Z1<-as.factor(Z1)
    Z2<-factor(Z1, labels=c("t2", "t1"))
    #creador de los nuevos tratamientos
    if (length(levels(Z1))>1) {
      res <- data.frame(F, Z1, Z2)
      L1 <- subset(res$F, res$Z1 == "t1")
      H1 <- list()
      for (i in 1:length(L1)) H1[[i]]<-which(base1[,fam1] == L1[i])
      H1 <- unlist(H1)
      L2 <- subset(res$F, res$Z2=="t1")
      H2 <- list()
      for  (i in 1:length(L2)) H2[[i]]<-which(base2[,fam2] == L2[i])
```

```
    H2 <- unlist(H2)
    new.t.1 <- rbind(base1[H1, ], base2[H2, ])
    new.t.2 <- rbind(base1[-H1, ], base2[-H2, ])
  } else {
    new.t.1 <- base1
    new.t.2 <- base2
  }
  if (H0 == 1){
    rta<-carving.obs(as.formula(formula), vars = vars, fam1 = fam1, fam2 = fam2,
                     base1 = new.t.1, base2 = new.t.2,
                     Btype = Btype, Gtype = Gtype, scale = scale)
    rta
  } else {
    if(H0 == 2) {
      Ba <- estB(as.formula(formula), base = base1, type = Btype)
      Bb <- estB(as.formula(formula), base = base2, type = Btype)
      Ga <- estG(new.t.1, vars, fam = fam1, type = Gtype, scale = scale)
      Gb <- estG(new.t.2, vars, fam = fam2, type = Gtype, scale = scale)
      Za <- Ga%*%scaleV(Ba)
      Zb <- Gb%*%scaleV(Bb)
      angB <- c(acos((t(Ba)%*%Bb)/ sqrt( (t(Ba)%*%Ba)*(t(Bb)%*%Bb))))
      angZ <- c(acos((t(Za)%*%Zb)/ sqrt( (t(Za)%*%Za)*(t(Zb)%*%Zb))))
      LB <- estL(Ba,Bb)
      LZ <- estL(Za,Zb)
      rta <- c(angB, angZ, LB, LZ, abs(Ba-Bb), abs(Za-Zb), abs(c(Ga)-c(Gb)))
      #nombrador
      Bdif<-paste("dif.b", 1:length(Ba), sep=".")
      Zdif<-paste("dif.z", 1:length(Ba), sep=".")
      g1<-paste(rep(c(1:length(Ba)), each= length(Ba)), rep(c(1:length(Ba)),
length(Ba)), sep=".")
      g2<-paste("dif.G", g1, sep=".")
      nom<-c("ang.b", "ang.z", "lr.b", "lr.z", Bdif, Zdif, g2)
      #salida
      names(rta)<-nom
      rta
    } else {
      Ba <- estB(as.formula(formula), base = new.t.1, type = Btype)
      Bb <- estB(as.formula(formula), base = new.t.2, type = Btype)
      Ga <- estG(base1, vars, fam = fam1, type = Gtype, scale = scale)
      Gb <- estG(base2, vars, fam = fam2, type = Gtype, scale = scale)
      Za <- Ga%*%scaleV(Ba)
      Zb <- Gb%*%scaleV(Bb)
      angB <- c(acos((t(Ba)%*%Bb)/ sqrt( (t(Ba)%*%Ba)*(t(Bb)%*%Bb))))
      angZ <- c(acos((t(Za)%*%Zb)/ sqrt( (t(Za)%*%Za)*(t(Zb)%*%Zb))))
      LB <- estL(Ba,Bb)
      LZ <- estL(Za,Zb)
      rta <- c(angB, angZ, LB, LZ, abs(Ba-Bb), abs(Za-Zb), abs(c(Ga)-c(Gb)))
      #nombrador
      Bdif<-paste("dif.b", 1:length(Ba), sep=".")
      Zdif<-paste("dif.z", 1:length(Ba), sep=".")
      g1<-paste(rep(c(1:length(Ba)), each= length(Ba)), rep(c(1:length(Ba)),
length(Ba)), sep=".")
      g2<-paste("dif.G", g1, sep=".")
      nom<-c("ang.b", "ang.z", "lr.b", "lr.z", Bdif, Zdif, g2)
      #salida
      names(rta)<-nom
      rta
    }
  }
}
data.frame(t(replicate (N, carving.int(formula = formula, vars = vars, fam1 =
fam1, fam2 = fam2,
                                       base1 = base1, base2 = base2, Btype =
```

```
Btype, Gtype = Gtype,
                                          scale = scale, H0 = H0))))
}


#EXAMPLE
EvsL<-carving.rand(N= num_iter, formula = relw~rcrece+rR+rf + (1|fam), vars=
c("rcrece","rR", "rf"),
                   fam1 = "fam", fam2 = "fam", base1 = A, base2 = B,
                   Btype="lmer", Gtype="G", scale = TRUE, H0 = 1)


###-----------------------------------------------###
### ESTIMATE P VALUES
###-----------------------------------------------###

###ARGUMENTS
# obs: result from carving.obs
# pseudo: result from carving.rand

###DETAILS
# estimate the probability, under the null hypothesis, of obtaining higher values
than the observed ones.

### OUTPUT
# A named vector containing the P values from a  carving fork analysis. Include the
significance of the
# angle between selection gradient vectors (ang.b), the angle between response to
selection vectors (ang.z),
# the ratio between selection gradient vector lengths (lr.b), the ratio between
response to selection
# vector lengths (lr.z), the absolute differences between each selection gradient
(dif.b.1, dif.b.2, etc.),
# the absolute differences between each response to selection (dif.z.1, dif.z.2,
etc.) and the absolute
# differences between corresponding cells in G matrices (for example dif.G.1.1
indicate differences between
# the variances of trait 1; dif.G.3.4 indicate differences between the covariances
of traits 3 and 4).


carving.P <- function (obs, pseudo) {
  n <- ncol(pseudo)
  P <- numeric(length = n)
  for (i in 1:n)
  P[i] <- length(pseudo[,i][pseudo[,i]>obs[i]])/length(pseudo[,i])
  names(P) <- names(obs)
  P
}

#EXAMPLE

carving.P(obs=OBS, pseudo=EvsL)


###-----------------------------------------------###
### HISTOGRAMS
###-----------------------------------------------###

### ARGUMENTS
# obs: result from carving.obs
# pseudo: result from carving.rand
# Main: if TRUE, the firts four histograms are ploted, corresponding to the angle
```

```
between selection
# gradient vectors (ang.b), the angle between response to selection vectors (ang.z),
the ratio between
# selection gradient vector lengths (lr.b) and  the ratio between response to
selection vector lengths (lr.z).
# If FALSE, use select to indicate which column to plot from pseudo
# ... other arguments to be passed to hist function

### OUTPUT
# An histogram or a matrix of histograms of pseudo-values.
# Observed values are indicated with a verticla red line


carving.plot<- function (obs, pseudo, main = TRUE, select, ...){
  if(main == TRUE){
    layout(matrix(1:4,2,2))
    for (i in 1:4){
      hist(pseudo[,i], main=colnames(pseudo)[i], xlab=NULL)
      abline(v=obs[i], col="red")
    }
  } else {
    layout(1)
    hist(pseudo[, select], main=colnames(pseudo)[select], xlab=NULL)
    abline(v=obs[select], col="red")
  }
}


#EXAMPLES
carving.plot(obs=OBS, pseudo=EvsL, main=TRUE)
carving.plot(obs=OBS, pseudo=EvsL, main=FALSE, select = 6)


################################################################################
###
#### END OF ROUTINE
################################################################################
###
```

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EVOLUTIONARY ECOLOGY OF PLANT DEFENCES

Plant traits that predict resistance to herbivores

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Summary

1. Although secondary metabolites are recognized as fundamental to the defence of plants against insect and mammalian herbivores, their relative importance compared to other potential defensive plant traits (e.g. physical resistance, gross morphology, life-history, primary chemistry and physiology) are not well understood.

2. We conducted a meta-analysis to answer the question: What types of genetically variable plant traits most strongly predict resistance against herbivores? We performed a comprehensive literature search and obtained 499 separate measurements of the strength of covariation (measured as genetic correlations) between plant traits and herbivore susceptibility – these were extracted from 72 studies involving 19 plant families.

3. Surprisingly, we found no overall association between the concentrations of secondary metabolites and herbivore susceptibility – plant traits other than secondary metabolites most strongly predicted herbivore susceptibility. Specifically, genetic variation in life-history traits (e.g. flowering time, growth rate) consistently exhibited the strongest genetic correlations with susceptibility. Genetic variation in gross morphological traits (e.g. no. branches, plant size) and physical resistance traits (e.g. latex, trichomes) were also frequently correlated with variation in herbivore susceptibility, but these relationships depended on attributes of the herbivores (e.g. feeding guild) and plants (e.g. longevity).

4. These results call into question the conventional wisdom that secondary metabolites are the most important anti-herbivore defence of plants. We propose the hypothesis that herbivores select most strongly on genetic variation in life-history, morphological and physical resistance traits, but the greater pleiotropic effects of genes controlling these traits impose strong constraints on their evolution. Meanwhile, secondary metabolites could have evolved to be important defensive mechanisms not because they have the largest effect on herbivores, but because the constraints on their evolution are the weakest.

Key-words: antibiosis, co-evolution, genetic covariance, plant defence, plant resistance, plant-insect, secondary chemistry

Introduction

'The examples cited of [herbivorous] insects … clearly demonstrate the function of secondary substances in these plants as means of repelling or attracting insects.'

p. 1470 Fraenkel 1959 *Science*

'The observed patterns clearly point to the critical importance of plant biochemistry in governing the [co-evolutionary] relationships between the two groups…. Of secondary, but still possibly major importance, are mechanical plant defenses…'

p. 605 Ehrlich & Raven 1964 *Evolution*

Interactions between plants and herbivores are among the most dominant species interactions in nature. Plants form the basal resource of virtually all food webs, and herbivores consume 10–15% of the plant biomass produced annually in both natural and managed ecosystems (Cyr & Pace 1993).

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This herbivory can have cascading ecological and ecosystem-level effects (Crawley 1983; Bardgett, Wardle & Yeates 1998; Wimp & Whitham 2001; Stark, Julkunen-Tiitto & Kumpula 2007). These ecological interactions have fueled ongoing co-evolution between plants and herbivores, whereby plants have evolved an arsenal of defences that reduce the amount and impact of herbivory, and herbivores have evolved countermeasures to thwart these defences (Ehrlich & Raven 1964; Futuyma & Slatkin 1983; Karban & Baldwin 1997; Karban & Agrawal 2002).

As the opening quotations imply, the earliest appreciation for the role of plant traits in providing resistance against herbivores came with the recognition that secondary metabolites (e.g. terpenoids, glucosinolates, tannins) and physical plant traits (e.g. latex, trichomes) influence the feeding patterns of arthropod herbivores (Dethier 1941; Fraenkel 1959; Krieger, Feeny & Wilkinson 1971). More recent research has found, for instance, that higher concentrations of glucosinolates and greater densities of trichomes in *Arabidopsis thaliana* reduced herbivory by two flea beetle species (Mauricio 1998). These traits can also evolve as adaptive defences since there exists heritable variation for glucosinolate and trichome levels, and herbivores selected for an increase in these levels (Mauricio & Rausher 1997). Similar patterns associated with the functional role and evolution of plant secondary chemistry have been observed in several plant-herbivore systems in a micro-evolutionary (within-species) context (Fordyce & Malcolm 2000; Kessler, Halitschke & Baldwin 2004; Agrawal 2005; Despres, David & Gallet 2007; Mitra *et al*. 2008; Johnson *et al*. 2009a). These patterns are not universal, however, as increased concentrations of secondary compounds can confer susceptibility to specialist and generalist herbivores (Mithen, Raybould & Giamoustaris 1995; Agrawal, Gorski & Tallamy 1999; Lankau 2007; Bidart-Bouzat & Kliebenstein 2008).

Macroevolutionary (between-species) patterns associated with the evolution of chemical and physical plant traits, and the co-evolution of their herbivores, also provide some of the strongest support for the hypothesis that these traits are adaptive defences (Ehrlich & Raven 1964; Berenbaum 1990; Agrawal 2007). For example, the innovation of laticifers and cardiac glycosides in *Asclepias* provided novel physical and chemical defences effective against most herbivores, which contributed to the rapid diversification of the clade (Farrell, Dussourd & Mitter 1991; Farrell & Mitter 1998). Recent evidence shows that the initial evolution for increased latex and cardenolides was followed by a decline in the concentrations of these traits (Agrawal & Fishbein 2008), probably because of counter adaptations and diversification in specialist herbivores (Holzinger & Wink 1996; Farrell & Mitter 1998). Based on these types of data (also see Becerra 1997, 2003), it seems likely that chemical and physical plant traits play important ecological and evolutionary roles in defence as proposed by early pioneers in the field (Dethier 1941; Fraenkel 1959; Ehrlich & Raven 1964). Nevertheless, the role of these traits might not be as straightforward as originally believed.

Many empirical studies have found that plant traits without obvious associations with resistance can influence the preference and performance of herbivores. For example, variation within and between plant species for physiological traits such as water content and nitrogen concentration are correlated with the performance of many herbivore species (Scriber & Feeny 1979; Mattson 1980; White 1984; Agrawal 2004; Johnson 2008). Phenological traits can also have large effects on herbivory, as in *Helianthus annuus* where late flowering individuals experience reduced damage by weevils and moths (Pilson 2000). Studies like these have shown that a large diversity of traits, including primary and secondary chemistry, physiological, morphological and life-history traits, all relate to a plant's resistance to herbivores. As such, it is increasingly recognized that a plant's defence may depend on the effects of these traits acting in concert (Coley, Bryant & Chapin 1985; Kursar & Coley 2003; Agrawal & Fishbein 2006; Agrawal 2007).

Despite these advances, we lack an understanding of the traits that are most strongly associated with resistance against herbivores, and the relative importance of different types of traits involved in defence (Karban & Baldwin 1997; Stamp 2003). This information is needed to fully understand the ecology and evolution of plant defence, and is necessary to broaden current applications of plant-defence theory to agricultural systems. In this quantitative review, we attempt to fill this gap by answering the following questions: At a general level, does variation in secondary chemistry (SM) correlate with resistance more strongly than all non-secondary metabolite plant traits (NSM)? On a narrower but related level, what plant traits (secondary chemistry, morphology, life-history, primary chemistry and physiology) are the best predictors of resistance to herbivores? Are certain functional groups of herbivores, such as chewers and piercing-sucking feeders, influenced more strongly by particular types of plant traits? And finally, does variation in plant form (e.g. herbaceous versus woody) moderate effects of certain classes of plant traits on herbivores? To answer these questions at the microevolutionary scale, we collated data from published studies that estimated genetic correlations between genetic variation in plant traits and herbivore susceptibility using an ecological genetics design (Table S1), and then synthesized these data with meta-analysis. The paucity of data at the macroevolutionary scale (Table S2) prevented a robust meta-analysis, but we also provide a preliminary quantitative review of the trends found among existing datasets at this scale.

Materials and methods

DATA MINING, STUDY SELECTION CRITERIA AND EFFECT SIZE ESTIMATION

We compiled and synthesized a comprehensive dataset of published studies testing pairwise correlations between trait values and herbivore susceptibility. A study was included in our dataset if it estimated variation in at least one plant trait and one measure of herbivore susceptibility. Relevant studies were identified by querying Thomson Scientific's Web of Science online database (ISI; http://apps.isiknowledge.com) using the following keyword searches: 'resistance', 'mean famil*', 'plant resistance', 'genetic var*', 'plant damage', 'genetic correlatio*', 'antibiosis', 'antixenosis', 'herbivor*',

'plant defens*', 'plant defence*', 'plant-insect', 'insect damage', 'herbivor* damage' and 'plant trait'. We also examined studies cited in the papers identified above as well as those reported in a previous meta-analysis (Leimu & Koricheva 2006).

We then narrowed our selection criteria to studies only reporting genetic correlations between plant traits and susceptibility to herbivores – these studies have an advantage over research testing phenotypic correlations because their results can be directly interpreted in an evolutionary context (Lande & Arnold 1983; Rausher 1992). These studies typically used the means of families (e.g. full-sib, paternal half-sibs), genotypes, isogenic lines, clones, accessions, and cultivars as their unit of replication. Plant susceptibility to herbivores was estimated according to the amount of damage, the preference and performance of individual herbivores. Insects can damage plants in many different ways and so we utilized herbivore damage data that included any measure of the quantity of tissue removed by herbivores, including: the number or % of damaged leaves, severity of aphid damage, biomass removed, leafminer sting/leaf area (see Supporting Information Table 1 for a complete list of response variables). Gall and miner density were also included as 'damage' because the presence of such herbivores implies foliar damage. We defined herbivore preference as any measure of herbivore abundance or density in which herbivores were allowed to naturally colonize or choose between plants. Herbivore performance included the growth rate of individual herbivores or populations, herbivore mass, survival, number of hatched larvae and insect maturation time. When resistance (1–relative damage), antibiosis (a reduction in herbivore performance) or antixenosis (a reduction in herbivore preference) were reported, the sign was inverted to reflect plant susceptibility. Since we found no difference in effect sizes among these three classes of herbivore susceptibility (i.e. damage, preference and performance; between-group heterogeneity test using a traditional fixed-effects meta-analysis:Q_b = 2·96, d.f. = 2, P = 0·23), we combined these data and do not distinguish among them when reporting results. In general, a positive effect size describes that on average, an increase in the value of a plant trait is associated with an increase in susceptibility to herbivores.

Genetic correlations were quantified as Pearson product-moment correlation coefficients (r). When Spearman correlations (ρ) were reported, we transformed these data with $r = 2\sin(\pi\rho/6)$ when the sample size of the study was $N < 90$, and we did not transform the data when $N > 90$ since $r \approx \rho$ at this level of replication (Lajeunesse in press). If the coefficient of determination R^2 was reported we took the square root to estimate r. When authors reported a range of R^2 values we used the highest value as per Leimu & Koricheva (2006). We also did not include studies reporting partial coefficients from multiple regression analyses. Very few studies reported results using multiple regressions, and although they contain information valuable for our review, it was too difficult to extract r from partial correlations given that they are dependent on which traits were included in the final model. Only a fully reported model would be useful to extract these data. However, when possible, raw pairwise r was recovered by contacting the corresponding authors of the publication. Finally, all r correlations were transformed into Z-score effect sizes prior to analyses (following Rosenthal 1991).

Our final dataset included K = 499 genetic correlations from 66 studies published between 1983–2010 (Table S1, Appendix S1). These correlations were reported for 40 species (including plant varieties and hybrids) from 19 plant families. These published studies also included 65 herbivore species from 33 families and 15 orders.

TRADITIONAL AND PHYLOGENETICALLY-INDEPENDENT META-ANALYSIS

The Z-transformed correlation coefficients were pooled using both traditional and phylogenetically-independent meta-analysis

Table 1. Phylogenetically-independent meta-analysis of genetic correlations between plant traits and susceptibility of plants to herbivory. Pooled effect sizes ($\bar{Z}_+^P$) and review sample sizes (K^P in brackets) are reported for two major subgroups: (a) contrast between secondary (SM) and non-secondary metabolites traits (NSM), and (b) contrast among traits described as physical, gross morphology, life–history, and primary chemistry and physiology. Significant non-zero genetic correlations (i.e. effect sizes) are in bold and are based on 95% confidence intervals, andQ_B^Ptest evaluating between-group differences are reported in Table S5

	NSM trait subgroups					
	SM	NSM	Physical	Gross morphological	Life-history	Primary chemistry and physiology
Feeding guild						
Browsers	0·159 (2)	0·108 (3)	0·004 (2)	**0·273 (2)**	**−0·237 (1)***	0·064 (2)
Chewers	−0·035 (15)	**−0·107 (25)**	−0·136 (14)	**−0·145 (19)**	**−0·240 (10)**	−0·162 (5)
Endophytes	0·096 (3)	0·086 (7)	**−0·362 (1) ***	0·278 (5)	0·049 (3)	0·001 (2)
Piercing/sucking	0·132 (4)	0·131 (18)	0·133 (6)	0·230 (9)	−0·062 (5)	−0·026 (2)
Herbivore specificity						
Specialist	−0·041 (13)	**−0·171 (28)**	**−0·190 (13)**	−0·011 (17)	**−0·417 (10)**	0·005 (5)
Generalist	0·035 (13)	−0·072 (28)	−0·107 (11)	−0·076 (14)	−0·078 (10)	−0·084 (5)
Plant longevity						
Annual	−0·141 (5)	**−0·207 (17)**	−0·130 (5)	**−0·168 (13)**	**−0·441 (9)**	**−0·266 (1)***
Biennial	**0·413 (2)**	−0·039 (1) *	0·030 (1) *	0·101 (1) *	−0·065 (1)*	**−0·309 (1)***
Perennial	0·044 (13)	0·162 (24)	0·019 (13)	0·223 (12)	0·164 (6)	0·058 (7)
Plant life–form						
Herbs	0·011 (13)	**−0·086 (29)**	−0·069 (14)	−0·034 (20)	**−0·244 (14)**	−0·116 (5)
Woody plants	0·070 (6)	0·208 (10)	−0·110 (5)	**0·338 (5)**	0·274 (2)	0·068 (4)

*These effect sizes should be interpreted with caution because they were derived from single species and thus are the statistical equivalent of a pooled effect derived from a traditional fixed-effects meta-analysis.
NSM, non-secondary metabolite, SM, Secondary metabolites.

(see Hedges & Olkin 1985; Lajeunesse 2009). All traditional meta-analyses (regression weighted by the within-study variances) were performed using METAWIN (version 2·1; Rosenberg, Adams & Gurevitch 2000). In these analyses, the unit of replication was individual *Z*-scores for specific correlations among plant traits, but for many studies multiple *Z*-scores were available for multiple categories of plant traits. These multiple effect sizes extracted from single studies were treated as independent because we were interested in testing for potential moderator effects among multiple plant trait categories. Finally, our results from phylogenetically-independent and traditional meta-analyses were similar, and so we focus our interpretation and description of results on the phylogenetically-independent methods. All results from traditional analyses are provided online (Tables S3 and S4).

To control for the evolutionary history of plants, we applied Lajeunesse's (2009) method to integrating phylogenetic history into meta-analysis using a weighted GLS approach. First, a phylogenetic hypothesis of the 42 plant species was assembled from a modified megatree of all major plant groups based on APG III. (2009) using PHYLOMATIC (Webb & Donoghue 2004). The internal branch-length (BL) distances of this phylogeny (i.e. temporal ordering of nodes) were based on the estimated divergence times of major plant lineages compiled by Hedges, Dudley & Kumar (2006) and Hedges & Kumar (2009). Species lacking phylogenetic information were placed as polytomies at the root of their family or genus should multiple species have the same genus/family (see Fig. S1). We then converted this ultrametric tree into a phylogenetic correlation matrix (P) that has the standardized shared BL distance of each species in off-diagonals and ones in the main diagonal (Grafen 1989; Rohlf 2001). These correlations are then used to modify the weighted regression scheme of meta-analysis (see Lajeunesse 2009; Lajeunesse, Jennions & Rosenberg in press). For each moderator variable (i.e. for each categorical factor or predictor that could explain structure in the variation of effect sizes), a subset tree was used to estimate P for each category – this P contains only the relevant species for which effect size data was available and conserves all the BL information found in Fig. S1. Finally, for many studies, there were multiple correlations available for each moderator category for a given species. To resolve this issue, we pooled these multiple effect sizes prior to our phylogenetically-independent meta-analysis. This pooling resulted in one representative (pooled) effect size and variance for each species within a given moderator category, and also resulted in smaller sample sizes for each moderator category given that the unit of the review was now individual species (K^{P} = number of species). These multiple effect sizes for each species were pooled using a traditional meta-analysis with a fixed-effects model (following Lajeunesse, Jennions & Rosenberg in press).

For both the traditional and phylogenetically-independent meta-analysis, the statistical significance of pooled correlations ($\bar{Z}_{+}$) between plant traits and herbivore susceptibility was assessed using the bias-corrected 95% bootstrap confidence intervals (CI) generated from 4999 iterations. Pooled effects sizes were considered statistically significant if CI did not overlap zero (Adams, Gurevitch & Rosenberg 1997). We tested the explanatory power of moderator effects (details below) using the between-group Chi-squared test Q_{B}(Hedges & Olkin 1985). A significant Q_{B} (Q_{B}^{P} for phylogenetically-independent test) indicates that the moderator grouping is a significant explanatory variable for heterogeneity among effect sizes. Finally, a random-effects model was assumed for all analyses (following Gurevitch & Hedges 1999).

MODERATOR EFFECTS AND HYPOTHESIS TESTS

To identify which category of plant traits best predicted the correlations to herbivore susceptibility, we pooled effect sizes into moderator subgroups of plant traits among five major functional categories: (i) secondary metabolites (SM); (ii) physical traits; (iii) gross morphology; (iv) life-history; and (v) primary chemistry and physiology. SM included any compound not directly involved in the primary function of a plant's physiology, growth or resource acquisition, which included the activity of enzymes directly involved in secondary metabolite production (e.g. myrosinase). Physical traits were non-chemical leaf and stem characteristics that could prevent insect herbivores from damaging a plant (e.g. trichome density, leaf toughness). Gross morphological traits included large physical structures (e.g. biomass, height, number of branches) and the size of plants. Life-history included traits that described the rate of growth, phenology and investment in reproductive structures. Finally, primary chemistry and physiological traits included concentrations of elements or plant processes directly involved in growth and resource acquisition. We were also interested in whether the strength of correlations between plant traits and herbivore susceptibility could depend on aspects related to the biology of either the plants or herbivores. Each insect species was categorized according to: feeding guild (browsers, chewers, endophytes like miners, gallers and seed predators, piercing/sucking), herbivore type (vertebrate vs. invertebrate), herbivore specificity (specialist: one or two plant families; generalist: three or more plant families). We also categorized plant species according to their longevity (annual, biennal, perennial) and life-form (herbs or woody plant).

To test the relative importance of secondary metabolites as resistance traits, we initially contrasted secondary metabolites (SM) vs. non-secondary metabolite (NSM) plant attributes as factors that could explain variation in susceptibility to herbivores. We then tested whether this result was conditional on: feeding guild, host specificity, plant longevity and plant life-form. To more precisely identify the types of traits that best predict herbivore susceptibility, we performed the same analyses but contrasted SM, physical traits, gross morphological traits, life-history traits, and primary chemistry and physiology traits.

Finally, we examined for publication bias using funnel plots (Hunter & Schmidt 2004; Fig. S2). In the absence of publication bias, it is expected that the variation around the overall mean effect would have the shape of a symmetric funnel – where the variation in effect sizes decrease with increasing within-study sample sizes and that the effect size is independent of sample size (Palmer 1999). We tested for a bias against nonsignificant (null) results by visually inspecting funnel plots for a characteristic gap in the lower inner area of the funnel, which would suggest that nonsignificant results were missing (Hunter & Schmidt 2004). Finally, we tested for the independence of effect sizes and their sample sizes using a Spearman's rank correlation, and assessed their overall distribution using a weighted histogram and a normal quantile plot (Fig. S3).

Results

BIAS AND VARIATION AMONG CORRELATIONS

We found no evidence for publication bias in our data (Fig. S2). Effect sizes were symmetrically and normally distributed (Figs S2 and S3), and the correlation between sample size and *Z*-transformed effect sizes was negligible (Spearman correlation; $R_{s} = 0{\cdot}04$, d.f. = 498, $P = 0{\cdot}381$).

Importantly, there was significant variation among effect sizes (phylogenetically-independent within-study heterogeneity test assuming a fixed-effects model: Q_T^P = 1353·0, d.f. = 41, P < 0·0001). This is evidence that variation among the effect sizes was not due to sampling error and it provides justification for: exploring which types of plant traits provide the strongest predictors of herbivore susceptibility; whether genetic correlations varied as a function of herbivore and plant attributes; and for assuming a random-effects model for pooling correlations (see Gurevitch & Hedges 1999).

WHAT PLANT TRAITS PREDICT RESISTANCE TO HERBIVORES?

Overall, genetic correlations between SM and herbivore susceptibility were not significantly stronger than correlations between NSM and herbivore susceptibility (phylogenetically-independent between-group test:Q_B^P = 0·07, d.f. = 1, P = 0·786; Table S3). When NSM were further subdivided, a significant difference was detected among plant trait categories (Q_B^P = 10·77, d.f. = 3, P = 0·013; Table S3), but genetic variation in life-history traits was the only consistently significant predictor of herbivore susceptibility ($\bar{Z}_+^P$ = −0·22, 95% CI: −0·37 to −0·06, K^P = 16). Correlations involving SM and other traits were weaker and did not differ from zero.

ARE CERTAIN FUNCTIONAL GROUPS OF HERBIVORES INFLUENCED MORE STRONGLY BY PARTICULAR TYPES OF PLANT TRAITS?

Genetic correlations between plant traits and herbivore susceptibility depended on the feeding guild of the focal herbivore (Q_B^P = 15·58, d.f. = 3, P = 0·001; Fig. 1). On average, genetic variation in SM was not related to the performance of herbivores from any feeding guild, while NSM had relatively strong correlations with chewing insects, mammalian browsers and endophytes (Table 1). Specifically, gross morphological and life-history traits negatively correlated with chewing insect performance (Table 1). Life-history traits also negatively correlated with damage by mammalian browsers while gross morphology was positively related to damage. The strongest negative genetic correlations across all feeding guilds were those observed between physical plant traits and susceptibility to endophytic herbivores (Table 1). Trait classes did not significantly vary in their ability to predict damage by piercing-sucking herbivores (Table 1).

The effects of plant traits on herbivore susceptibility did not vary between specialist and generalist feeding herbivores (Q_B^P = 0·07, d.f. = 1, P = 0·785). However, in the case of specialist herbivores, while genetic variation in SM was not consistently correlated with susceptibility, correlations with NSM were significantly negative and nonzero (Fig. 2). Among NSM traits, physical and life-history traits were both negatively correlated with susceptibility, while there was no clear association with other traits. No plant trait consistently correlated with susceptibility to generalist herbivores (Table 1).

DOES VARIATION IN PLANT ATTRIBUTES LEAD TO DIFFERENTIAL EFFECTS OF CERTAIN CLASSES OF PLANT TRAITS ON HERBIVORES?

The association between plant traits and herbivore susceptibility greatly depended on whether a plant species was annual, biennial or perennial (i.e. plant longevity) (Q_B^P = 11·12, d.f. = 2, P = 0·004). Within annuals, only genetic

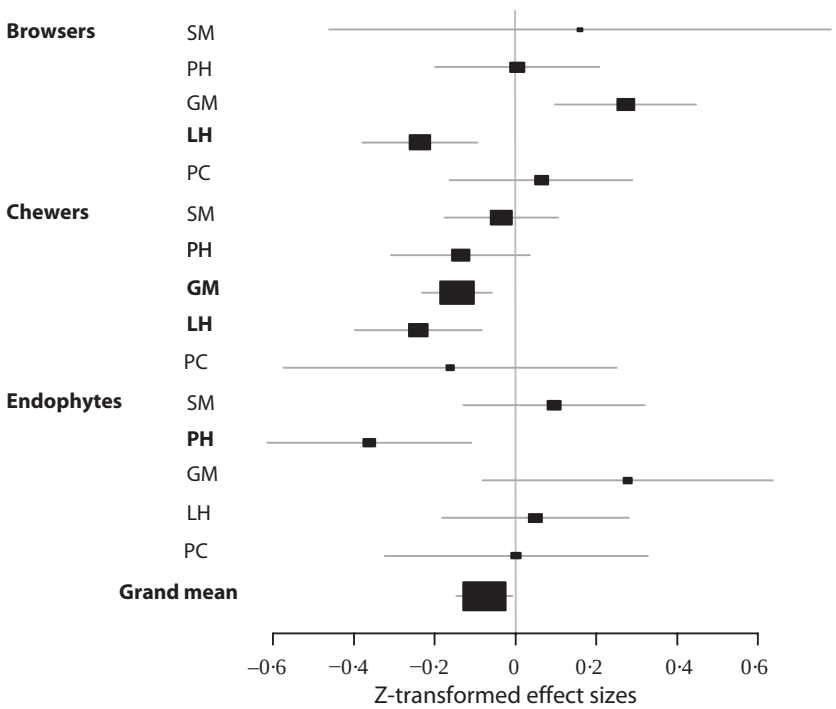

Fig. 1. The difference among the strength of the correlation between susceptibility to herbivory and plant traits among feeding guilds. Trait categories are abbreviated as follows: Secondary metabolites (SM), physical leaf and stem traits (PH), gross morphological (GM), life-history (LH) primary chemistry and physiology (PC). A positive effect size describes that on average, an increase in the value of a plant trait is associated with an increase in susceptibility to herbivores. The effect sizes for piercing-sucking insects did not significantly differ from zero and so they are not shown (see Table 1). The size of the square around each mean effect size is proportional to the weight of this mean effect in the overall meta-analysis; that is, it indicates which trait category contributed the most to the grand mean (Lewis & Clarke 2001). Bold abbreviations indicate non-zero mean effects.

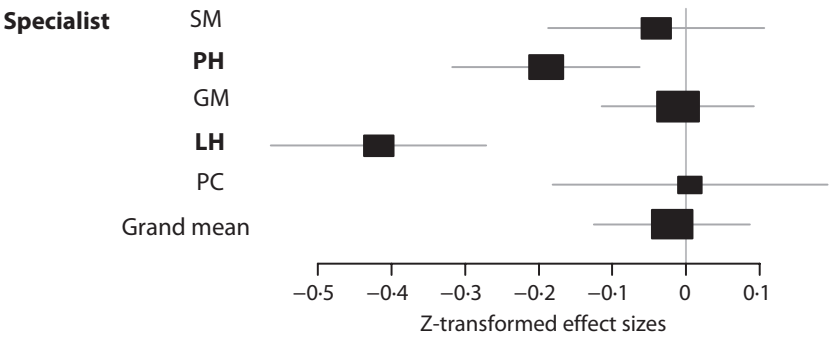

Fig. 2. The differences in effect sizes of genetic correlations between plant traits and plant susceptibility for specialist herbivores. Effect sizes for generalist herbivores not shown because they did not significantly deviate from 0 (see Table 1). The abbreviations and interpretation of results are the same as Fig. 1.

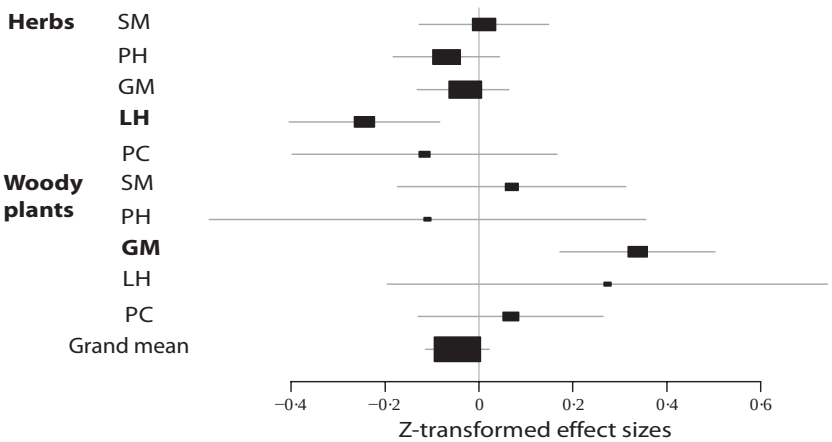

Fig. 3. Comparison of the strength of correlation between plant traits and plant susceptibility among herbaceous and woody plants. The abbreviations and interpretation of pooled effects are the same as in Fig. 1.

variation in NSM was significantly related to herbivore susceptibility, where gross morphology, life-history and primary chemistry exhibited the highest negative correlations (Table 1). In biennials, SM significantly correlated with susceptibility, but on average these correlations were positive. For perennial plants, gross morphology and life-history traits exhibited the highest correlations but all confidence intervals overlapped with zero (Table 1).

Finally, plant life-form (herbaceous vs. woody plants) also explained differences among genetic correlations between plant traits and susceptibility to herbivores (Q_B^P = 4·81, d.f. = 1, P = 0·028). Life-history traits were significantly negatively correlated with susceptibility in herbaceous plants while gross morphology positively correlated with susceptibility in woody plants (Table 1; Fig. 3). SM provided a non-significant and weak predictor of herbivore susceptibility across life-forms (Table 1; Fig. 3).

RELATIONSHIP BETWEEN PLANT TRAITS AND HERBIVORE SUSCEPTIBILITY AT A MACROEVOLUTIONARY SCALE

To understand whether our results observed at the microevolutionary scale could also be replicated at a macroevolutionary scale, we performed a second traditional meta-analysis where correlations between plant traits and herbivore susceptibility were estimated at the interspecific levels instead of the intraspecific level. Few datasets are available at this scale (6 studies which used 6 plant species and 90 effect sizes; Table S2) and so we interpret them with caution.

Similar to our results at the microevolutionary scale, variation in NSM were negatively related to susceptibility ($\bar{Z}_+$ = −0·33, 95% CI = −0·49 to −0·17, K = 78), whereas variation in SM did not differ from zero ($\bar{Z}_+$ = −0·02, 95% CI = −0·24 to 0·23, K = 12). Within NSM, physical traits were negatively related to susceptibility ($\bar{Z}_+$ = −0·44, 95% CI = −0·60 to −0·23, K = 42), while other traits showed no overall relationship. Unfortunately life-history traits could not be evaluated due to a very small review sample size. Despite the deterrent effect of physical traits, there was no overall difference among the mean pooled effects (Q_B = 0·31, d.f. = 3, P = 0·959).

Discussion

The most striking result of our meta-analysis is that plant secondary metabolites did not significantly predict resistance to mammalian and insect herbivores. By contrast, genetic variation in life-history, gross morphology, physical leaf and stem traits, and primary chemistry and physiology were most strongly related to herbivore susceptibility. This result is surprising because it is widely believed that the primary function of secondary metabolites is to defend plants against herbivores and pathogens. Our findings call into question a paradigm that has pervaded the thinking and direction of research on the evolution of plant defences for five decades (Fraenkel 1959; Ehrlich & Raven 1964; Rosenthal & Janzen 1979; Fritz & Simms 1992; Karban & Baldwin 1997; Stamp 2003; Berenbaum & Zangerl 2008). We argue below that secondary metabolites are still important in the evolution of defence, however, perhaps not for the reasons commonly invoked.

Our results further suggest that the recently advocated 'syndromes' approach to the study of plant defence evolution might be the key to understanding why the world is green (Kursar & Coley 2003; Agrawal 2007).

WHY DO SECONDARY METABOLITES NOT PREDICT SUSCEPTIBILITY TO HERBIVORES?

Although it has long been recognized that many types of traits are involved in defence against herbivores, it is widely believed that secondary metabolites play the dominant role in the ecology and evolution of plant defence (Rosenthal & Janzen 1979; Bennett & Wallsgrove 1994; Theis & Lerdau 2003; Berenbaum & Zangerl 2008; Orians & Ward 2010). Why then does our review of ecological genetics experiments, which estimate the strength of genetic correlations between secondary chemistry and herbivore susceptibility, not support this view? We offer three potential explanations.

Firstly, individual plants contain a vast array of secondary compounds and specific secondary metabolites that might have evolved to defend plants against a specific herbivore or specific groups of herbivores (Bennett & Wallsgrove 1994; Harborne & Williams 2000; Theis & Lerdau 2003; Macel, Van Dam & Keurentjes 2010; J.P. Salminen, unpublished data). Although some classes of chemicals have been portrayed as having general effects on herbivores (e.g. condensed tannins, see Feeny 1976), other chemicals appear to be effective against specific subsets of natural enemies (Linhart & Thompson 1999; Macel *et al.* 2005; Leiss *et al.* 2009; J.P. Salminen, unpublished data), or are most effective in combination with a specific mixture of other secondary metabolites. Moreover, specialist insect herbivores often evolve counter-adaptations to overcome or even benefit from specialized chemical defences (Karban & Agrawal 2002; Despres, David & Gallet 2007). Thus, correlations between herbivore susceptibility and genetic variation in the amount of a specific chemical, or an entire class of chemicals (e.g. cardenolides), can range from negative to positive, and may therefore be expected to show little or no relationship with the level of secondary metabolites.

Secondly, co-evolution and non-adaptive evolutionary processes may cause most secondary metabolites to have no biological function (Jones & Firn 1991). The classic view of co-evolution predicts that plant and insect populations are locked into a co-evolutionary arms-race, where novel defensive chemicals experience strong positive selection because they allow plants to escape herbivory (Ehrlich & Raven 1964). Herbivore populations may subsequently evolve counter-adaptations to overcome these novel defences, which in turn, would again selectively favor novel plant defence chemistry. This co-evolutionary process is thought to have generated the wide diversity of chemical defences observed in many plant clades (Berenbaum & Feeny 1981; Becerra 1997; Farrell & Mitter 1998; Wink 2003; Agrawal & Fishbein 2008), as well as variation in the levels of specific chemical compounds (Zangerl & Berenbaum 2005; Agrawal *et al.* 2009). If such an arms-race renders specific defensive compounds ineffective against herbivores, then this would lead to the gradual accumulation of chemical diversity – provided the cost of maintaining non-functional metabolites is not too high. If this accumulation process is common, then most secondary metabolites are perhaps relics of past co-evolutionary interactions.

Gene duplications – including chromosome doubling, tandem duplication, and RNA-mediated movement of genes to other parts of the genome – are also likely to be an important mechanism for adaptive and non-adaptive evolution of chemical diversity in plants. Gene duplication can lead to the adaptive evolution of new secondary metabolic functions (Rausher 2006; Des Marais & Rausher 2008), or the non-adaptive divergence of two gene copies (Innan & Kondrashov 2010). Even when one duplicated gene diverges neutrally from a functional gene copy, it might still result in the production of enzymes that catalyze biosynthetic reactions that produce non-functional secondary metabolites. If we accept the argument that most plants do contain an abundance of biological inactive secondary metabolites (Jones & Firn 1991), then detecting those chemicals involved in defence will be akin to finding a 'needle in a haystack'. In which case, we will require the use of newly developed genomic and metabolomic technologies that are not well suited to conventional ecological genetics approaches (Barakat *et al.* 2009; Macel, Van Dam & Keurentjes 2010).

The third explanation for our findings is that traits other than secondary metabolites have larger effects on the preference and performance of herbivores. The relatively strong and consistent genetic correlations involving life-history variation, gross morphology and physical plant traits indicate that this is at least a partial explanation (Table 1). Since increased herbivory must on average result in negative fitness effects, our results imply that herbivores select these traits more strongly than on secondary metabolites. For example, a field experiment that measured genetic variation and selection on all of the classes of traits reviewed here found that the strength of directional selection was 2·6–4·5 stronger on life-history variation (plant longevity) and gross morphology (plant biomass) than on specific secondary metabolites, although the relative contribution of herbivory to the measured selection gradients was unclear (Johnson *et al.* 2009a). Therefore, in systems where herbivores are a potent agent of natural selection, phenology, growth rate, the thickness and hairiness of leaves, and the size and architecture of plants, may evolve as adaptive defences against herbivory. This argument is not a new one. The importance of these traits have been recognized and studied over several decades and they have been explicitly incorporated into the most influential theories of plant defence evolution (Feeny 1976; Coley 1980; Coley, Bryant & Chapin 1985; Herms & Mattson 1992). Nevertheless, the recognition of traits such as phenology and gross morphology as defences runs against the existing paradigm that the ecology and evolution of secondary metabolites represent the *most important* plant defence against herbivores.

The three explanations offered above are not mutually exclusive and all are likely to have contributed to our results.

Indeed, secondary metabolites can be relatively specialized in their functions and are not restricted to a role in defence; many secondary metabolites have no apparent biological function; and, variation in secondary chemistry within natural populations often has less of an effect on herbivores than variation in non-chemical traits. Does this mean less focus should be paid to the defensive role of plant secondary chemistry? Not necessarily, but perhaps a more balanced and pluralistic approach is needed.

DO SECONDARY METABOLITES PLAY A ROLE IN PLANT DEFENCE?

Secondary metabolites do play a role in plant defence. However, the findings of our review lead us to conclude that their role in anti-herbivore defence is more complex than often appreciated. We propose that life-history, morphology and physical leaf and stem traits typically have larger effects on the preference and performance of herbivores than secondary metabolites. However, these traits, critical to a plant's fitness, are controlled by many genes from multiple primary and secondary biosynthetic and physiological pathways, and are subject to selection by many biotic and abiotic factors. Therefore it is likely that there is strong stabilizing selection that maintains an optimal level within populations for these traits and the genes controlling them. Herbivores likely impose selection on life-history and plant morphology, but they are one selective agent among many, and the ability for plant populations to respond might be limited. In other words, the evolutionary constraints acting on non-secondary metabolic traits are strong and natural selection by herbivores may result in little evolutionary response.

We propose the hypothesis that secondary chemistry is important in plant defence, not because of large effects that specific chemicals have on herbivores, but because the evolutionary constraints acting on these traits are relatively weak compared to those acting other traits. The production of secondary metabolites does involve many genes within branching pathways, and there are often many pleiotropic effects of mutations in these genes (e.g. Rausher 2006). Nevertheless, the pleiotropic effects of genes involved in the biosynthesis of secondary metabolites are likely smaller than traits associated with life-history or morphological variation. Even very weak selection can lead to large adaptive phenotypic changes in the levels and diversity of defensive chemicals over long periods of time. If secondary chemistry is important in defence not because of its large effects but because of weak selective constraints, then we predict that the importance of secondary metabolites in defence would be more evident when comparing the effects of secondary metabolites on herbivores among plants species that reflect macroevolutionary timescales. Although recent studies support this prediction (Agrawal *et al*. 2009; Johnson, Smith & Rausher 2009b), our review of existing macroevolutionary datasets does not, but given the scarcity of data across a small number of systems, we believe the prediction remains to be rigorously tested.

Conclusions

We propose that in a microevolutionary context, plant secondary chemistry has had a secondary role in defending plants against herbivores – second to life-history, morphology and physical resistance traits. We further argue that plant secondary metabolites may still evolve to be potent defences against herbivory over macroevolutionary timescales, not because of their large effects on herbivores but because of the relatively weak selective constraints acting on these traits. Our findings support recent calls for a reevaluation and pluralistic approach to the study of plant defence evolution, which considers the role of traditional and non-traditional resistance traits, as well as correlations between them, on herbivores (Kursar & Coley 2003; Agrawal 2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. The hypothesized phylogenetic relationships of the 41 plant species from 20 different families used in our phylogenetically-independent meta-analysis.

Figure S2. From the traditional meta-analysis: Funnel plot of *Z*-transformed effect sizes against their within-study sample size (K = 498). White dots denote secondary metabolites (SM) and black dots, non-secondary metabolites (NSM) including physical, gross morphological, life-history and primary chemistry and physiology traits.

Figure S3. From the traditional meta-analysis: (a) weighted frequency histogram of *Z*-transformed correlations between plant traits and herbivore susceptibility. White bars denote secondary metabolites (SM) and black bars, non-secondary metabolites (NSM) including physical, gross morphological, life-history and primary chemistry and physiology traits. (b) Normal quantile plot testing normal distribution.

Table S1. Database for meta-analysis at microevolutionary level.

Table S2. Database for meta-analysis at macroevolutionary level.

Table S3. Differences among plant trait categories in the strength of pooled genetic correlations ($\bar{Z}_+$) between plant traits and susceptibility of plants to herbivory. The number of pooled effects are K, and the Q_B test evaluates differences on the strength of the correlation among plant traits. All analyses designated with ^{P} are phylogenetically-independent.

Table S4. Traditional meta-analysis of correlations between traits and susceptibility of plants to herbivory. Pooled effect sizes and sample sizes (in brackets) are reported for two major subgroups: (a) contrast between secondary (SM) and non-secondary metabolite traits (NSM), and (b) contrast among traits described as physical (PH), gross morphology (GM), life-history (LH), and primary chemistry and physiology (PC). Q_b tests differences in the strength of the correlation among plant traits.

Table S5. The phylogenetically-independent between-group heterogeneity test (Q_b^P) for differences among categories of plant traits potentially related with plant susceptibility to herbivory (see pooled effects for each group in Table 1). The between-group heterogeneity test for differences among feeding guild, herbivore specificity, plant longevity and plant life-from can be found in the text.

Appendix S1. References cited on the meta-analyses databases

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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Table S3. Differences among plant trait categories in the strength of pooled genetic correlations ($\bar{Z}_+$) between plant traits and susceptibility of plants to herbivory. The number of pooled effects are *K*, and the Q_B test evaluates differences on the strength of the correlation among plant traits. All analyses designated with $^{\mathbf{P}}$ are phylogenetically independent.

	traditional meta-analysis				**phylogenetically-independent meta-analysis**			
grouping categories	K	$\bar{Z}_+$	LCI	UCI	$K^{\mathbf{P}}$	$\bar{Z}_+^{\mathbf{P}}$	LCI	UCI
plant traits	Q_b = 3.15, d.f. = 1, P = 0.076				$Q_b^{\mathbf{P}}$ = 0.07, *d.f.* = 1, P = 0.7858			
secondary metabolites	132	0.035	-0.036	0.101	19	0.037	-0.089	0.162
non-secondary metabolites	367	-0.043	-0.093	0.005	39	0.058	-0.026	0.141
non-secondary metabolites subgroups	Q_b = 9.40, d.f. = 3, P = 0.052				$Q_b^{\mathbf{P}}$ = 10.77, *d.f.* = 3, P = 0.0131			
physical	92	**-0.073**	**-0.158**	**-0.002**	19	-0.051	-0.198	0.096
gross morphological	182	0.017	-0.057	0.096	25	0.076	-0.014	0.166
life history traits	72	**-0.137**	**-0.268**	**-0.018**	16	**-0.215**	**-0.373**	**-0.057**
primary chemistry and physiology	21	-0.083	-0.264	0.066	9	-0.092	-0.316	0.013

Table S4. Traditional meta-analysis of correlations between traits and susceptibility of plants to herbivory. Pooled effect sizes and sample sizes (in brackets) are reported for two major subgroups: (a) contrast between secondary (SM) and non-secondary metabolite traits (NSM), and (b) contrast among traits described as physical (PH), gross morphology (GM), life history (LH), and primary chemistry and physiology (PC). Q_b tests differences in the strength of the correlation among plant traits.

	Traditional meta-analysis					
			NSM subgroups			
sub categories	**SM**	**NSM**	**PH**	**GM**	**LH**	**PC**
feeding guild			(Q_B = 30.21, d.f. = 3, P < 0.0001)			
browsers	0.163 (21)	**0.181** (65)	-0.058 (14)	**0.324** (44)	-0.237 (2)	0.024 (5)
chewers	-0.003 (99)	**-0.143** (208)	**-0.147** (57)	**-0.133** (98)	-0.153 (46)	-0.182 (7)
endophytes	0.084 (6)	0.097 (29)	-0.362 (3)	0.256 (15)	0.114 (8)	-0.016 (3)
piercing/sucking	0.170 (6)	0.073 (65)	0.207 (18)	0.107 (25)	-0.187 (16)	-0.046 (6)
herbivore specificity			(Q_B = 4.44, d.f. = 1, P = 0.035)			
specialist	-0.025 (40)	**-0.087** (227)	**-0.147** (57)	-0.001 (117)	**-0.314** (39)	0.042 (14)
generalist	0.061 (76)	-0.001 (79)	0.135 (21)	-0.033 (37)	-0.032 (15)	-0.310 (6)
plant longevity			(Q_B = 42.06, d.f. = 2, P < 0.0001)			
annual	**-0.123** (18)	**-0.218 (**127)	**-0.151** (17)	**-0.187** (65)	**-0.230** (44)	small K
biennial	**0.271** (21)	-0.032 (37)	-0.088 (5)	0.077 (19)	-0.049 (7)	-0.260 (6)
perennial	0.017 (93)	**0.091** (203)	-0.047 (70)	**0.198 (**98)	0.205 (21)	0.046 (14)
plant life-form			(Q_B = 23.25, d.f. = 1, P < 0.0001)			
herbs	0.025 (102)	**-0.119** (247)	-0.061 (69)	**-0.129** (102)	**-0.171** (63)	-0.124 (13)
woody plants	0.094 (30)	**0.170 (**120)	-0.110 (23)	**0.278** (80)	0.208 (9)	0.002 (8)

Table S5. The phylogenetically-independent between-group heterogeneity test ($Q_b^{\mathbf{P}}$) for differences among categories of plant traits potentially related with plant susceptibility to herbivory (see pooled effects for each group in Table 1). The between-group heterogeneity test for differences among feeding guild, herbivore specificity, plant longevity and plant life-form can be found in the text.

	SM vs. NSM			**NSM subgroups (PH vs. GM vs. LH vs. PC)**		
	$Q_{\mathrm{B}}^{\mathbf{P}}$	**d.f.**	***P***	$Q_{\mathrm{B}}^{\mathbf{P}}$	**d.f.**	***P***
feeding guild						
browsers	0.02	1	0.8761	**20.29**	**3**	**0.0002**
chewers	0.71	1	0.3989	1.17	3	0.7600
endophytes	0.01	1	0.9203	**9.85**	**3**	**0.0199**
piercing/sucking	0.01	1	0.9748	2.12	3	0.5479
herbivore specificity						
specialist	2.38	1	0.1227	**22.67**	**3**	**<0.0001**
generalist	0.66	1	0.4154	0.02	3	0.9990
plant longevity						
annual	0.35	1	0.5559	**8.53**	**3**	**0.0362**
biennial	**4.82**	**1**	**0.0282**	**19.22**	**3**	**0.0003**
perennial	0.78	1	0.3769	1.56	3	0.6681
plant life-form						
herbs	1.46	1	0.2275	4.95	3	0.1750
woody plants	0.51	1	0.4773	6.19	3	0.1024

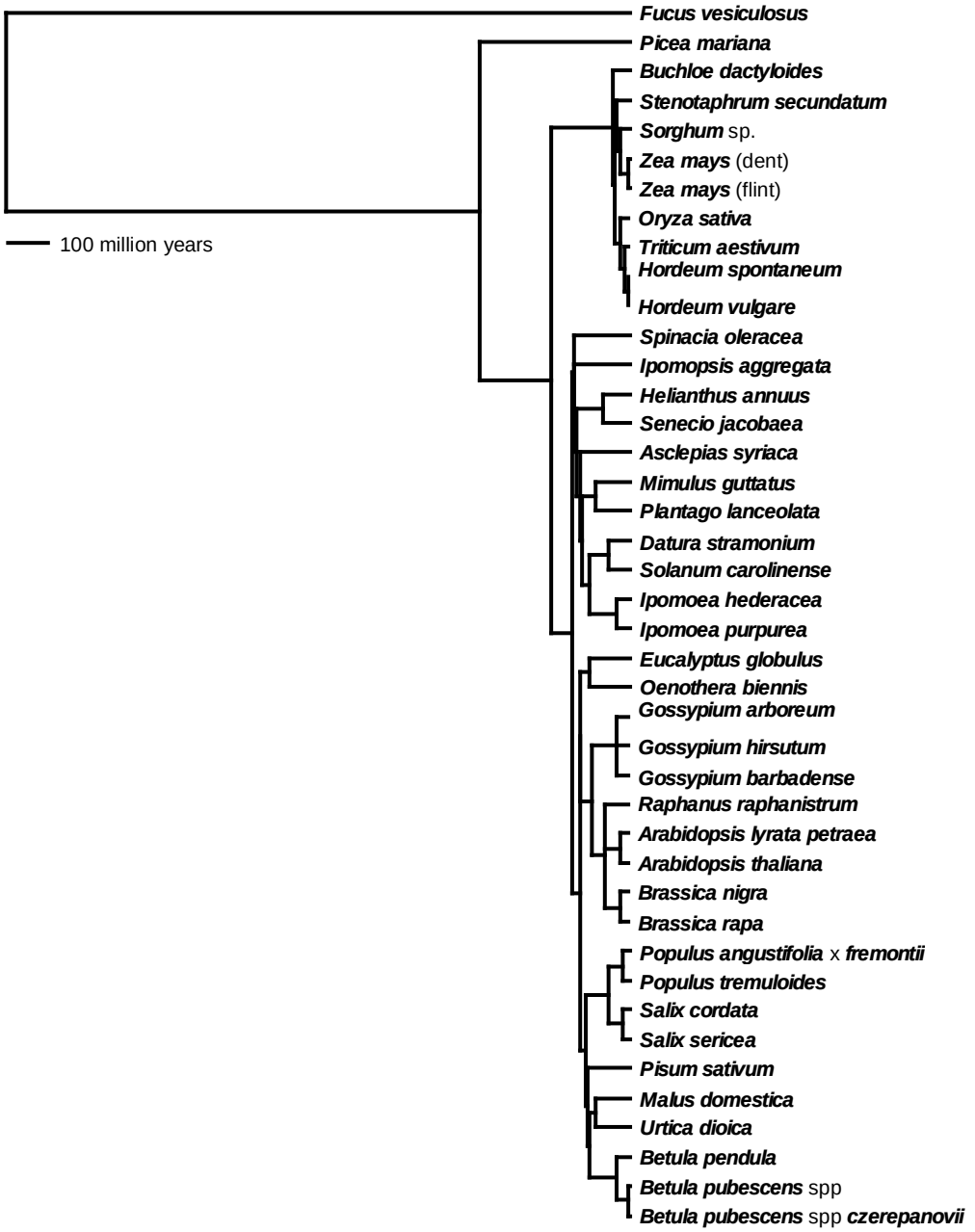

Figure S1. The hypothesized phylogenetic relationships of the 41 plant species from 20 different families used in our phylogenetically-independent meta-analysis.

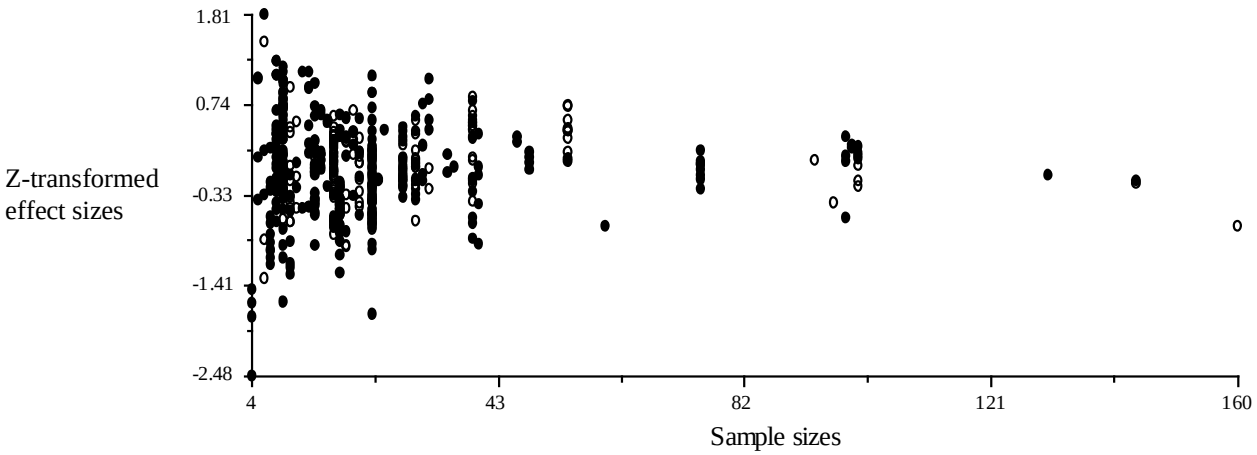

Figure S2. From the traditional meta-analysis: Funnel plot of Z-transformed effect sizes against their within-study sample size (K = 498). White dots denote secondary metabolites (SM) and black dots, non-secondary metabolites (NSM) including physical, gross morphological, life history and primary chemistry and physiology traits.

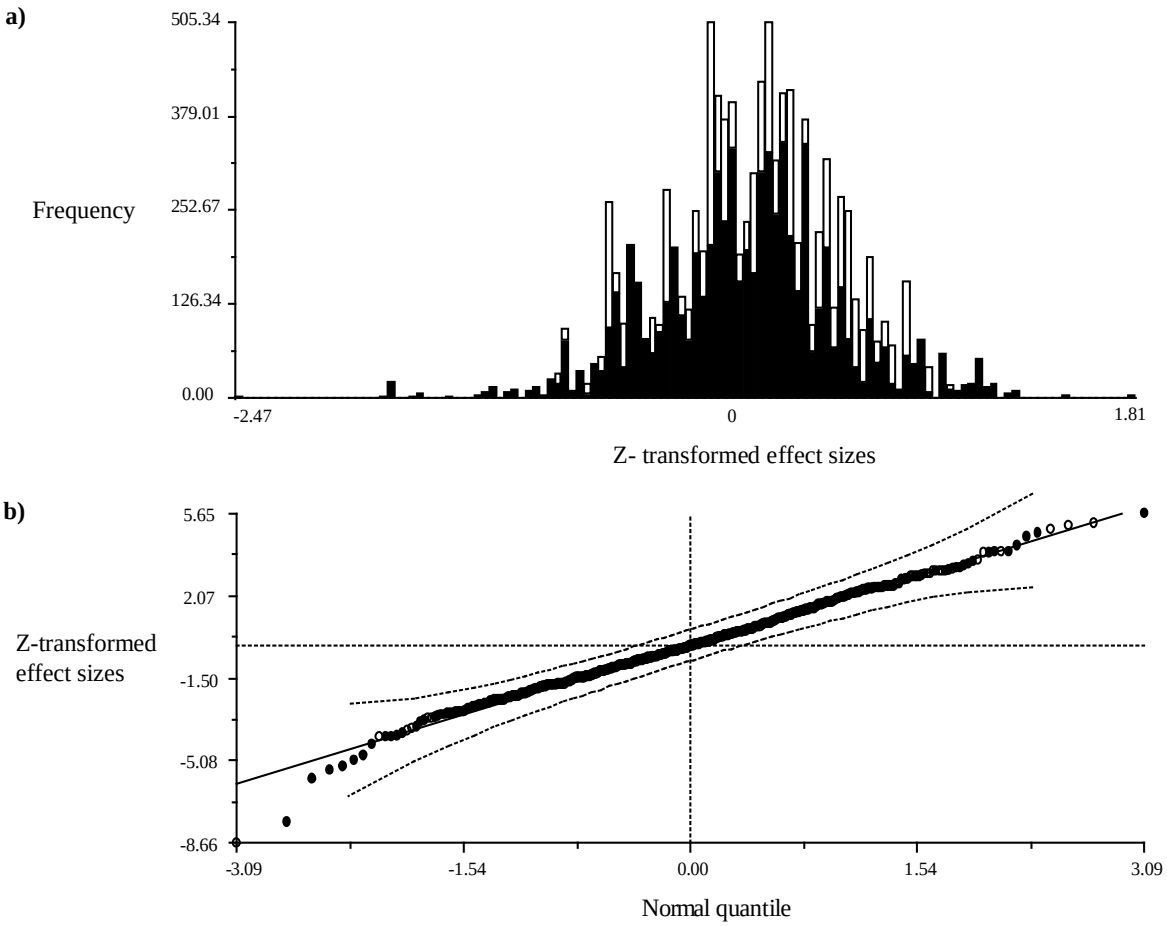

Figure S3. From the traditional meta-analysis: **a)** weighted frequency histogram of *Z*-transformed correlations between plant traits and herbivore susceptibility. White bars denote secondary metabolites (SM) and black bars, non-secondary metabolites (NSM) including physical, gross morphological, life history and primary chemistry and physiology traits. **b)** Normal quantile plot testing normal distribution.

DISCUSIÓN GENERAL

Los resultados más relevantes de esta tesis se pueden resumir en tres puntos. En primer lugar, la evolución simultánea de la resistencia y tolerancia es posible ya que estas estrategias defensivas pueden funcionar de manera complementaria. Sin embargo, esta funcionalidad puede ser condicionada por la presencia/ausencia de los agentes selectivos, lo que probablemente está relacionado con la especificidad del herbívoro (***i.e.*** generalista/especialista). En segundo lugar, se mostró que la importancia de la selección difusa en un atributo puede ser atenuada cuando se evalúa, no el gradiente de selección (β_i; versión univariada), sino el vector de selección (**β**; versión multivariada) y cuando la interacción **G × E** también ocurre. Finalmente, la expresión multivariada del fenotipo y los cambios selectivos en otros atributos no defensivos cuando el ambiente de herbivoría ha sido manipulado, señala que el fenotipo defensivo es una respuesta más general. Esta idea fue confirmada en el último capítulo, al detectar que los atributos de historia de vida y de morfología gruesa de las plantas también están involucrados en la defensa de plantas. Esto nos abre la pregunta de en dónde comienza y termina una adaptación.

Los dos primeros capítulos señalan la importancia de considerar ambientes ecológicos más realistas, que tomen en cuenta al menos más de un par de especies en interacción. Varios trabajos previos han hecho este señalamiento, sin embargo, pocos habían demostrado directamente el efecto sobre la respuesta a la selección, y menos asumido la importancia de la aproximación del análisis multivariado. La concepción de que la selección difusa (capítulo 1) es más frecuente que la pareada es completamente

entendible, empero, la importancia evolutiva de este tipo de selección puede ser condicionada cuando: a) se estudia desde una perspectiva multivariada (capítulo 2) y b) cuando los patrones difusos selectivos se evalúan en términos de la respuesta a la selección. De esta manera, la importancia de la selección difusa puede ser reducida si el cambio en el valor adaptativo de un atributo (cambio en el gradiente selectivo β_i) a pesar de ser significativo no altera el vector de selección (**β**), que considera el valor adaptativo de otros atributos. De igual manera, si el efecto ambiental es más fuerte a través de la plasticidad sobre **G** que sobre **β**, la selección difusa también tendría un efecto menor de lo que en otras situaciones se ha considerado. Por otro lado, el que se detecte selección difusa podría no tener implicación alguna si dicho patrón selectivo no afecta la respuesta evolutiva (***i.e.*** si no hay evolución difusa). Aun con estas consideraciones en mente, es factible pensar que la (co)evolución difusa es probablemente más frecuente que la pareada (Futuyma y Slatkin 1983). Esta conclusión general no sólo indica que para entender la evolución de ciertos atributos (por ejemplo los defensivos) es necesario considerar otros agentes selectivos, sino que nuestra capacidad predictiva sobre los procesos micro (co)evolutivos es reducida. De esta manera, cambios en el régimen selectivo (**β** × **E**) y/o en la expresión del fenotipo (***i.e.*** plasticidad fenotípica; **G** × **E**) definen la escala, tanto temporal como espacial (ej. mosaico geográfico), a la cual nuestras predicciones pierden valor predictivo. Por tanto, la pregunta: ¿cuál es la capacidad predictiva de la ecuación que predice la respuesta a la selección (***i.e.*** la ecuación del criador)? En realidad debe ir dirigida a cuestionar cuál es la estabilidad del contexto ambiental y la plasticidad fenotípica de los atributos considerados, cuando se construye una predicción sobre la evolución del fenotipo. Muy

probablemente en un ambiente controlado y bajo un esquema bien definido de selección artificial (***i.e.*** una degradación ambiental controlada ***sensu*** Fisher (1930), la ecuación del criador ($\mathbf{\Delta z} = \mathbf{G} \times \boldsymbol{\beta}$) permitirá hacer predicciones a más largo plazo, incluso sin importar cuán plástico sea un atributo. Por lo tanto, la construcción de una buena predicción que avizore un patrón macro (co)evolutivo de filogenias congruentes entre especies interactuantes debe considerar, ***a priori***, interacciones pareadas entre especies y un ambiente selectivo estable (Gomulkiewicz ***et al.*** 2003). Mientras tanto, desde la óptica macro (co)evolutiva, la falta de un patrón de filogenias congruentes podría favorecer la conclusión errónea de que el proceso coevolutivo no ha jugado un rol importante para entender la evolución de los fenotipos bajo interacción (Gould 1988). De esta reflexión se desprenden algunas preguntas: ¿Qué señal deberíamos detectar para considerar que un patrón (co)evolutivo fue generado por (co)evolución difusa? Y, de la misma manera en que se buscan interacciones fuertes entre especies para aplicar la hipótesis de coevolución pareada ¿Qué tipo de interacciones deberíamos de estudiar para comenzar a entender la importancia evolutiva de las interacciones multiespecíficas? Probablemente especies y atributos que suelen estar vinculados en interacciones multiespecíficas (desde la perspectiva de redes, especies nodales en una comunidad) deban de ser el foco para estudiar, a nivel micro y macro (co)evolutivo, fenómenos altamente frecuentes como la evolución difusa y no necesariamente súper espectaculares pero poco comunes como la coevolución entre pares.

Una predicción sencilla se ha propuesto sobre el tipo de defensa que debería evolucionar cuando las plantas enfrentan a herbívoros generalistas y especialistas. En

particular se espera que las especies especialistas sean menos afectadas por metabolitos secundarios que las especies generalistas, ya que la especialización de los primeros puede haber superado las barreras defensivas de la planta hospedera (Whittaker y Feeny 1971); evidencia sobre esta predicción la confirman (Ali y Agrawal 2012). De esta manera, se espera que si el valor adaptativo de la resistencia decae (por ejemplo, al decaer el valor defensivo de los metabolitos secundarios) al enfrentar a herbívoros especialistas, el valor adaptativo de la tolerancia se incremente (Agrawal y Fishbein 2008; Garrido ***et al.*** 2012). Este patrón fue observado en el capítulo I, donde cambios en los valores adaptativos de las estrategias defensivas son condicionados por la presencia del herbívoro generalista, ***Epitrix parvula*** y el especialista, ***L. daturaphila***. Si el valor adaptativo de la resistencia y tolerancia está condicionado no sólo por la presencia/ausencia de especies, sino por el nivel de especialización de éstas, se puede proponer la selección y evolución difusa como un mecanismo ecoevolutivo que pudiera restringir el proceso de escalada armamentista (Garrido y Fornoni 2006). En particular se predice una escalada armamentista cuando el valor defensivo y adaptativo de la resistencia es alto y por ende, favorecido para enfrentar a los herbívoros. Esta predicción es esperable ya que la resistencia afecta la adecuación de los herbívoros y por consiguiente favorece la evolución de contraadaptaciones (Garrido y Fornoni 2006). Por otro lado, dado que la tolerancia no afecta la adecuación de los herbívoros, no promueve la evolución de contraadaptaciones y por lo tanto tampoco la generación de un proceso de carrera armamentista. De manera interesante el capítulo III también apoya la visión de que las plantas utilizan atributos de historia de vida (ej. tasa de crecimiento, tiempo a la reproducción e inversión en estructuras

reproductivas) para enfrentar el ataque de especialistas, atributos de historia de vida que desde la aproximación de este capítulo (III) pueden ser considerados como estrategias defensivas, lo cual nos plantea la discusión acerca de dónde comienza y termina una adaptación.

Los capítulos II y III no sólo invitan a considerar ambientes selectivos más realistas (capítulo II) y fenotipos defensivos más complejos (capítulos II y III), sino a cuestionarnos dónde comienza y termina una adaptación, en particular las adaptaciones defensivas. Este punto es fundamental por muchas razones (Wagner 2001), siendo una de ellas que obviamente la propia delimitación de la adaptación bajo estudio determinará nuestra capacidad para entender y predecir su evolución. Esta capacidad predictiva está condicionada, en primer lugar, por nuestra capacidad de definir la adaptación y posteriormente de entender su naturaleza contextual, es decir, cómo interactúa con otros caracteres y con el ambiente ecológico. El capítulo III señala qué atributos de historia de vida condicionan el nivel de resistencia de las plantas. De esta manera, la presencia de una correlación significativa (tanto negativa como positiva) entre algún atributo de historia de vida (ej. tasa de crecimiento) y la cantidad de daño indicaría que dicho caracter tiene un componente defensivo. El capítulo II, por su parte, muestra cómo el valor adaptativo de atributos de historia de vida cambia en función de la manipulación de herbívoros (i.e. de agentes selectivos), lo que indica que dichos atributos tienen un componente defensivo pero muy probablemente vía la tolerancia. De esta forma, los límites que definen una adaptación defensiva como la resistencia o la tolerancia pueden ser cuestionados, y ser un componente más a considerar cuando los modelos teóricos fallan al tratar de explicar lo que vemos en la

naturaleza. Así, en esta tesis se considera importante rescatar la aproximación de analizar de manera simultánea y multivariada los patrones selectivos, la arquitectura del fenotipo y la respuesta a la selección en condiciones ecológicas cambiantes, no sólo para comenzar a estudiar la evolución multiespecífica o difusa, sino para evaluar la estabilidad de lo que definimos como una adaptación (Lewontin 2001).

Así como el estudio de las restricciones genéticas ha sido incluido en el programa adaptacionista (Conner 2012) es importante considerar de manera explícita las restricciones ambientales que pueden condicionar la evolución de los fenotipos en sí y la de las interacciones ecológicas en las que se encuentran involucrados. Teniendo esto en mente, en el capítulo II se diseñó la prueba del "trinche de asado" (carving fork analysis), que permite integrar la estructura genética de los fenotipos (matriz **G**), los patrones selectivos y evaluar su interacción con el ambiente cuando éste es manipulado. Todo en conjunto permite evaluar la importancia de restricciones ecológicas y genéticas al proceso de evolución por selección natural. Habiendo adquirido y valorado esta visión de evolución multivariada en un ambiente multiespecífico, cabe la pregunta de si por la forma en que estudiamos los costos adaptativos no deberían de ser vistos desde una perspectiva similar. Una aproximación como la presentada en esta tesis puede ayudar a entender de manera más integral cómo las especies evolucionan enfrentando cambios vertiginosos producidos directa o indirectamente por el hombre.

De la misma manera en que es muy poco probable que las especies evolucionen en condiciones ambientales estables como las producidas en centros de crianza y mejora de especies (Lush 1934), es muy poco realista querer hacer predicciones

ecoevolutivas en comunidades libres de efectos antropogénicos. Actualmente, el ***Homo sapiens*** es una especie ***clave*** en la determinación de la estructura de los ecosistemas y comunidades. La forma en la que está modificando su entorno hace necesario abrir el panorama de investigación teórica y aplicada para poder entender este nuevo fenómeno ecológico que tiene claros efectos evolutivos (Palumbi 2001). Dicho contexto señala la importancia por entender y predecir los procesos microevolutivos de las interacciones ecológicas bajo presiones antropogénicas, como en el caso de la evolución de fenotipos resistentes y/o tolerantes a herbicidas (Baucom y Mauricio 2008), insecticidas (National Research Council 2000) y antibióticos (Hughes y Datta 1983), o la evolución de fenotipos altamente capaces de invadir nuevas comunidades, como ocurre en el caso de las especies invasoras (Lee 2002). En este contexto aplicado, el estudio de la dinámica ecoevolutiva impulsada por una rápida evolución (Thompson 1998), necesita de las herramientas generadas por la ciencia básica para plantear soluciones sustentables a más largo plazo sobre el manejo de la problemática ambiental. Por ejemplo, es muy probable que fallen aquellos planes de manejo de especies invasoras basados únicamente en análisis demográficos que no consideran la variación fenotípica entre los individuos invasores. Por lo tanto, se debe favorecer una visión más integrada tal y como la que propone Lee (2002), donde se indica la necesidad de explorar "el papel que juegan las restricciones genéticas, la expresión de la varianza genética aditiva, la epistasis, los procesos de hibridización, el efecto de pocos genes y posiblemente la reorganización genómica, en explicar el potencial invasivo de las especies". Por ejemplo, es lógico que los atributos de las especies delimiten su rango de distribución y su capacidad de invadir nuevos hábitats (Tsutsui ***et***

al. 2000). Por ejemplo, entender el papel que juega la tolerancia (Fornoni 2011) y en general la plasticidad fenotípica parece ser fundamental para entender la capacidad invasiva de una especie (Lee 2002; Ghalambor ***et al.*** 2007).

Finalmente, la invasión de una especie, al igual que la invasión de una variante fenotípica (ej. la evolución de fenotipos más resistentes en una población) depende no sólo de su naturaleza intrínseca, sino del contexto en donde se encuentran o expresan y cómo estos contextos interactúan de manera dinámica. El estudio en paralelo de las interacción entre especies (ambientes multiespecíficos), entre atributos (arquitectura genética de los fenotipos) y entre procesos ecológicos y evolutivos (dinámica ecoevolutiva) constituye un área fértil para construir hipótesis alterativas sobre cómo evolucionan los fenotipos en un ambiente multidimensional en constante degradación.

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