UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE ECOLOGÍA
BIOLOGÍA EVOLUTIVA

VARIACIÓN, VALOR ADAPTATIVO Y PLASTICIDAD
DE LA INTEGRACIÓN FENOTÍPICA

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS
PRESENTA:

MARÍA DE JESÚS XÓCHITL DAMIÁN DOMÍNGUEZ

TUTORA PRINCIPAL DE TESIS: DRA. KARINA BOEGE PARÉ
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MÉXICO, CD. MX. , JUNIO 2022.

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M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted, que el Comité Académico del Posgrado en Ciencias Biológicas, en su reunión ordinaria del día 14 de febrero de 2022, aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante: **DAMIAN DOMÍNGUEZ MARÍA DE JESÚS XOCHITIL,** con número de cuenta **301167879,** con la tesis titulada: **"VARIACIÓN, VALOR ADAPTATIVO Y PLASTICIDAD DE LA INTEGRACIÓN FENOTÍPICA",** bajo la dirección de la **DRA. KARINA BOEGE PARÉ**, Tutora Principal, quedando integrado de la siguiente manera**:**

Presidente:	DR. JUAN SERVANDO NUÑEZ FARFÁN
Vocal:	DRA. NATALIA IVALÚ CACHO GONZÁLEZ
Vocal:	DRA. JULIETA ALEJANDRA ROSELL GARCÍA
Vocal:	DRA. AURORA GAXIOLA ALCANTAR
Secretario:	DR. JUAN ENRIQUE FORNONI AGNELLI

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

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 19 de mayo de 2022

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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The best scientist is open to experience and begins with romance — the idea that anything is possible.

Ray Bradbury

In science, it often happens that scientists say, 'You know that's a really good arguments; my position is mistaken,' and then they would actually change their minds and your never hear that old view from them again. They really do it. It doesn't happen as often as it should, because scientists are human, and change is sometimes painful. But it happens every day. I cannot recall the last time something like that happened in politics or religion.

Carl Sagan

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RESUMEN

La selección natural es un mecanismo evolutivo que influye en los componentes de adecuación de los organismos, actuando de forma simultánea en numerosos rasgos que expresa un organismo. Aunque la idea de que la de selección natural es un fenómeno multivariado no es ajena a la ecología evolutiva, la mayor parte de los estudios se han enfocado en analizar el valor adaptativo de los rasgos de forma independiente, o bien, en realizar análisis de numerosos caracteres de los individuos con un enfoque univariado. Esta perspectiva es completamente razonable dadas las limitaciones logísticas y experimentales, sin embargo, las conclusiones obtenidas de los estudios con enfoques univariados tienen la limitación que supone el obviar las relaciones entre rasgos o las correlaciones entre pares de rasgos. Por tanto, un enfoque multivariado nos permitiría analizar de manera apropiada a los fenotipos complejos y los factores que influyen en su potencial evolutivo.

Un enfoque para entender la evolución de fenotipos complejos es utilizando el concepto de integración fenotípica, que se define como la red de relaciones multivariadas entre rasgos morfológicos, fisiológicos o conductuales que define ciertos módulos funcionales de los organismos. En este trabajo se emplea al arbusto *Turnera velutina* Presl (Passifloraceae) como un modelo para el estudio de la integración fenotípica como rasgo complejo. A través de un enfoque experimental y descriptivo se analizaron las implicaciones de la variación en la magnitud y los patrones de integración de atributos foliares de entre familias maternas de una población experimental establecida en La Mancha, Veracruz. Adicionalmente, se determinó la influencia de agentes de selección como la precipitación y la disponibilidad de luz en la modificación de los niveles y patrones de integración. Para ello se midieron en las plantas de estudio rasgos foliares que describen la estrategia funcional de las plantas, como la masa foliar específica, la densidad de tricomas, los contenido de azúcar en el néctar extrafloral, cianogénicos, clorofila, el isótopo δ13C y la razón Carbono:Nitrógeno.

Entre los principales resultados, destaca que se detectaron niveles significativos de variabilidad en la magnitud y los patrones de integración de los rasgos funcionales dentro y entre poblaciones. A nivel poblacional, los fenotipos más integrados crecieron más rápido y produjeron más flores que los menos integrados. El patrón de variación entre poblaciones mostró una relación inversa entre la magnitud de la integración y algunas variables ambientales como la precipitación en los meses más secos y cálidos. Por último, la norma de reacción que describe la plasticidad de la magnitud de la integración mostró que las estrategias funcionales de las hojas se integran y convergen en el morfoespacio en condiciones de baja disponibilidad de luz.

Estos resultados demuestran que la magnitud de la integración puede considerarse un carácter complejo sobre el que actúa la selección natural, moldeando grupos de rasgos que constituyen las estrategias funcionales que las plantas despliegan dentro o entre

poblaciones. Los factores ambientales, como la precipitación y la disponibilidad de luz modifican los niveles de integración y los patrones de correlaciones entre caracteres, optimizando estrategias funcionales que dependen del contexto selectivo. Por último, las evidencias apuntan a que las asociaciones entre rasgos son un importante componente del potencial evolutivo de los organismos, un componente que no puede ser determinado sin la consideración y uso de un enfoque multivariado apropiado.

ABSTRACT

Natural selection is a multivariate process. This evolutionary mechanism influences fitness' components acting simultaneously upon numerous traits of the organism. Although this idea is not new in evolutive ecology, most studies have focused on analyzing the adaptive value of traits individually or assessing different characters with an univariate approach. This method is reasonable given the logistic and experimental restrictions of many study systems. However, the conclusions obtained from research considering univariate approaches have limitations underscoring relationships among traits or the correlations between pairs of traits. Thus, a multivariate approach on the study of natural selection should enhance our understanding of complex phenotypes and the factors affecting their evolutionary potential.

An approach to study complex phenotypes is using of the concept of phenotypic integration, which represents the network of relationships among morphological, physiological, or behavioral traits that define particular functional modules of an organism. In this work we used the *Turnera velutina* Presl (Passifloraceae) shrub as model to study phenotypic integration of leaf traits as a complex character. With experimental and descriptive approaches, we analyzed the meaning of the variation in the magnitude and patterns of phenotypic foliar integration among maternal families in an experimental population established in La Mancha, Veracruz state. Additionally, we tested the influence of selection pressures as precipitation and light availability on phenotypic integration levels and patterns. In the studied plants, we measured foliar traits to determine the functional strategy of plants, including leaf mass per area, trichome density, sugar nectar in the extrafloral nectar, cyanogenic content, chlorophyll content, $\delta 13C$, and C:N.

We detected significant levels of variation in the magnitude and patterns of foliar integration of functional traits within and among populations. At a population level, the more integrated phenotypes grew faster and had more flowers. The integration patterns among populations showed an inverse relationship between the integration magnitude and the precipitation in the driest and warmest months. Finally, the reaction norm describing the plasticity in the magnitude of foliar integration revealed that functional strategies are integrated and converged in the morphospace when plants grew under light limitation conditions.

These results confirm that phenotypic integration can be considered a complex character upon which natural selection acts, defining sets of traits behind plant functional strategies within or among populations. Environmental factors as precipitation or light availability can modify the level of integration and the correlation patterns among characters, optimizing functional strategies in response to selective context. At last, the evidence of this work supports the idea that associations among traits are an important component of the evolutionary potential of organisms, an overlooked component unless a multivariate approach is used.

INTRODUCCIÓN GENERAL

Los organismos son sistemas complejos con fenotipos que son mucho más que la suma de sus rasgos individuales. Sin embargo, tradicionalmente los estudios evolutivos han analizado estos fenotipos como una colección de rasgos aislados desde una perspectiva univariada (Pigliucci 2004) o considerando el control de las correlaciones entre atributos (Lande y Arnold 1983). Sin embargo, desde hace tiempo, trabajos como el de Paul V. Terentjev (1931) muestran la preocupación por entender el papel funcional y adaptativo de rasgos individuales que covarían o están relacionados para expresar un fenotipo complejo (p.e. Pléyades de correlación). Este enfoque fue retomado alrededor de 1960 cuando Everett Olson, Robert Miller (1958) y Raisa Berg (1960) reinterpretaron este concepto como el de integración fenotípica denotando una asociación funcional y morfológica entre rasgos que forman parte de una estructura común, hasta integrar factores genéticos, ontogenéticos o adaptativos que resultan en la tendencia a la covarianza entre rasgos particulares (Zelditch 1988, Herrera et al. 2002, Wagner y Zhang 2011, Murren 2012, Ordano et al. 2008, Armbruster 2014). Actualmente, las propiedades emergentes de las matrices de covarianza entre rasgos fenotípicos han sido abordadas desde el estudio de los dos componentes de la integración, la magnitud y el patrón de integración (Armbruster 2014, Fig. 1). Por un lado, la magnitud de la integración expresa la intensidad media de las covarianzas o correlaciones entre múltiples rasgos. Si los rasgos del fenotipo se organizan sobre un número reducido de ejes de variación indicando que están fuertemente asociados, su integración será alta (Wagner 1984, Cheverud et al. 1989). Por otro lado, el patrón de la integración indica cómo se asocian los rasgos (Waitt y Levin 1993, Nicotra et al. 1997, Relyea 2001). En las últimas dos décadas, estas propiedades se han empleado de manera recurrente para evaluar la integración fenotípica como un rasgo intrínseco de las especies con efectos directos sobre la evolvabilidad, produciendo respuestas coordinadas en varios rasgos bajo presiones selectivas (Hallgrimsson et al. 2009, Wagner et al. 2007, Goswami et al. 2014).
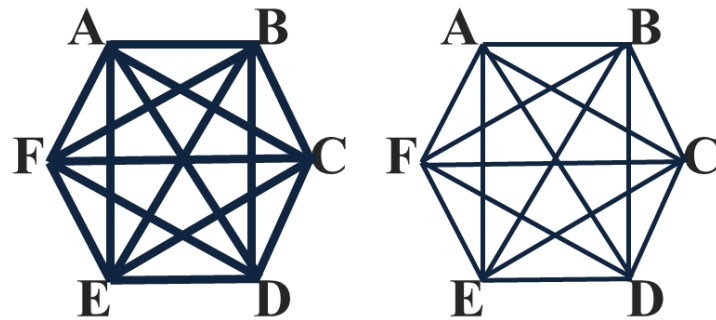
Figura 1. La integración fenotípica se define a través de las correlaciones entre atributos en un fenotipo hipotético. Las letras A-F representan diferentes atributos y las líneas que las unen correlaciones de diferente intensidad de acuerdo con el grosor. Izquierda. Fenotipo altamente integrado debido a la fuerza de las correlaciones entre atributos. Derecha. Fenotipo con baja integración y correlaciones débiles entre los rasgos que describen al fenotipo.

A pesar de que el concepto Integración fenotípica no es nuevo, supone un cambio en el enfoque de análisis de los fenotipos porque la adaptación es un proceso inherentemente multivariado que requiere, a su vez, de un enfoque multivariado (Pigliucci 2004, Blows et al. 2007, Arnold et al. 2008, Murren 2012). Mientras el promedio y la varianza proveen información crítica sobre la tendencia general del cambio en rasgos individuales bajo selección, emplear las covarianzas (integración fenotípica) permite analizar la ruta evolutiva de los caracteres complejos y cómo los cambios relativos de un rasgo están acompañados de cambios simultáneos en otros atributos, definiendo, por ejemplo, las estrategias funcionales de un organismo (Pigliucci 2004, Hansen y Houle 2008, Goswami et al. 2014). Existen dos hipótesis sobre el papel de la integración sobre la ruta evolutiva de los fenotipos. Por un lado, se espera que la integración de un fenotipo cambie de acuerdo con un ajuste de las relaciones funcionales de los rasgos que lo conforman a través de un incremento en la variabilidad coordinada de dichos rasgos, por lo que la selección natural podría actuar sobre ciertos rasgos que covarían entre sí (Björklund 1996, Bontemps et al. 2017). En este caso,

favorecer la covarianza entre ciertos rasgos facilitaría seguir las líneas de menor resistencia evolutiva para una población. Sin embargo, las correlaciones entre rasgos representan una restricción en el sentido de que, si dos rasgos están positiva o negativamente asociados, hay zonas del espacio fenotípico a las que la población no puede acceder o lo hace de forma limitada cuando el eje principal de variación es perpendicular a la dirección de la selección (Schluter 1996, Klingenberg 2005). Por ejemplo, si dos especies tienen el mismo valor para la media y la varianza de dos rasgos, pero muestran un patrón opuesto de covarianzas, dichas especies ocuparán diferentes regiones del espacio fenotípico (Laughlin y Messier 2015).

En años recientes se ha incrementado el número de estudios que reconocen la importancia de la covarianza entre rasgos a través de los componentes de la integración. A través de esta perspectiva se ha abordado el estudio de disyuntivas entre funciones (Bonser 2006, Pigliucci 2003), las estrategias de historia de vida (Wright et al. 2004, Chave et al. 2009), las tendencias filogenéticas de la integración (Marroig y Cheverud 2001, Pérez et al. 2007, Felice et al. 2018), la variabilidad de la integración entre poblaciones (Waitt y Levin 1993, Herrera et al. 2002) y su potencial como rasgo adaptativo (Ordano et al. 2008, Lázaro y Santamaría 2016) en una variedad de sistemas y empleando rasgos morfológicos, fisiológicos o conductuales (Conner et al. 2014). Los resultados de estos estudios nos han permitido concluir que, si bien la integración fenotípica influye en la evolvabilidad de los rasgos, esto sólo ocurre cuando la dirección de las covarianzas se alinea con las líneas de menor resistencia (Goswami et al. 2014). Por ejemplo, en el caso de las plantas, la principal presión selectiva sobre la integración morfológica de las flores parece estar asociada con el acople anatómico entre los vectores de polinización y el acceso a los órganos reproductivos (Benitez-Vieyra et al. 2018), sin descartar otras fuerzas selectivas como el sistema reproductivo (Rubini-Pisano 2020). En contraste, los estudios de integración fenotípica en órganos vegetativos como las hojas han tomado como modelo de estudio los rasgos fisiológicos, encontrando que los cambios en grupos de estos atributos promueven cambios en las estrategias funcionales de estos rasgos a través del área de distribución de las especies, en respuesta a la variación en factores como la temperatura, la precipitación o la

disponibilidad de nutrientes (Boucher et al. 2013, Salgado-Negret et al. 2015, Seguí et al. 2017, Benavides et al. 2021).

La mayoría de los estudios previamente mencionados se han centrado en analizar la variabilidad de integración fenotípica a nivel intraespecífico (Ordano et al. 2008, Benítez-Vieyra et al. 2018) o interespecífico (Waitt y Levin 1993, Ordano et al. 2008, Benavides et al. 2021), pero son muy pocos los trabajos que han evaluado los niveles de integración fenotípica en individuos pertenecientes a la misma población y los efectos de esa variación sobre la adecuación de las plantas (p.e. Lázaro y Santamaría 2016, Bontemps et al. 2017). En consecuencia, no tenemos evidencias de los intervalos de variación, de los impactos de la integración sobre la adecuación de los individuos, ni del cambio de este carácter complejo bajo diferentes escenarios selectivos. El aporte del presente trabajo, en este contexto, constituye en determinar estos aspectos de la integración fenotípica, utilizando las hojas como módulos funcionales y empleando un enfoque experimental y descriptivo para determinar si la integración foliar es un carácter multivariado con un valor adaptativo y plástico y las implicaciones de este fenómeno en el contexto evolutivo.

Al ser organismos modulares, las plantas son un modelo ideal para el estudio de la integración fenotípica. Las hojas son órganos clave para la ganancia de recursos y su fenotipo se puede describir a partir de las relaciones entre sus rasgos funcionales. Los rasgos funcionales se definen como caracteres morfológicos, fisiológicos o fenológicos que tienen un efecto sobre la adecuación (Violle et al. 2007) y los patrones de covarianza entre ellos están asociados a ejes de variación que definen un continuo entre estrategias funcionales conocido como el espectro de economía foliar (Reich et al. 1997, Wright et al. 2004). En un extremo del continuo se encuentran especies con altas tasas fotosintéticas y de intercambio de gases, con hojas con una alta concentración de nutrientes, baja masa foliar específica y de corta longevidad, asociadas con especies de ganan recursos en un periodo de tiempo corto y son más productivas. El otro extremo del espectro está caracterizado por especies con características opuestas, que reflejan una estrategia de conservación de recursos con tasas fotosintéticas y concentración de nutrientes en las hojas comparativamente más bajas. Las estrategias funcionales se consideran como la respuesta fenotípica expresada por las

plantas en condiciones que resultan de las presiones selectivas, las restricciones biofísicas y anatómicas y la disponibilidad de recursos (Reich et al. 1997).

Las hojas de las plantas muestran una amplia variación morfológica y ambiental entre individuos y poblaciones. Las hojas del arbusto tropical *Turnera velutina* Presl. (Turneraceae) además, exhiben caracteres relacionados con la defensa contra herbívoros, como la presencia de nectarios extraflorales y la secreción de néctar extrafloral que colectan las hormigas de diferentes especies y que constituyen una defensa biótica dentro de la estrategia de defensa de esta especie (Ochoa-López et al. 2015). *T. velutina* está distribuida en México en playas y dunas costeras, en bosque tropical caducifolio y selva baja, desde el nivel del mar hasta los 1300 m de altitud (Arbo 2005). Adicionalmente, en un estudio previo se han identificado los rangos de variación de la integración fenotípica en plantas juveniles y de estadio reproductivo y su asociación con la biomasa bajo condiciones controladas (Damián et al. 2018, Anexo). Estas características en los rasgos foliares y la facilidad relativa para establecer poblaciones experimentales convierten a esta especie en un modelo ideal para evaluar los efectos de la variación de la integración fenotípica dentro y entre poblaciones.

Dada la importancia de las flores para la reproducción de las plantas, no resulta sorprendente que sean estas las estructuras sobre las que se ha evaluado el valor adaptativo de la integración fenotípica (Ordano et al. 2008, Lázaro y Santamaría 2016). No obstante, en las cuatro especies de la familia Rosaceae analizadas en Ordano et al. (2008) no se encontraron gradientes significativos de selección sobre la integración floral, pero en *Loniceras implexa* (Caprifoliaceae) se encontró un patrón de selección disruptiva entre la magnitud de la integración y el número de semillas (Lázaro y Santamaría 2016). En este trabajo, se investigó, por primera vez el valor adaptativo de la integración de las hojas, estructuras fundamentales para la sobrevivencia y crecimiento de las plantas. En el capítulo uno se evaluó la relación entre adecuación e integración empleando rasgos funcionales de las hojas de *T. velutina* en una población experimental establecida en las dunas costeras del Centro de Investigaciones Costeras La Mancha (CICOLMA) en el estado de Veracruz. La población experimental se estableció con individuos de 20 genotipos en los que se realizaron

las estimaciones de integración fenotípica y se aplicó un Análisis Jerárquico de Selección (Ordano et al. 2008). Este capítulo fue publicado como artículo requisito para el proceso de graduación (Damián et al. 2020).

La asociación positiva entre la integración y la adecuación de las plantas obtenida en el primer capítulo dio pie al análisis de los cambios de la integración ante diferentes condiciones ambientales. En el segundo capítulo la estimación de la integración se realizó a nivel de poblaciones en cinco sitios establecidos a lo largo de un gradiente de precipitación de 700 a 3200 mm de precipitación media anual. Además de mostrar un patrón positivamente relacionado con la disponibilidad de agua en los meses más cálidos y secos, se detectaron patrones particulares de integración en las poblaciones, que coinciden con las estrategias funcionales descritas en el espectro de economía foliar. Este capítulo se presenta en el formato de manuscrito que ha sido sometido a revisión en la revista *American Journal of Botany*.
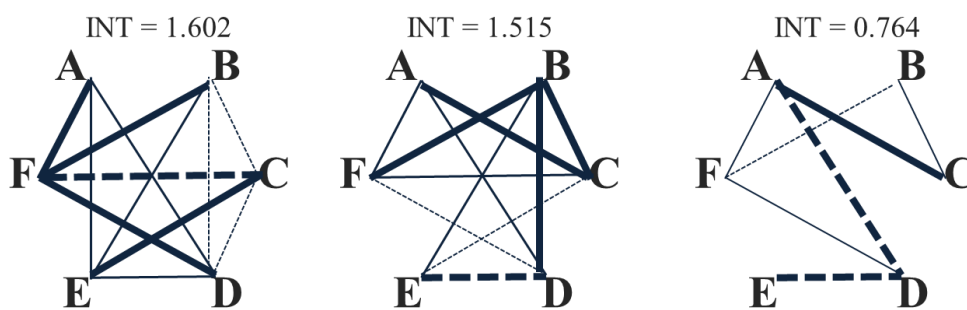
Figura 2. Plasticidad de los componentes de la integración fenotípica. Los diagramas representan patrones de correlación entre atributos en diferentes ambientes y el valor de la magnitud de la integración. Los esquemas de la izquierda y el centro tienen valores de integración fenotípica similares diferente patrón de correlación. El esquema de la derecha muestra cambios tanto en la magnitud como el patrón de las correlaciones.

Considerando los resultados previos, el capítulo tres se enfoca en analizar la capacidad de cambio de la magnitud y el patrón de la integración bajo dos condiciones de disponibilidad de luz (Fig. 2). Para este capítulo se tomaron como punto de partida las

diferencias en la magnitud de integración de las familias maternas evaluadas en el capítulo uno, por lo que una nueva población experimental fue generada con doce de esas familias en 2017 para evaluar la norma de reacción de la integración y determinar qué zonas del espacio fenotípico están asociadas con las dos condiciones lumínicas que experimentan las plantas. Este capítulo se presenta con el formato de un manuscrito para ser enviado a revisión a *Journal of Evolutionary Biology*. Finalmente, se anexa la publicación que muestra los detalles del cambio de la integración fenotípica y sus implicaciones para plantas de *T. velutina* en dos estadios ontogenéticos (Damián et al. 2018) como antecedente al presente trabajo.

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Capítulo 1. Natural selection acting on integrated phenotypes: covariance among functional leaf traits increases plant fitness.

Xóchitl Damián, Sofía Ochoa-López, Aurora Gaxiola, Juan Fornoni, César A. Domínguez and Karina Boege. 2020. New Phytologist, 225: 546-557.

Natural selection acting on integrated phenotypes: covariance among functional leaf traits increases plant fitness

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Summary

• Plant functional strategies are usually accomplished through the simultaneous expression of different traits, and hence their correlations should be promoted by natural selection. The adaptive value of correlations among leaf functional traits, however, has not been assessed in natural populations.

• We estimated intraspecific variation in leaf functional traits related to the primary metabolism and anti-herbivore defence in a population of *Turnera velutina*. We analysed whether natural selection favoured the expression of individual traits, particular combinations of traits or leaf phenotypic integration.

• Patterns of covariation among traits were related to water and nitrogen economy, and were similar among genotypes, but the magnitude of their phenotypic integration differed by 10-fold. Although families did not differ in the mean values of leaf functional traits, directional selection favoured low nitrogen content and low chemical defence, high content of chlorophyll, sugar in extrafloral nectar and trichome density. Families with higher phenotypic integration among leaf traits grew faster and produced more flowers.

• We suggest that the coordinated expression of leaf traits has an adaptive value, probably related to optimisation in the expression of traits related to water conservation and nitrogen acquisition.

Introduction

Adaptation is an inherently multivariate process (Lande, 1979; Blows, 2007) resulting from the action of natural selection upon the simultaneous expression of morphological and physiological characters (Relyea, 2001; Ghalambor *et al.*, 2003). Hence, covariance among traits should play a key role influencing the tempo and mode of evolution of multivariate phenotypes (Gould & Lewontin, 1979; Lande, 1979). Depending on the orientation of the multivariate axis of maximum genetic and phenotypic variation, correlations among traits (standardised estimates of covariation) can influence the rate and direction of phenotypic evolution (Schluter, 1996; Lynch & Walsh, 1998; Blows *et al.*, 2004). Correlations themselves can be targets of natural selection, thus favouring the evolution of adaptive associations among specific sets of traits (Cheverud, 1996). In this context, examining the properties of the genetic variance–covariance matrix of leaf traits (Arnold, 1992) and its relationship with fitness should provide insights into how phenotypes evolve. In addition, this approach can facilitate the detection of possible constraints and synergies on multivariate evolutionary responses.

Because plants have a modular organisation, intra-individual trait variances and covariances can be estimated. This constitutes an ideal system to test the adaptive value of covariation among functional traits at a microevolutionary scale. For example, sets of traits are involved in interactions with pollen vectors and fruit dispersal agents in flowers and fruits, respectively (Ordano *et al.*, 2008; Valido *et al.*, 2011), resource acquisition in leaves (Wright *et al.*, 2004; Díaz *et al.*, 2016), or sap transport and mechanical support in stems (Chave *et al.*, 2009; Montes-Cartas *et al.*, 2017). Specifically, leaves simultaneously express traits in a coordinated way to achieve different functions. For instance, leaf area, toughness and chlorophyll (Chl) content are positively related to carbon acquisition through photosynthesis (Génard *et al.*, 2008), while combinations of different chemical defences reduce foliar damage by herbivores (Johnson *et al.*, 2009). In addition, both resource acquisition and defence functions commonly have a nonindependent expression due to resource trade-offs, multifunctionality of traits and/or regulatory processes (Mason & Donovan, 2014; Züst & Agrawal, 2017). Optimisation theory predicts that particular phenotypes should be favoured by natural selection when the ratio between the benefits and costs of their expression is maximized (Richardson, 1994). Given that multiple traits often share functions, natural selection should promote the simultaneous optimisation of such traits considering both direct and indirect costs and associated trade-offs. As a result, the

expression of functional strategies (Grime, 1979; Reich *et al.*, 2003), namely specific combinations of functionally associated traits, should promote greater fitness than others.

The covariation of plant functional traits has been studied through descriptions of functional strategies in different species and ecosystems (Wright *et al.*, 2004; Díaz *et al.*, 2016). However, the microevolutionary processes shaping these strategies have not been fully described (Donovan *et al.*, 2011; Moles, 2018). Interspecific patterns of covariation among functional traits constitute a first line of evidence of the restrictions in the expression of particular combinations of traits (Díaz *et al.*, 2016). For example, the expression patterns of foliar attributes associated with the leaf economics spectrum (LES, *sensu* Wright *et al.*, 2004) show that species converge in functional strategies within a continuum from conservative to acquisitive resource use. These strategies are represented by the expression of sets of related traits. In particular, species with acquisitive strategies are characterised by a low investment in mass per leaf area (LMA), and nitrogen-rich, short-lived leaves. By contrast, species with conservative strategies have long-lived leaves, high LMA, low leaf nitrogen concentrations (N_{mass}) and slow growing rates. A second line of evidence is the intraspecific variation in multivariate functional strategies along environmental gradients (Boucher *et al.*, 2013; Brouillette *et al.*, 2014). For instance, *Helianthus anomalus* populations growing in dry locations with low nutrient availability have higher leaf N_{mass}, lower water use efficiency (WUE) and early flowering compared with populations growing on more humid and fertile soils (Brouillette *et al.*, 2014). Although this evidence suggests an adaptive explanation for the covariation among functional traits (Arntz & Delph, 2001; Donovan *et al.*, 2011; Reich, 2014), there are no formal experimental assessments of the intraspecific variation of functional strategies and their adaptive value within plant populations.

One approach to study multivariate functional strategies is to consider the phenotype as a vector of correlated traits (Collyer & Adams, 2007). The concept of phenotypic integration allows the magnitude and pattern of covariation in the expression of different traits to be analysed (Arnold, 2005). The magnitude of integration indicates the tendency or disposition for covariation (Armbruster *et al.*, 2014) and provides insight into the flexibility of associations among multiple traits. Accordingly, greater values of integration mean stronger correlations among traits. A second property of the phenotypic integration concept is the pattern of these correlations. The analysis of the patterns of phenotypic integration allows the detection of differences in trait association and fine-scale relationships among traits, revealing the potential constraints acting on the independent evolution of different foliar functions (Reichert & Höbel, 2018). For example, a recent analysis of the functional strategies related to resource-storage and acquisition during the domestication of wheat, detected that the number of significant correlations (a proxy of integration magnitude; Nicotra *et al.*, 1997) was greater in wild ancestors than in domesticated crops (Roucou *et al.*, 2018). Ancestral varieties were integrated phenotypes with strong relationships among above- and below-ground traits. The authors suggested that artificial selection decoupled functions of capture and resource use,

resulting in crop genotypes with correlation patterns that better optimise nutrient acquisition. Hence, quantitative measures of the magnitude of individual phenotypic integration of functional traits (Wilson & Nussey, 2010), coupled with estimations of plant fitness, offer an opportunity to assess its adaptive value under natural conditions.

Until now, most efforts testing the adaptive value of covariance in plants have been conducted only on flower morphology as an adaptation to optimise pollination (Ordano *et al.*, 2008; Benítez-Vieyra *et al.*, 2014; Lázaro & Santamaría, 2016), whereas very limited information is available for leaf traits (Bontemps *et al.*, 2017). The main goal of the present study was to experimentally test whether plant fitness is related to the degree of covariation among particular sets of leaf functional traits. First, we assessed whether genetic families of *Turnera velutina* growing in natural conditions differed in the magnitude and pattern of phenotypic integration of their leaves. We then evaluated whether natural selection acted on the mean values of individual leaf traits, on particular combinations of traits and on the magnitude of their phenotypic integration. We hypothesised that phenotypic integration of functional traits should be the result of the optimisation in the expression of multiple traits as functional strategies related to resource acquisition and plant defence. Hence, we expected that families with higher integration of foliar traits should have high vegetative and reproductive performance. Assessing the variability in phenotypic integration of functional traits and its relationship to plant fitness may highlight the adaptive value of complex phenotypes or functional modules beyond the selective value of single traits.

Materials and Methods

Study system

Turnera velutina Presl (Passifloraceae) is an endemic Mexican shrub (Arbo, 2005) that grows in tropical dry forests and in sand-dune vegetation under the shade of trees. Flowering occurs mostly during the rainy season (Torres-Hernández *et al.*, 2000). Fruits are capsules with an average of 36 ant-dispersed seeds (Sosenski *et al.*, 2017). *T. velutina* produces extrafloral nectaries on both sides of the leaf petiole. Several species of ants consume extrafloral nectar and protect the plant by reducing leaf damage caused by the main specialist herbivore *Euptoieta hegesia* Cramer (Lepidoptera: Nymphalidae; Cuautle & Rico-Gray, 2003). A recent study has shown that extrafloral nectar can also distract ants from entering the flowers and disturbing pollinators (Villamil *et al.*, 2018, 2019). *T. velutina* displays multiple defensive traits against herbivores besides extrafloral nectar, such as trichomes, toughness and hydrogen cyanide (Ochoa-López *et al.*, 2015). In addition, ontogenetic changes in phenotypic integration of physiological, defensive and morphological leaf traits have been previously reported in this species (Damián *et al.*, 2018).

An experimental population of *T. velutina* was established in a coastal sand dune at the field station Centro de Investigaciones Costeras La Mancha (CICOLMA), located along the coast of Veracruz, Mexico (19°35′N, 96°22′W, < 100 m above sea level).

The climate in this area is warm and subhumid with mean annual precipitation of 1286 mm, which occurs mostly between June and September. Mean annual temperature ranges between 24°C and 26°C, with minimum and maximum temperatures of 15°C and 41°C, respectively (CONAGUA, 2016). Plants from the experimental population were obtained by first generating an F_1 generation ($n = 300$ plants) from 20 maternal plants obtained from their natural sand dune habitat. An F_2 generation (2000 seeds per family) was obtained through self-pollination of F_1 plants (for further details, see Ochoa-López et al., 2018). In August 2014, 1200 3-wk-old seedlings of the 20 genetic families were transplanted in 20 1×1 m plots in four sites where *T. velutina* naturally occurs. Each plot had between three and 10 plants per genetic family. This design allowed plants to interact with their natural herbivores, pathogens, pollinators and competitors, dealing with the variation in environmental conditions over 2 yr. The advantage of using genetic families from an experimental population allowed us to reduce error measurements while estimating leaf functional traits, and to assess the potential genetic basis of the covariation among those traits.

Data collection

Leaf traits were measured in October 2015 when 70% of the plants in the experimental population had reached their reproductive stage. All traits were measured on the first four apical fully expanded leaves of plants from 13 genetic families, which had at least nine reproductive individuals, the minimum number required for integration analyses with the number of studied traits ($n = 151$ plants and 641 leaves). To quantify sugar content in extrafloral nectaries (SEFN), we first rinsed them with distilled water and isolated the branches with micro perforated plastic bags, allowing the exchange of moisture and heat while preventing ant activity on the branches for at least 20 h. SEFN was estimated following Ochoa-López et al. (2018), adding 2 µl of distilled water to each extrafloral nectary and reabsorbing the mix of water. Diluted nectar was placed in a hand-held refractometer (0–50°Brix, Reichert 137531L0, Munich, Germany) to quantify sugar concentration (C). After recording sugar concentration, the mix of nectar and water was reabsorbed using 5 µl capillary tubes (Blaubrand intraMARK, Brand, Germany) and its volume (V) was estimated by measuring the length of the nectar column. Sugar content was estimated as sugar ($µg\,µl^{-1}$) = [C (°Brix) × V (µl)]/100 (Heil et al., 2000).

To assess the cyanogenic potential of leaves, hydrogen cyanide content (HCN, $µg\,g^{-1}$ dry weight) was quantified through a colorimetric assay (Schappert & Shore, 1995; Ochoa-López et al., 2018). Briefly, we cut six leaf discs (0.6 cm^2) from each leaf, and half of them were dried at 60°C for 72 h to quantify dry mass while the remaining three were stored in an Eppendorf tube. After crushing the leaf tissue inside the tubes with 7 µl chloroform, we introduced a 2.5 × 1 cm filter paper previously soaked in a solution of 0.5% picric acid and 5% $NaCO_2$ avoiding direct contact between leaf material and the filter paper. Samples were incubated in darkness at room temperature for 24 h. After this period, one 0.6 cm^2 disc was cut from each filter paper strip and was stored in

the fridge at 4°C in a new Eppendorf tube with 1 ml of 50% ethanol. We measured the absorbance of this elution at 590 nm (ELx808; BioTx Instruments Inc., Winooski, VT, USA) in three replicate 250 µl samples per tube. After averaging the readings, we estimated HCN content using the formula HCN = (optical density − 0.04789659)/0.000652 ($R^2 = 0.91$, $P < 0.001$), obtained from a standard curve with this protocol using sodium cyanide instead of leaf tissue (Ochoa-López et al., 2018).

Trichome density was calculated as the average density of trichomes in two 14 mm^2 discs of each leaf. LMA was estimated as the dry mass (recorded after oven drying the leaves at 60°C for 72 h) divided by leaf area, which was estimated with a portable scanner (CI-202 CID Inc., Cama, WA, USA). Chl content per unit leaf area ($µg\,cm^{-2}$) was calculated as the mean value of five measurements (on both sides of the leaf avoiding the middle vein plus a measure in the leaf tip) taken with a CCM-200 Chl content meter (Opti-Sciences, Tyngsboro, MA, USA), using the equation Chl content ($µg\,cm^{-2}$) = $-0.093 + 1.36 \times \sqrt{(\text{ICC units})}$ ($R^2 = 0.79$, $P < 0.001$) (Damián et al., 2018). ICC is the index of chlorophyll content, which comes from the readings obtained with the CCM-200 chlorophyll content meter (Damián et al., 2018). Dry material was ground in a TissueLyser II (Qiagen) and the carbon ($\delta^{13}C$) isotopes and total nitrogen and carbon content in leaf samples were analyzed by mass spectrometry (Thermo Scientific Delta V Advantage IRMS with EA-2000Flash Elemental analyzer), the $\delta^{13}C$ (‰) values are relative to the Pee Dee Belemnite (VPDB). Analyses were performed at the Laboratory of Biogeochemistry and Applied Stable Isotopes (LABASI, PUC), Chile. We calculated carbon : nitrogen ratio (C : N) by dividing the carbon content over the nitrogen content in 1 g of dry leaf mass.

Plant fitness

We recorded three variables as estimators of vegetative performance and reproductive output of each plant. We estimated relative growth rates on individual plants as RGR = $\log_e (h_f - h_0)/550$ d. Initial height was recorded for seedlings in 2014 as h_0 and their final height (h_f) was measured in February 2016. Between October 2014 and February 2016, we counted the number of flowers produced by each plant and the resulting fruits were collected. We quantified the total number of seeds produced by each plant and calculated the average seed production for each genetic family.

Variability of functional traits among genetic families

Differences in the mean value of foliar traits were assessed with a multivariate analysis of variance (MANOVA), considering Family as a principal source of variation. To fit statistical assumptions of normality, trichome density, LMA and nitrogen content were log transformed, and a reciprocal transformation was used for C : N. To describe the range of expression of foliar traits we estimated their coefficients of variation (CV). In addition, the main axes of variation in the simultaneous expression of leaf traits were examined through a principal component analysis (PCA) performed on the correlation matrix of the phenotypic trait values of all plants (R Core Team, 2014). The loadings from this PCA

were further used to explore if natural selection favoured particular trait combinations representing the functional strategies of leaves (see the 'Selection analysis' section).

Variation in the magnitude and patterns of phenotypic integration among genetic families

The magnitude of phenotypic integration was calculated for each genetic family using the index of phenotypic integration, INT (Wagner, 1984; Cheverud et al., 1989), which calculates the variance among the eigenvalues (λ_i) of the correlation matrix. High variance among eigenvalues means that most traits are correlated and thus the first principal component (PC) accounts for most of the variation (high phenotypic integration). By contrast, low variance among eigenvalues indicates that the variation within the matrix is evenly distributed among all PCs (low phenotypic integration). PCAs were conducted on R v.3.3.3 using the PHENIX package (R Core Team, 2014; Torices & Muñoz-Pajares, 2015) to calculate INT for each maternal family. Because variation in the correlation structure of traits can result from differences in resource acquisition among individuals of different size (Magwene, 2001; Torices & Muñoz-Pajares, 2015) and/or age (Damián et al., 2018), INT estimations were based on partial correlation matrices after controlling for plant size, using the length of the main stem recorded at each sampling date. INT values were corrected as INT = (Var (λ_i) − (number of traits − 1)/ number of individuals per genetic family) and transformed to percentage of the total maximum integration. Total maximum integration equals the number of traits in the correlation matrix (i.e. the maximum integration was 8) and was considered significant if the 95% confidence interval did not include zero (Herrera et al., 2002). We considered nonoverlapping confidence intervals among families as evidence of genetic variation in phenotypic integration within the studied population. The relationship between each individual trait and the INT estimations was assessed using Pearson correlation tests.

Differences in the structure of the variance–covariance matrices were assessed with the random skewers method (Calsbeek & Goodnight, 2009). This method measures the extent to which two matrices respond similarly to a common perturbation, which essentially simulates the response of a complex phenotype from two maternal families to a common selection gradient (Lande, 1979). Matrices were multiplied by random selection vectors (β) following the response to selection equation (Lande & Arnold, 1983), so that $R_1 = \mathbf{G}_1 \beta$ and $R_2 = \mathbf{G}_2 \beta$. The correlation between R_1 and R_2 was then calculated, and this process was reiterated over 1000 random vectors to produce an average correlation between response vectors. The correlation value represents an estimate of similarity among matrices (Teplitsky et al., 2014). The average correlations range from zero, if matrices are completely different, to one, if both matrices are similar. The magnitude of the response vectors was calculated as the vectors' length ratio (Maubecin et al., 2016). This ratio assesses the similarity in matrix size; if two matrices are equal, the magnitude of the response is expected to be the same for both matrices, and the ratio will be equal to one. We used the R script from Maubecin

et al. (2016) to evaluate the correlation between responses to selection.

Selection analysis

To determine the influence of individual traits and their joint expression on plant fitness, we performed a hierarchical selection analysis (Ordano et al., 2008). This approach relies on the use of independent multiple linear regression analyses (Lande & Arnold, 1983) to assess how relative plant fitness is predicted by different levels of trait associations, from independent traits (no association), sets of particular combinations of traits and/or the joint expression of all traits (i.e. the magnitude of phenotypic integration). More details can be found in Ordano et al. (2008). To assess natural selection on individual traits, we used their average value estimated from the four leaves sampled for each plant. These values were standardised to $\bar{x} = 0$ and $\sigma = 1$ and fitness was relativized for each plant with the population mean fitness (Lande & Arnold, 1983). Plant size was detected as a significant factor influencing our fitness estimates ($R = -0.17$ to 0.91, $0.001 < P < 0.13$), and hence it was included as a covariable in the multiple regression analysis when assessing selection for individual and combinations of traits.

Standardised selection differentials (S), accounting for the effects of both direct and indirect selection acting on a trait, were estimated as the covariance between relative fitness and the standardised trait values (Price, 1970). To determine the direct effect of selection on each trait, we estimated directional selection gradients (β) as the partial regression coefficients from multiple linear regressions on each level. Independent analyses were performed for each fitness estimator. Quadratic and correlative selection gradients were not assessed due to the large number of traits and low number of genetic families. However, to assess natural selection on the joint expression of foliar traits and the strength of their correlations we evaluated the influence of the magnitude of leaf phenotypic integration (INT) on plant fitness using a linear regression analysis. Family INT values were used as the response variable in this analysis, while relative fitness was included as the independent one ($n = 13$). Last, to further assess the particular combinations of foliar traits favoured by natural selection, we used the scores from the first three PCs as independent variables. These PCs were chosen because together they explained c. 70% of the total variation.

Data availability

Data are available from figshare 10.6084/m9.figshare.9334241. v1 (Damián et al., 2019).

Results

Variability of functional traits and their phenotypic integration among genetic families

The MANOVA revealed that mean values of functional traits did not differ among genetic families (Wilks' $\lambda = 0.915$,

Table 1 Mean values ± SD (CV) of foliar traits in the 13 genetic families of *Turnera velutina* established in coastal dunes.

Trait	Mean ± SD	CV
Sugar content (μg μl^{-1})	0.022 ± 0.015	66.481
HCN (μg g^{-1} dry weight)	3884.318 ± 352.974	9.087
Trichome density (number mm^{-2})	19.533 ± 1.368	7.005
LMA (g m^{-2})	0.368 ± 0.043	11.605
Chlorophyll content (μg cm^{-2})	7.259 ± 0.295	4.070
Nitrogen content (μg mg^{-1} dry mass)	44.237 ± 2.979	6.735
δ^{13}C (‰)	−29.901 ± 0.372	−1.244
C : N ratio	11.408 ± 0.419	3.676

HCN, hydrogen cyanide content; LMA, leaf mass per area.

$F_{8,142} = 1.64$, $P = 0.12$, Table 1). The CVs for foliar traits were below 10%, with exception of SEFN, which had a CV of 66.5% (Table 1). The magnitude of phenotypic integration, however, was significantly different from zero and varied among families, indicating the presence of genetic variation within our experimental population (Figs 1, 2). The mean value of INT was 11.4-% (INT = 0.911 ± 0.476 SD), ranging between 2% and 23%. INT was negatively correlated with nitrogen content ($r = −0.562$, $P = 0.046$, Supporting Information Table S1). The paired comparisons of variance–covariance matrices with the random skewers procedure showed high similarity in structure and size among matrices, with correlation values above 0.95.
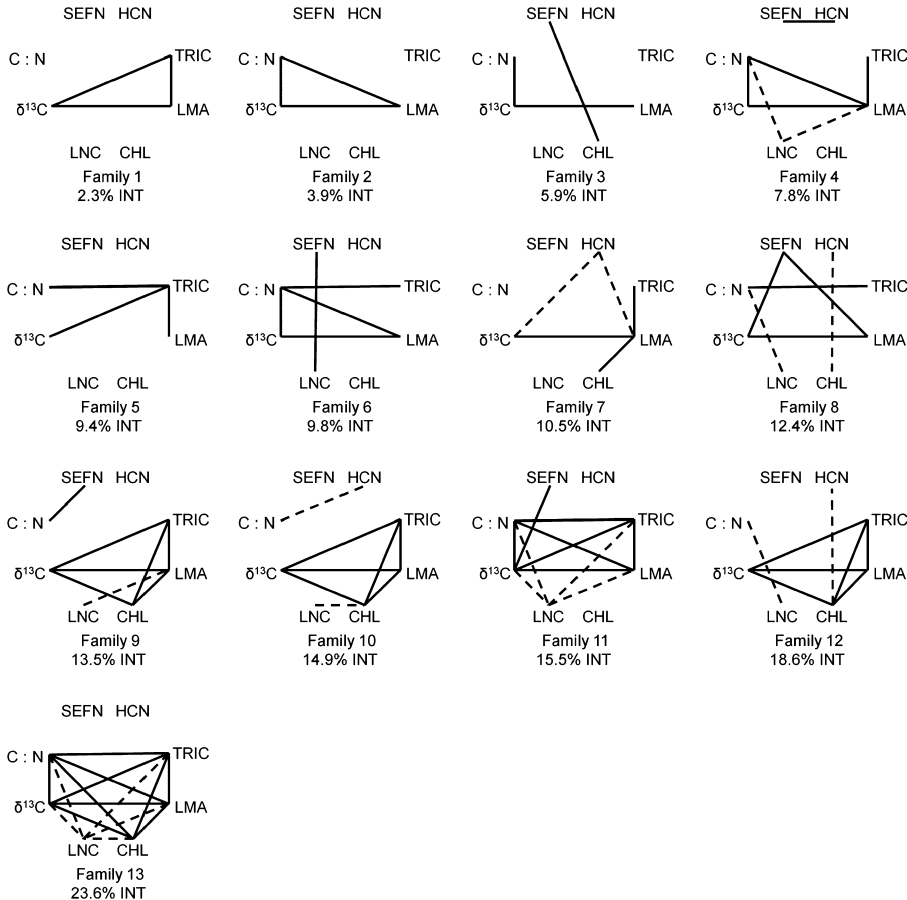
Correlation patterns estimated with the mean value of leaf traits for each genetic family showed strong positive correlations among trichome density, LMA and δ^{13}C, and a negative association between Chl content and C : N (Table 2). The only negative correlations where those including nitrogen content or HCN. Whereas correlations among trichome density, LMA and δ^{13}C were present in almost all families, correlations among SEFN and other traits were significant only for one family (Fig. 2; Table S2).

Selection analyses

Relative growth rate (RGR) was significantly correlated with flower number and seed production but the latter two were not correlated (Table S3). Significant selection differentials were detected for some foliar traits (Table S4). Chl content, nitrogen content and C : N were significantly correlated with growth rate, while SEFN, LMA, nitrogen content and δ^{13}C were positively related to flower number. Total seed production was associated with SEFN, Chl content and δ^{13}C. However, these relationships may be the result of both direct and indirect effects of each trait on plant fitness, due to their correlations. Partial regression coefficients from the selection analysis identified the direct effects of such characters and suggested that the individual expression of defensive traits had direct but opposite effects on plant fitness (Table 3). Compared with the intensity of selection observed on

Fig. 1 Variation in the magnitude (% of maximum INT) and patterns of phenotypic integration among functional traits in maternal families of *Turnera velutina*. Correlograms are based on partial correlations after controlling for plant size (see the Materials and Methods section). Only significant ($P < 0.05$) positive (lines) and negative (dashed lines) correlations are presented. δ^{13}C, carbon isotope concentration; C : N, carbon : nitrogen ratio; CHL, chlorophyll content; LMA, leaf mass per area; HCN, hydrogen cyanide content; LNC, leaf nitrogen content; SEFN, sugar content in extrafloral nectar; TRIC, trichome density.
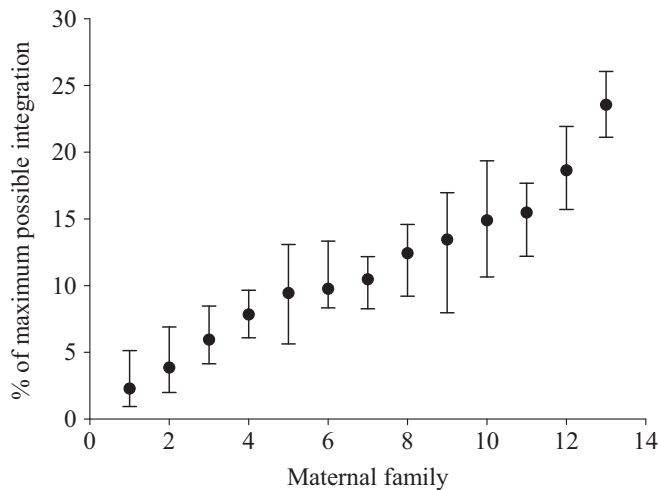
Fig. 2 Variation in the magnitude of phenotypic integration (INT ± 95% confidence interval) for 13 maternal families of *Turnera velutina*.

the reproductive components of fitness, the strength of natural selection acting on vegetative growth was weak (Table 3). While a negative directional gradient was detected for HCN ($\beta = -0.005 \pm 0.002$) on growth rate, more intense and positive directional gradients were found for SEFN ($\beta = 0.216 \pm 0.089$) and trichome density on seed production ($\beta = 0.214 \pm 0.078$). Functional traits related to nitrogen showed significant selection gradients for vegetative and reproductive performance. In particular, growth rate was favoured by leaf Chl content ($\beta = 0.005 \pm 0.002$) but negatively influenced by leaf nitrogen content ($\beta = -0.005 \pm 0.002$) (Fig. 3). By contrast, C : N was positively associated with seed number ($\beta = 0.170 \pm 0.077$). Interestingly, the magnitude of phenotypic integration (INT) was positively associated with two fitness components, growth rate ($\beta = 0.019 \pm 0.007$; Fig. 4a) and flower number ($\beta = 0.208 \pm 0.093$; Fig. 4b).

To further assess which combinations of correlated traits were favoured by natural selection, we explored the relationship between plant fitness and the scores of the two main PCs from the PCA performed with phenotypic values of all plants. We identified significant selection gradients acting on the two main axis of variation of PC1 and PC2 (Table 3). Flower production was negatively related to the combination of traits from PC1 ($\beta = -0.095 \pm 0.035$; Fig. 4d). This axis of variation was

explained by the joint expression of trichome density, LMA and δ^{13}C (Table 4). The combination of traits from PC1 had also a negative effect on seed production ($\beta = -0.176 \pm 0.032$; Fig. 4e) but a positive influence of traits defining PC2 ($\beta = 0.122 \pm 0.050$; Fig. 4f). PC2 was related to high values of cyanide content and C : N ratio and low values of nitrogen content in leaves (Table 4).

Discussion

The main contribution of this work is a first empirical demonstration of the adaptive value of the coordinated expression of functional leaf traits, estimated through the covariance between the magnitude of their phenotypic integration and plant fitness components. Although the patterns of correlations among functional traits were similar among genetic families, the magnitude of their phenotypic integration was significantly different. Selection analyses showed that not only did the expression of individual functional traits or their joint expression in specific combinations (in particular those related to leaf and nitrogen economy) influence plant fitness, but so too did the magnitude of their correlations. The data presented here suggest that leaf phenotypes exhibit intrapopulation variation in their covariance patterns that can have an adaptive value, which may be overlooked when only assessing the mean values of leaf traits.

Intraspecific variation in the phenotypic integration of leaves

The magnitude of leaf phenotypic integration in *T. velutina* genetic families ranged from 2% to 20%. Although our sample size was rather small, the observed range of phenotypic integration values falls within that observed in a previous study with *T. velutina* under glasshouse conditions and twice the maternal families used in this study (Damián *et al.*, 2018). The other available study documenting intraspecific variation of phenotypic integration of leaf functional traits reports that foliar integration of *Polygonum viviparum* can fluctuate between 10% and 30% among different populations (Boucher *et al.*, 2013). These studies suggest that phenotypic integration, as a complex trait, exhibits significant variation within and among populations, a first requisite for natural selection to act on those genetic variants.

Table 2 Correlation matrix among foliar traits of *Turnera velutina* (*n* = 13 maternal families).

Foliar traits	Sugar content	HCN content	Trichome density	LMA	Chl content	Nitrogen content	δ^{13}C
HCN content	0.168						
Trichome density	*0.593*	0.332					
LMA	**0.849**	0.297	*0.589*				
Chlorophyll content	0.158	0.392	0.228	0.123			
Nitrogen content	0.205	−0.210	0.293	0.136	0.023		
δ^{13}C	*0.630*	0.425	**0.723**	**0.798**	0.391	0.374	
C : N ratio	0.470	−0.137	0.284	0.362	*−0.653*	−0.064	0.028

Bold values denote significant correlations after Bonferroni correction. Italic values were significant at $P < 0.05$.
HCN, hydrogen cyanide content; LMA, leaf mass per area.

Table 3 Selection gradients (β ± SE) from hierarchical selection analysis acting on three levels of functional trait association.

Level of analysis	Fitness estimator		
	RGR	Flower number	Seed number
Individual traits	HCN −0.005 ± 0.002	ns	SEFN 0.212 ± 0.089
	Chl 0.005 ± 0.002	ns	Trichomes 0.214 ± 0.078
	Nitrogen −0.005 ± 0.002	ns	C : N 0.170 ± 0.077
Phenotypic integration	INT 0.019 ± 0.007	INT 0.208 ± 0.093	ns
Combination of traits	ns	PC1 −0.095 ± 0.035	PC1 −0.176 ± 0.032
	ns	ns	PC2 0.122 ± 0.050

Only significant factors at $P < 0.05$ in each model are presented. Full results are available in Table S4.
HCN, hydrogen cyanide content; INT, magnitude of phenotypic integration; ns, nonsignificant; PC1, scores from principal component 1; PC2, scores from principal component 2; RGR, relative growth rate; SEFN, sugar content in extrafloral nectar.

Genotypic variation in the magnitude of INT can arise from differences in the correlation strength among foliar traits, as a result of differences in phenotypic plasticity of particular traits and their correlations. For example, *Fagus selvatica* trees are exposed to heterogeneity in water availability and this variation in conditions seems to favour several water intake strategies and different combinations of functional traits (Bontemps *et al.*, 2017). In *T. velutina*, individuals seem to have the ability to combine ecophysiological functions in different ways, which can be advantageous under different contexts. We hypothesise that genotypic variation in the magnitude of foliar integration can be subject to selection pressures from variable and heterogeneous conditions in coastal dunes such as soil depth, sand burial, shade provided by canopy or wind exposure (Conti *et al.*, 2017).

We are aware that the number of genetic families used in this study was quite small and acknowledge that estimations of INT magnitude could change as a result of the influence of environmental factors on correlations among traits within the studied population (Arnold, 1992; Boucher *et al.*, 2013) or differential selection pressures exerted across populations (Herrera *et al.*,

2002; Lázaro & Santamaría, 2016). Although the relevant point of our study was to detect the relationship between phenotypic integration and plant fitness under the specific environmental conditions in our study site, we acknowledge that explicitly testing the influence of environmental heterogeneity with more genotypes, across different seasons and among different populations warrants further investigation. This additional information would provide insights into the stability of the variance–covariance matrices between functional traits and the proximate causes of phenotypic integration variability.

Natural selection: from individual traits to integrated phenotypes

Natural selection acted on functional traits through their individual or combined effects. This can be attributed to a mixture of direct and indirect selection due to the strong correlations observed among functional traits. Nonetheless, the direct action of natural selection on individual traits was particularly relevant for those attributes related to defence against herbivores. Genetic
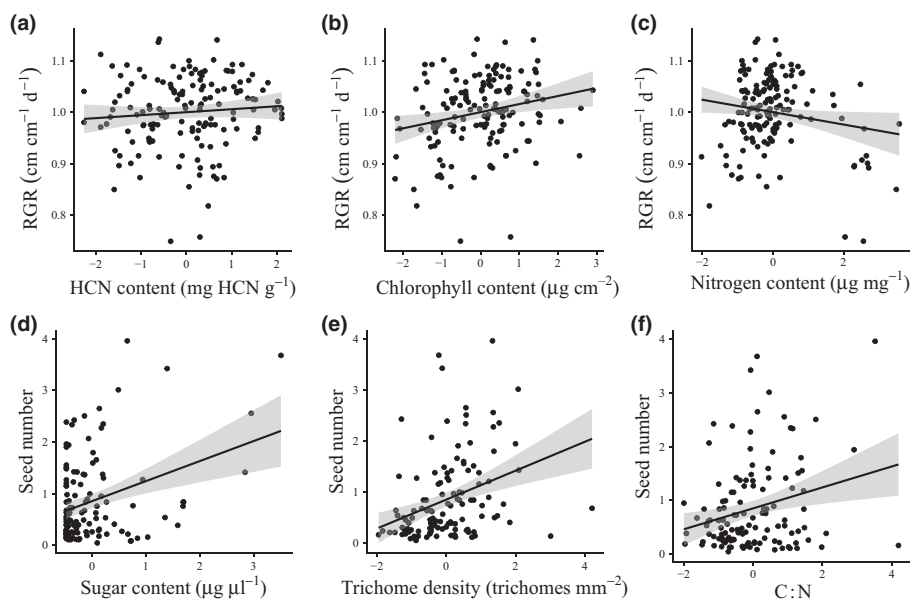

Fig. 3 Selection gradients on individual foliar traits assessed in the experimental population of *Turnera velutina*. Graphs show the confidence interval at 95%. HCN, hydrogen cyanide content; RGR, relative growth rate; C : N, carbon : nitrogen ratio.
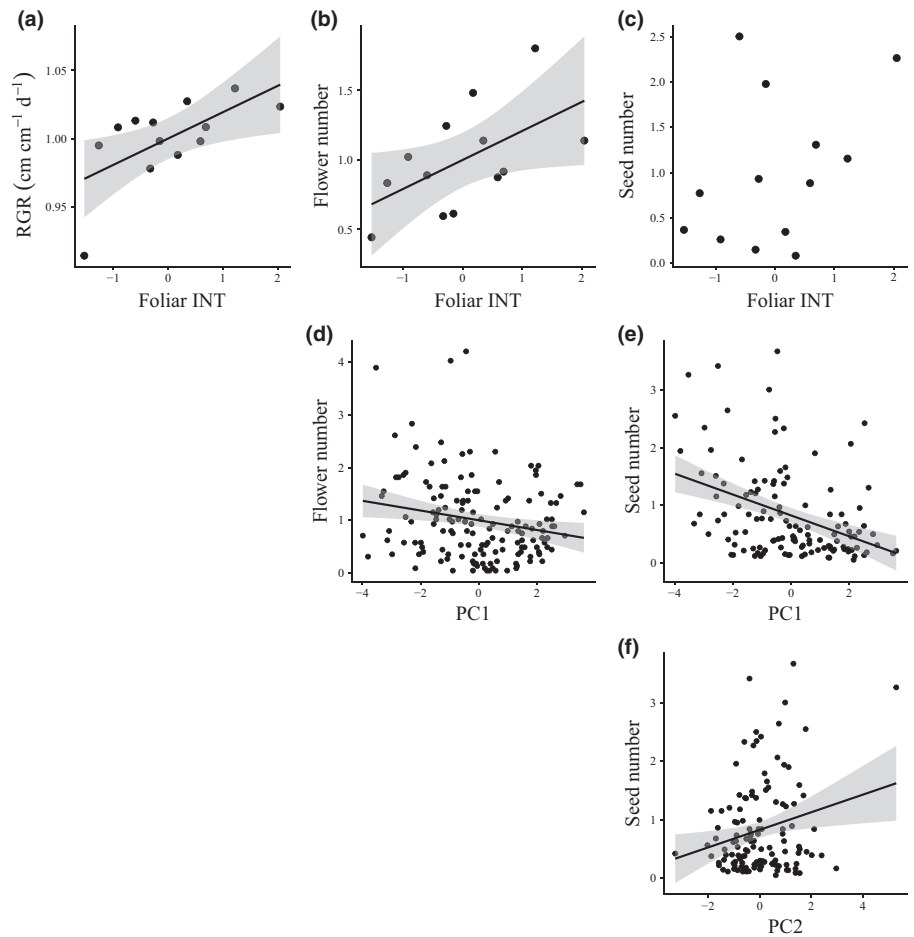
Fig. 4 Selection gradients on phenotypic integration magnitude (INT) and combination of functional traits in the experimental population of *Turnera velutina*. Graphs show the confidence interval at 95%. PC1 was interpreted as a water economy axis, while PC2 was related to nitrogen metabolism. PC1, scores from principal component 1; PC2, scores from principal component 2; RGR, relative growth rate.

families with higher levels of biotic and physical defences produced more flowers and more seeds. A positive selection on the amount of sugar in extrafloral nectar could be explained by its role in attracting ants as a biotic defence against caterpillars (Ochoa-López *et al.*, 2018), in seed dispersal (Cuautle *et al.*, 2005) or in preventing ants from disturbing pollinators (Villamil *et al.*, 2018, 2019). Trichome density on leaves can act as an additional defence (Ochoa-López *et al.*, 2018) and/or as a

Table 4 Results from PCA performed in foliar traits of *Turnera velutina* with plants from 13 genotypes in which INT was estimated.

Foliar traits	PC1	PC2	PC3
Eigenvalue	1.719	1.165	1.017
Variance explained	0.369	0.170	0.129
Cumulative variance	0.369	0.539	0.668
Sugar content	−0.126	0.399	0.568
HCN content	0.191	0.570	0.262
Trichome density	−0.460	−0.146	−0.072
LMA	−0.514	0.048	0.038
Chlorophyll content	−0.301	−0.340	0.558
Nitrogen content	0.185	−0.491	0.003
$\delta^{13}C$	−0.500	−0.004	−0.014
C : N ratio	−0.311	0.367	−0.539

HCN, hydrogen cyanide content; INT, magnitude of phenotypic integration; LMA, leaf mass per area; C : N, carbon : nitrogen ratio.

mechanism to reduce water loss at the leaf level (Woodman & Fernandes, 1991). Although we do not have information about the reflectance and absorbance properties of *T. velutina* leaf trichomes, evidence from other species indicates that similar trichome density and length can produce a heat-dissipating boundary layer (Bickford, 2016). Hence, leaf trichomes may have a multiple functional role (herbivore defence, reflecting radiation, lowering temperature and reducing water loss), thus stressing the multivariate nature of selection acting on this trait. Surprisingly, leaf nitrogen content had a negative adaptive value. This result can be explained by the relationship between low nitrogen content and drought tolerance in environments with low water availability (Ramírez-Valiente *et al.*, 2014). For example, low nitrogen content can favour greater above-ground growth in dry years in the cork oak *Quercus suber*, compared to mesic years (Ramírez-Valiente *et al.*, 2014). In addition, plants with high contents of nitrogen could be unfavoured by selection if herbivores prefer their highly nutritious foliage. Cyanogen content was also negatively selected, which coincides with the ontogenetic switch from chemical to biotic defences during development of *T. velutina* (Ochoa-López *et al.*, 2018; S. Ochoa-López *et al.*, unpublished). Interestingly, individual traits showed weak influence on vegetative growth, as indicated by lower values of selection gradients, in comparison to traits promoting higher seed production. This could imply indirect effects of individual traits on reproductive

output not recorded on the present study or that small differences in plant growth among genotypes can scale up into greater differences in reproductive output.

Although different individual leaf traits influenced plant fitness, we report that the strength of their correlations, measured as the magnitude of their phenotypic integration, can influence plant reproductive success. In this study, stronger correlations (e.g. greater INT) among *T. velutina* leaf traits such as LMA (Bermúdez & Retuerto, 2014; Bontemps *et al.*, 2017), trichomes (Woodman & Fernandes, 1991) and $\delta^{13}C$ (Bontemps *et al.*, 2017), suggest a strategy related to water use efficiency. We consider this relationship as a first evidence of an adaptive value of the joint expression of particular traits defining leaf functional strategies. These strategies have been described for different plant species as a function of variation in multiple traits (Reich, 2014). At an interspecific level, they have been identified as alternative trait combinations resulting from the optimisation of resource acquisition and allocation to different functions (Marks & Lechowicz, 2006). Empirical evidence of variation in functional strategies within populations also highlights the role of the covariation among traits in the evolution of integrated phenotypes (Bontemps *et al.*, 2017).

To further understand which particular combination of traits had a positive relationship with growth rate, flower number and seed production, we analysed the PCs for which we found significant selection gradients. A negative selection on PC1 suggests that plants optimising water balance through a combination of resources invested in leaves (LMA), transpiration efficiency ($\delta^{13}C$) and trichome density (Hoof *et al.*, 2008) produced more flowers. It has been shown that the expression of these traits is strongly related to fitness in other species (Donovan & Ehleringer, 1992; Dudley, 1996). In addition, we found evidence of natural selection favouring high investment in biotic defence and leaf quality (significant positive directional selection on PC2). This component was related to a higher investment in sugar content in extrafloral and trichome density and higher carbon allocation (high C : N ratio). This evidence matches previous works reporting that combinations of traits can be targets of selection (Johnson *et al.*, 2009; Boege, 2010). For example, plant fitness has been found to be favoured by particular combinations of water-related traits in *Fagus sylvatica* (Bontemps *et al.*, 2017); floral traits involved in pollinator attraction of *Prunus mahaleb* (Ordano *et al.*, 2008); sets of chemical traits associated with early flowering in *Oenothera biennis* (Johnson *et al.*, 2009); and with volatile organic compounds determining the floral bouquets of *Penstemon digitalis* (Parachnowitsch *et al.*, 2012). Although these studies highlight the adaptive value of particular trait combinations, there is only one other study reporting a significant relationship between INT and fitness. Lázaro & Santamaría (2016) found that plants of *Lonicera implexa* with low floral integration produced more seeds per flower. This negative relationship was interpreted as evidence of selection on intrafloral integration, particularly on the set of correlated traits related to pollinator accessibility (see Ordano *et al.*, 2008). In the case of *T. velutina* leaves, functional traits seem to be more integrated than morphological traits because the former can have multiple functions. We

detected that genotypes with greater phenotypic leaf integration had higher growth rates and increased reproductive output. However, it is likely that under different environments these genotypes could express new variants of their functional strategies, as a result of the optimisation of specific functions in response to new environmental or biotic conditions. Hence, environmental changes could trigger new patterns in the correlation structure and in the magnitude of leaf phenotypic integration. This issue warrants further investigation.

Phenotypic integration and leaf functional strategies

From the magnitude and patterns of phenotypic integration of leaf traits in *T. velutina*, we suggest that this species has a conservative resource strategy. A high LMA coupled with high $\delta^{13}C$ values can reduce water stress (Bermúdez & Retuerto, 2014) and increase WUE (Bontemps *et al.*, 2017; Messier *et al.*, 2018) in the coastal dune environment. The strong association between these traits and trichome density can further reduce water loss through stomata and buffer air temperature (Galmés *et al.*, 2007). Hence, it seems plausible that trichomes also play a role in WUE besides their antiherbivore defensive function (Valverde *et al.*, 2001). Positive correlations between LMA and $\delta^{13}C$ have been previously related to WUE (Navas *et al.*, 2010; Bontemps *et al.*, 2017). For example, in 24 tree species of a temperate community this correlation was consistently found in populations from the same species and among species, suggesting that this relationship may be fundamental for water and carbon economy at different scales (Messier *et al.*, 2018).

In summary, we have shown that variation of functional strategies within populations can be analysed using the concept of phenotypic integration. Our approach to studying the adaptive value on individual traits, combinations of traits and on their covariance structure illustrates that beyond mean values, natural selection is a multivariate process that can favour optimisation of functional strategies through the expression of multiple traits. We are aware that the selected traits may not reflect all physiological processes occurring within leaves, but we suggest that the analyses presented here highlight that key information to understand phenotypic evolution can be missed if we focus only on individual traits or their pairwise combination. Moreover, this issue is not exclusive for plants, as tight correlations among functional traits are also common in animals. For example: colour polymorphism is integrated with territorial behaviour in *Uta stansburiana* enhancing functionality of alternative reproductive strategies (Sinervo & Lively, 1996); toepads and claws are functionally integrated facilitating access to new niches in *Anolis* lizards (Yuan *et al.*, 2019); and covariance between morphological and behavioural traits represents individual differences in personality in *Parus major* (Moiron *et al.*, 2019). Hence, we suggest that future studies assessing the evolution of functional strategies should incorporate the multivariate relationships among traits within populations. In this way, we can advance our understanding of how suites of traits influence plant performance and their phenotypic evolution.

Author contributions

XD, SOL, KB, JF and AG designed the experiments. XD and SOL conducted fieldwork. XD processed and analysed the data. XD and KB led the writing of the paper with critical input from JF, AG, SOL and CAD.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Pearson correlations between phenotypic integration magnitude and mean value of foliar traits.

Table S2 Partial correlations among functional traits in maternal families of *Turnera velutina*.

Table S3 Correlations between fitness components at plant and maternal family level.

Table S4 Results from selection analysis on individual traits, INT values and combinations of traits (scores of principal components 1 and 2).

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Capítulo 2. Phenotypic integration of leaf traits increases in a dry-wet environmental continuum

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Phenotypic integration of leaf traits increases in a dry-wet environmental continuum

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Supporting Information: Table S1

ABSTRACT

- *Premise of the study*: Species established along environmental gradients face a diversity of challenges to survive and reproduce. Adjustments in functional traits are expected throughout a coordinated change of several traits either reinforcing or constraining the multi-trait phenotypic space that species can occupy in different environmental conditions.

- *Methods:* We used five populations of the tropical shrub *Turnera velutina* sampled across a precipitation range to explore the association between water availability and changes in phenotypic integration of leaf traits defining functional strategies of plants. We measured leaf traits related to plant primary metabolism and defense against herbivores and analyzed their variation estimating the magnitude and patterns of their integration.

- *Results:* We found that populations established in the drier and warmer conditions had the lowest integration magnitude, while populations in the wettest end of the range were strongly integrated. This change coincided with shifts from conservative towards resource acquisitive functional plants strategies, suggesting an ecological flexibility displayed according to the environmental conditions in particular populations. Additionally, we identified traits with high variability at the leaf and individual levels, in particular those involved in herbivore defense. In contrast, differences among populations were associated with leaf mass and trichome density.

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- *Conclusions:* We suggest that the variability of foliar phenotypic integration is the result of environmental filters selecting for sets of traits rather than individual traits, which highlight the adaptive value of particular trait combinations in different environments. Phenotypic integration is a valuable tool to identify the functional variation expressed by species, and to understand species responses to changing environmental conditions.

Key Words: correlation patterns; functional strategies; functional traits; intraspecific variation; phenotypic integration; precipitation gradient; *Turnera velutina*

INTRODUCTION

Intraspecific differentiation in phenotypes can be a consequence of geographic variation in natural selection agents. Therefore, plant species experiencing a broad range of conditions across environmental gradients can exhibit wide intraspecific differences in their phenotypes (Vasseur et al. 2018, Kuppler et al. 2020). For example, plants adapted to local environments are likely to display a unique combination of leaf functional traits representing functional strategies (Reich 2014), which leads to an adaptive phenotypic divergence among populations and a distinction of ecotypes (Lowry 2012). These ecotypes are often differentiated by suites of correlated traits sharing genetic, developmental, and/or physiological relationships (Khasanova et al. 2019), as a result of different selective regimes (imposed by resource availability and/or biotic interactions) and evolutionary or biophysical trade-offs among traits (Reich et al. 1997, Agrawal 2020).

Complex phenotypes are characterized by correlations among multiple traits, either as the result of pleiotropy, gene linkage or optimization processes causing traits to evolve together as character complexes (Chen & Lüberstedt 2010) providing adaptive advantages under specific environments (Armbruster 1985, Kingsolver & Wiernasz 1987; Armbruster & Schwaegerle 1996, Björklund 2004). For example, in dry environments small leaves with high leaf mass per area (LMA) are often correlated with water economy via reduced water loss relative to carbon gain (Muir et al. 2014). The influence of different environmental conditions on the covariances among characters may promote specific combinations of traits favored only in particular populations due to environmental filtering (Armbruster & Schwaegerle 1996). Hence, populations with shared environmental challenges should converge toward similar functional strategies and trait covariances. Because the evolutionary potential of populations may differ under contrasting environmental conditions (Sherrard et al. 2009), describing the covariance structure and their changes as a function of environmental factors is required to understand the ecological and evolutionary constraints contributing with the ability of species to succeed across habitats through different functional strategies. In the case of plant resource acquisition strategies, although there is robust empirical evidence of variation in functional traits at the interspecific level (Wright et al. 2004), scarce information is available of intraspecific trait covariation of leaf functional traits behind such strategies (Bégin & Roff 2004, Laughlin & Messier 2015).

An approach to study coupled changes in multiple traits is using the concept of phenotypic integration (Nicotra et al. 2007, Pigliucci 2003). This concept reflects the presence and intensity of correlations among traits through the depiction of the magnitude

and pattern of their covariances (Pigliucci & Preston 2004). In plants, for instance, strong

covariation among traits is the result of biophysical and/or selection processes favouring an

efficient acquisition and use of resources (Reich 2014). Genetic covariances between traits

arise also due to either pleiotropic effects or linkage disequilibrium (Falconer & MacKay

1996, Lynch & Walsh 1998). Hence, the estimation of phenotypic integration across

populations along geographical ranges allows the analysis of changes in functional

relationships among different traits. Additionally, the magnitude of phenotypic integration

associated with environmental factors improve our understanding of species responses and

divergent selection and environmental filtering on multi-trait functional strategies across

populations (Boucher et al. 2013, Salgado-Negret et al. 2015, Seguí et al. 2017). Until now,

several studies have described changes in the patterns of phenotypic integration and its

magnitude through different environmental gradients (Boucher et al. 2013, Hermant et al.

2013, Salgado-Negret et al. 2015, Seguí et al. 2017, Delhaye et al. 2020), however the

mechanism behind these changes is still unclear.

Water availability has been identified as the most important factor affecting plant

phenotypes (Meng et al. 2015, Siepielski et al. 2017). Water scarcity usually favors thick and

dense leaves which often have high leaf mass per area (LMA), leaf dry matter content

(LDMC), and high water use efficiency (Reich et al. 1999, Poorter et al. 2009, Pérez-

Harguindeguy et al. 2013, Lohbeck et al. 2015). It is thus expected that water limitation

may promote conservative strategies that confer tolerance to drought and reduce loss of

turgor at low leaf water potential. On the contrary, water availability favors increased

expression of resource acquisition traits, such as low LMA, large thin leaves, and high

specific root length (Poorter &Evans 1998, Wright et al. 2017, Shovon et al. 2019). Hence, populations from the same species can display a variety of adaptive functional strategies and foster ecotypic differentiation (Ramírez-Valiente et al. 2018, Brouillette et al. 2014, Vasseur et al. 2018). Environmental variation is considered a selective force that can drive or constrain life histories (Levins 1968, Schaffer 1974). Thus, plant adaptations that promote growth and survival in water limited environments or periods are critical for plant persistence in particular locations (Reed et al. 2010).

Water limitation in arid or semiarid environments can favour traits promoting water use efficiency through strong correlations between foliar N, LDMC, and LMA (Ackerly 2004, Caruso 2004). The simultaneous expression of these traits can constitute an adaptive mechanism to cope with water limitation, hence favoring high values of phenotypic integration (Schlichting & Pigliucci 1998, Damián et al. 2020). On the other hand, in populations where water stress along the year is predicted to be less pronounced, lower phenotypic integration and a decoupling of the expression of traits is expected (Pérez-Barrales et al. 2014), as these traits are likely to be under selection by other environmental filters and thus, promoted by divergent selection among traits. For example, LMA varies strongly with water and light availability (Poorter et al. 2009, Niinements et al. 2015) and foliar N varies with soil nutrient availability irrespective of water availability (Richardson et al. 2005). Then, low values of integration can be attributed to decoupling of functions under less stressing conditions. (Flores-Moreno et al. 2019).

In this study, we explored the variation in leaf functional traits and their phenotypic integration in five populations of the shrub *Turnera velutina* across a precipitation gradient

in Mexico. First, we assessed changes in the simultaneous expression of functional traits related to water use and defenses against herbivores, predicting that plants growing in dry populations should display conservative functional traits and switch to acquisitive strategies in wet habitats. Second, we analyzed the covariance among functional traits to assess how their joint expression varies across heterogeneous landscapes and determine whether variation in water availability is a likely causal selective agent driving ecotypic differentiation in complex phenotypes (Wade & Kalisz 1990, Lowry et al. 2014). We predicted that populations in the dry end of the precipitation range would have higher levels of integration than plant populations growing under greater precipitation regimes.

MATERIAL AND METHODS

Study species and populations— *Turnera velutina* Presl. is a tropical shrub endemic to Mexico that can be found mainly in coastal sand dunes and tropical dry forests from sea level to 1300 m.a.s.l. (Arbo 2005). Plants have simple leaves, with extrafloral nectaries attached to the petiole. The growing season and flowering peak occur during summer months (Cuautle et al. 2005). Between June to August of 2016, we sampled plants in five populations along a precipitation gradient. The populations were located in the Pacific and Mexico Gulf coast and in the Oaxaca Sierra (Table 1, Fig. 1). Anthropogenic disturbance was present in Sontecomapan (SN) where plants grew in backyards of a small village, and in Santa Catarina (SC), where plants were established in areas near to cattle grazing and agriculture fields. The five populations studied are located through a precipitation gradient described by the values of the Mean Annual Precipitation (MAP) (Zedillo-Avelleyra 2017,

smn.conagua.gob.mx/es/climatologia/informacion-climatologica/informacion-estadistica-climatologica). We sought to assess the influence of precipitation and rain-related variables in the expression of foliar phenotypic integration of *T. velutina*. With this aim, we included the following variables obtained from the wordclim bioclimatic database (wordclim.org) for each site: mean annual precipitation, precipitation of the wettest and driest months, precipitation seasonality, and precipitation of wettest, driest, warmest, and coldest quarters.

Plant traits— We measured foliar traits using between 13 and 30 reproductive plants (those bearing buds, flowers, fruits, or their scars in leaf petioles; N = 121 individuals, Table 1) per population. To reduce the variability in leaf functional traits due to plant age (Damian et al. 2018), only reproductive plants were selected. In each plant, we selected six branches and measured the most apical and fully developed leaf with reproductive structures. When plants did not fulfil this condition, we used the available branches to collect 2-3 leaves per branch. In each leaf we measured functional traits previously reported to contribute with resource acquisition strategies or because their role as defense against herbivores (Ochoa et al. 2015, Damián et al. 2018). Leaf mass per area (LMA, g m^{-2}) was measured as the oven-dry mass at 70ºC for 72 hrs divided by the one-side area of a fresh leaf (CI-202 CID Inc, Cama, WA, USA). Chlorophyll content (µg cm^{-2}) was measured with a CCM-200 chlorophyll content meter (Opti-Sciences, Tyngsboro, MA, USA) and average values of four points along the leaf were used in the formula µg cm^{-2} = -0.093 + 1.36 × √(ICC units) (R^2 = 0.79, P < 0.001 (Damián *et al.* 2018). Compound samples were prepared mixing half of each of the six leaves measured per plant. Dry tissue from each

sample was ground with a Tissuelyser II (Qiagen Group) for 90 seconds at 2 Hz (120 oscillations per minute). Duplicates of each sample were used to estimate foliar traits. Carbon isotopes (δ^{13}C) and total foliar nitrogen and carbon were analysed by mass spectrometry (Thermo Scientific Delta V Advantage IRMS with EA-2000Flas Elemental analyser). The δ^{13}C (‰) values are relative to the Pee Dee Belemnite (VPDB). Analyses were performed at the Laboratory of Biogeochemistry and Applied Stable Isotopes (LABASI, PUC), Chile. We calculated carbon-to-nitrogen ratio (C:N) by dividing C over N content in 1 g of dry leaf mass.

The resistance of plants against herbivores was assessed considering indirect (sugar in extrafloral nectaries), chemical (cyanogenic potential) and physical (trichomes density) defenses (Ochoa-López et al., 2015). To collect extrafloral nectar, previously washed extrafloral nectaries were isolated from pollinators and patrolling ants with micro perforated plastic bags surrounding each branch. Extrafloral nectar was collected between 1100 and 1400 h, adding a 2 µl distilled water in each extrafloral nectary with a 5 µl micropipette. The mix was reabsorbed and sugar concentration (C) in ºBrix was measured with a 0-50º Brix hand-held refractometer (0-50° Brix, Reichert 137531L0, Munich, Germany). The volume mix (V) was estimated as the height column in 5 µl capillary tubes (Blaubrand intraMARK, Brand, Germany). Sugar content in extrafloral nectar (EFN) was estimated as sugar (µg µl^{-1}) = [C (°Brix) × V (µl)] /100 (Heil et al. 2000). Hydrogen cyanide content (HCN) was measured with a colorimetric test described in detail in Ochoa et al. (2018). Briefly, we cut six samples of fresh tissue of 14 mm^2 from each leaf, half of them were crushed with 7 µl formaldehyde in an eppendorf tube. In each tube, we introduced a

2.5 × 1 cm filter strip previously soaked in a solution of 0.5% with picric acid and 5% $NaCO_2$ avoiding contact with the tissue. After incubating samples for 24 hrs at room temperature and in the dark, we cut a 0.6 mm^2 disc from each paper strip and diluted it with 1 ml of 50% ethanol. We read the absorbance of three samples of 250 µl at 590 nm using a spectrophotometer (ELx808, BioTx Instruments Inc., Winooski, VT, USA). The average reading was used to estimate each plant sample HCN content = (optical density - 0.04789659)/0.000652 (R^2 = 0.91, $P < 0.001$), obtained from a standard curve (Ochoa-López *et al.*, 2018). The remaining three leaf discs were dried at room temperature and weighted to calculate HCN content /gr. Finally, trichome density was assessed using a stereoscopic microscope to count the number of trichomes per mm^2 in two circle areas of 14 mm^2 per leaf.

Variation in foliar traits— To quantify the extent of intraspecific variation in foliar traits and gain insight about plant responses along the precipitation gradient, we assessed the variation in traits across different levels (i.e., populations, individuals, or leaves) using mixed effects regression models. We used intercept models with random effects corresponding to the different levels of hierarchy (leaves nested in individuals, and individuals nested in populations). We then extracted the percentage of variance explained by each hierarchical level for each trait. Variance components were estimated using restricted maximum likelihood (REML). Secondly, we described differences among populations in the expression of functional traits. A MANOVA was used to determine differences in mean values of all functional traits among populations, and then individual ANOVAs were performed for each trait using population as the main source of variation

with posterior Tuckey HSD. To understand trait variation across environmental gradients, we examined the relationship of all functional traits with the MAP and the other climatic variables using Sperman's correlation coefficients with plants from all populations considered as replicates.

Estimation of phenotypic integration among populations— To identify the main axis of phenotypic variation among populations we performed a Principal Component Analysis (PCA) on the correlation matrix of all functional traits. Estimations of the magnitude of phenotypic integration (INT) by population were computed through the variance on the eigenvalues on the partial correlation matrix [var(λ_i)] using the PHENIX package (Torices & Méndez 2014, Torices & Muñoz-Pajares 2015). This estimation assesses the magnitude of phenotypic integration after controlling for differences in the size of sampled plants, in our case; plant height was treated as a covariable. A high variance among eigenvalues indicates high integration because most of the phenotypic variation is accounted for by the first principal components, and hence, traits are strongly associated. Confidence intervals at 95% were estimated after a bootstrapping procedure. To characterize the pattern of phenotypic integration and the potential trade-offs among plant functions we obtained the partial correlation matrices among functional traits for each population (Torices & Muñoz-Pajares 2015). Finally, correlation tests were used to assess the relationships among the INT magnitude and MAP, and the climatic variables obtained from worldclim database. All analysis were run in R ver. 4.1.0 (R Core Team, 2014).

RESULTS

Extent of trait variation within populations and along precipitation gradient— The

variance partition analysis shows that sugar content in extrafloral nectar and HCN content

varied mainly among leaves, accounting for 66 and 79% of the total variance, respectively.

For C:N, foliar N and δ^{13}C *ca.* 50% of the variation was explained either by the identity of

individuals or their populations (Fig. 2). In contrast, variation in trichome density and LMA

was mostly concentrated at the population level (Fig. 2). The MANOVA showed that

populations significantly differed in their mean trait values (Wilks'$\lambda_{4,112}$ =0.007, $P < 0.001$).

ANOVAs with *post hoc* Tuckey HSD indicated differences in the expression of functional

traits among populations (Table 1, Fig. 3). In the driest population, plants had the highest

mean values of traits related to water use strategies such as LMA and δ^{13}C; and the lowest

variability among the sampled populations. In the case of traits attributed to anti-herbivore

functions, TR population, located in the middle of the precipitation gradient, had the

highest values of HCN and the lowest values for trichome density. Finally, mean foliar N and

C:N did not show significant changes among populations. Variation in traits were strongly

associated to MAP, with exception of HCN content (Fig. 3, Table 1 Supporting information).

LMA and chlorophyll content showed a decreasing pattern across the precipitation gradient

(Fig. 3).

Changes in phenotypic integration among populations— Significant phenotypic

integration among leaf traits was found in all populations. Estimations of INT ranked

between 2.6% and 8.2% (Fig. 4a). The magnitude of INT had a positive, although marginally

significant association with MAP ($\rho = 0.632$, $P = 0.108$). However, significant relationships

were found between INT and precipitation of the driest month (Worldclim B14 ρ = 0.769, P = 0.051) and the precipitation of warmest quarter (Worldclim B18 ρ = 0.939, P = 0.007) (Table 2). We also found differences in the pattern of correlations among functional traits (Table 4). The less integrated population, CH, had only one significant correlation; sugar content in extrafloral nectar and C:N. In the driest extreme of the precipitation gradient, the SC population also had low values of INT with a distinctive negative correlation between sugar content in extrafloral nectar and HCN content, and both traits were correlated positively with foliar N and C:N. In comparison, a positive correlation between LMA and δ^{13}C was present in populations with grater precipitation. In the TR population, defensive traits were indirectly correlated via foliar N, while in the LM population, chlorophyll content was correlated with defensive traits and δ^{13}C. Finally, in the most integrated population, SN, correlations were mostly negative, with only a positive association between trichome density and δ^{13}C.

Populations did not show a clear separation in the phenotypic space as revealed by the PCA (Fig. 4b). The main axis of variation explained 39% of total variation and separated the TR population from the rest of the populations. This axis was associated with water economy and included a positive correlation with HCN content and negative correlations with LMA, trichome density and δ^{13}C. The PC2 explained 21% of the variation and was positively correlated with chlorophyll content. The PC3 represented an axis related with N economy and biotic defense investment. This component had positive loadings for sugar content in extrafloral nectar and C:N in combination with negative loadings for foliar N (Table 3, Fig. 4b).

DISCUSSION

We found that plants growing across a precipitation gradient showed contrasting levels of

foliar phenotypic integration. Populations in sites with the lowest precipitation, with driest

and warmer months, had the lowest phenotypic integration values and had strong

correlations among defensive traits and foliar nitrogen. On the wettest end of the gradient,

in contrast, we found the most integrated populations, characterized by negative trait

correlations. This pattern suggests changes in the resource use strategy to best suit the

current environmental conditions. Moreover, we detected that the main sources of

variation of foliar traits in *T. velutina* occurred at the leaf and individual levels. While traits

with greater variation at the leaf level where those related with defenses against

herbivores, other functional traits related to water and nitrogen economy were highly

variable at the individual and population levels.

 Extent of trait variation and trait shifts along environmental gradients— Variation of

foliar traits differed between individuals and populations and provided information about

some pressures behind the optimization of plant performance. The high variation found at

the individual and leaf level in anti-herbivore defense traits can be explained with the

optimal defense theory, which suggests that differences in the expression of defenses

within individual plants can be due to high concentration of chemical defenses in more

valuable tissues with greater risk of damage by herbivores (McKey 1979, Stamp 2003,

McCall & Fordyce 2010). Specifically, this theory predicts that younger leaves located at the

tip of branches should be heavily defended (McCall & Fordyce 2010). This is the case of the

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higher concentrations of cyanide content (HCN) in younger than older leaves across leaf

cohorts in *Eucalyptus cladocalyx* (Gleadow & Woodrow 2000) and *Ryparosa kurrangii*

(Weber & Woodrow 2008). In the case of *T. velutina*, although we selected fully expanded

leaves, variation in leaf age could have influenced the value and expression of functional

traits, including those characters related with defense (Niinemets, 2016, Barton et al.

2019). The high variation in extrafloral nectar at the leaf level could have been promoted

by increased herbivore damage in young leaves or in those associated with flowers (Barton

& Hanley 2013). In several plant species extrafloral nectar is secreted in higher volumes in

young and fully expanded leaves than in young and undeveloped foliar structures (Heil et

al. 2000). In *T. velutina*, previous reports indicate that nectar secretion is higher during

flower anthesis and seed dispersion (Villamil 2017). Alternatively, cyanogenic compounds

have been proposed to play a role under dry conditions because they can storage N to

maintain photosynthetic capacity under warm and dry conditions (Møller 2010, Kooyers et

al. 2014). Hence, intra individual variation in defenses can result from mixed selection

pressures involving herbivores, mutualists, and abiotic factors within and among

populations.

Another group of traits, particularly C:N, foliar N, and δ^{13}C; exhibited comparable

variation between individuals and populations (Fig. 2). This variation is likely to be

promoted by abiotic factors at a regional scale such as climatic factors or soil fertility

(Salazar et al. 2018) and heterogeneous conditions within populations (e.g., light levels and

disturbance regime), resulting in complex patterns in which phenotypes maintain certain

levels of plasticity to face a myriad of selection pressures to optimize their performance. On

the contrary, foliar traits such as chlorophyll content, LMA, and trichome density exhibited

the highest variation among populations. This result suggests that traits considered good

descriptors of a functional strategy at the species level can have wide variation when more

than one population is studied (Albert et al. 2010, Hulshof & Swenson 2010). Therefore, the

analysis of variation of these traits across populations allows us to infer functional

strategies of *T. velutina*. For example, populations at the lowest precipitation range had

high investment in foliar tissue through increased area and high chlorophyll concentrations.

This suggests that plants in dry sites favor water conservation and carbon storage over

growth (Dudley 1995, Etterson 2004). In contrast, SN and LM populations, which are at the

wettest end of the gradient, seem to favor high metabolic rates and acquisitive strategies

(Reich et al., 1997; Wright et al., 2004, Fig 4b). This pattern can be the result of plasticity in

functional strategies within a species, showing that the phenotypic space that a species can

potentially occupy can be expanded in response to abiotic factors (Bontemps et al. 2017,

Salazar et al. 2018). An alternative explanation is that plant strategies are the result of

adaptation to local conditions (de Villemereuil et al. 2017). To elucidate between both

explanations further information is needed, including assessments of genetic

differentiation and plant fitness among different populations under contrasting

environments (Kawecki & Ebert 2004). Our results contribute to the growing evidence that

individual traits consistently change along an axis of the resource-acquisitive or resource-

conservative strategies within species (Ramírez-Valiente et al. 2010, Brouillette et al. 2014),

in equivalent magnitudes to interspecific differences (Albert et al. 2010, Brouillette et al.

2014).

Shifts of phenotypic integration across a precipitation gradient— Although the

magnitude of phenotypic integration of leaf functional traits was quite low in comparison

with other species (Boucher *et al.* 2013, Salgado-Negret et al. 2015), and the number of

populations analyzed along the precipitation gradient was rather small, we found a positive

relationship between the magnitude of phenotypic integration and the precipitation in the

driest and warmer months. This result does not support our initial hypothesis that

environmental harshness reduces the suitable combinations of functional traits to survive

under stressful environments, hence increasing the magnitude of their phenotypic

integration. On the contrary, we detected a pattern in which dis-integrated phenotypes

were found at the driest end of the precipitation range. Several alternative hypotheses

explain this pattern. On one hand, the evolution against strong covariances can be

attributed to multiple selective pressures acting in opposite directions, which can constrain

the expression of traits strongly associated. This seems true for high values of HCN which

confer high resistance to drought stress in *Trifolium repens* (Kooyers et al. 2014) but also

reduced herbivory in populations of *T. velutina* (Zedillo-Avelleyra 2017). LMA is other

adaptive trait conferring advantages under shorter and drier growing seasons, representing

a resource-acquisition strategy aimed to maximize carbon gain (Givnish 2002). LMA also

mediates the interception of light enhancing photosynthetic capacity under low-light

conditions (Gratani & Varone 2004) and leaf toughness that limits the consumption of

tissue by herbivores (Coley 1983). In the artic-alpine herbaceous *Polygonum viviparum*, for

example, populations distributed in the middle of a temperature gradient showed the

highest magnitude of phenotypic integration, suggesting that in the extremes of the

gradient a combination of biotic and abiotic pressures constrained the phenotypic expression of vegetative traits lowering the integration magnitude (Boucher et al. 2013).

Alternatively, low values of integration are attributed to higher plasticity of individual traits (Gianoli & Palacio López 2009). This proposal is based on the rationale that correlations expressed between traits in a more integrated phenotype can reduce the variability of individual traits and restrict the expression of functional responses to the environment. Evidence for this pattern has been recorded in the plasticity to shade in *Convolvus chilensis* and plasticity to drought in *Lippia alba* (Gianoli & Palacio-López 2009), although the estimator for phenotypic integration was the number of significant correlations and the plasticity is referred to the reaction norm of individual traits, not the plasticity of integration taken as a complex trait. This hypothesis is supported by an analysis of integration in the sub-Antartic community in Kerguelen Islands, where it was found that the endemic species *Pringlea antiscorbutica* and *Lyallia kerguelensis*, restricted to less variable microhabitats, exhibit a high degree of phenotypic integration (Hermant *et al.* 2013). In these species, microhabitat conditions resulted in local specialization with individuals expressing only particular trait combinations, reducing the variability of individual traits and strengthen their covariances (Hermant *et al.* 2013). In this context, our results allow us to explore the possibility that low integration values can be favored by natural selection as part of the exploration of the phenotypic space of functional strategies. A question to be answered is if this is the result of plasticity in phenotypic integration as a complex trait.

The low number of covariances detected in the driest populations could be the result of high variability in functional traits, related to heterogeneous conditions that we did not measure, such as soil N and P availability. In this scenario, phenotypic plasticity of the functional strategy results advantageous to respond to environmental challenges and to secure survival. While variation and flexibility of functional strategies were displayed in the driest populations, in populations established in the wettest end of the gradient plants likely optimized their phenotype increasing their covariances and the hence the magnitude of their phenotypic integration. This result coincides with the case of *Aextoxicon punctatum*, a tree species established in fragments formed by differential fog supply from windward to leeward edges, here the integration level was higher in populations in the drier leeward zone than in the wetter edge (Salgado-Negret et al. 2015). The authors hypothesized that high canalization of hydraulic anatomy across forest fragments could lead to optimization in water transport and survival of phenotypes in driest sites throughout the correlation among LMA, vessel diameter, stomatal density and trichome density (Salgado-Negret et al. 2015).

Populations of *T. velutina* established in the driest site are also those exposed to greater seasonality in rainfall. In contrast, populations established in the wettest end of the range, LM and SN, have less restrictions in water availability and less seasonal changes throughout the year. Hence, we suggest that drier sites exert a selective pressure for *T. velutina* phenotypes to be flexible to face a greater range of environmental conditions, thus increasing the variability in individual traits and reducing their covariances and phenotypic integration. In contrast, the wettest sites should canalize for an optimal strategy with high

covariance among traits and higher phenotypic integration values, which should optimize

plant resource acquisition and defense throughout the year. Nevertheless, canalization of

these strategies in the populations in the wettest sites could be also a response to soil

nutrient availability as has been previously reported in other tropical communities (Kaspari

et al. 2008, Wright et al. 2011, Condit et al. 2013), but this warrants further investigation.

Furthermore, studies should also explore these patterns including more populations of *T.

velutina* to provide conclusive evidence on the influence of environmental gradients on

phenotypic integration and functional plant strategies, promoting the existence of different

ecotypes.

Regarding the patterns of phenotypic integration, trait covariances were

characteristic of each population across the precipitation gradient. Although an increase in

the magnitude of integration theoretically means that covariances increase in strength,

paired relationships between traits did not increase steadily and most of the covariances

were significant only for specific populations. For example, in Santa Catarina (the driest

site), correlation patterns were characterized for an association among the traits related

with defense against herbivory (i.e., SEFN, HCN content, and trichome density), in addition

to LNC and LMA (Table 4). In contrast, LMA and its association with trichome density and

δ^{13}C dominated the correlation patterns of Troncones, La Mancha and Sontecomapan

populations, in the middle of the gradient (Table 4). A particular case was the Chamela

population, in which only a negative association between SEFN and C:N was detected, but

with a greater magnitude of integration than in the Santa Catarina population (Table 4).

The analysis of the correlation patterns indicates that more integrated populations are

mainly driven by the relationship between traits related to water and potentially nitrogen economy (Fig. 4). However, it must be considered that water availability is just one component of microhabitat heterogeneity influencing the evolution of adaptive plasticity in physiological and morphological traits. Consequently, conflicts in selective pressures are likely to arise with other selective agents.

CONCLUSIONS

The central result of our study is that the strength of the covariances among foliar traits in *T. velutina* populations depends upon the abiotic environment and results in different functional strategies; from dis-integration in driest sites to an increase in the magnitude of integration in the wettest range of the gradient. This suggest that environmental filters do not select traits independently but rather select particular trait combinations promoting survival and plant fitness, which shape the evolutionary landscape at multivariate level. We also provide evidence of the intraspecific variability in functional strategies, highlighting the use of phenotypic integration approach as a useful tool to understand and describe the shape and space occupied by species within the available functional trait space (Laughlin & Messier 2017). Overall, we suggest that this approach is useful to understand how plant phenotypes respond to multiple selection pressures (Cheverud 1982, Lande & Arnold 1983, Walsh & Blows 2009) and the evolution of phenotypes under the climate changes expected in the years for come.

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AUTHOR CONTRIBUTIONS

XD, KB, and AG designed and conceptualized the experiment. XD carried out the fieldwork, data curation and analysis with input from KB and AG. All authors contributed to writing the manuscript. Funding acquisition: KB and AG.

DATA AVAILABILITY

Data set used in this study is available via: 10.6084/m9.figshare.19775293.v1 (Damián, 2022).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Linear regressions between precipitation variables and individual functional traits of *T. velutina*.

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TABLES

Table 1. Mean values ± sd and Coefficient of variation (CV%) of functional leaf traits of *Turnera velutina* from five populations located across a precipitation gradient. Different superscript letters indicate statistically significant differences between means. The last column shows results from ANOVAs with $F_{4,116}$ degrees of freedom for sugar content, hydrogen cyanide content, trichome density and chlorophyll content; and $F_{4,112}$ degrees of freedom for δ^{13}C, foliar N and C:N.

Site characteristics	Population					*F*
	Santa Catarina	Chamela	Troncones	La Mancha	Sontecomapan	
	(*N* = 13, SC)	(*N* = 22, CH)	(*N* = 30, TR)	(*N* = 20, LM)	(*N* = 27, SN)	
Location	17º32'14",	19º34',	17º46'26",	19º35'40",	18º33'24",	
	97º00'56"	105º06'	101º42'47"	96º22'51"	94º59'38"	
Sampling date	June 14-16	August 12-16	July 13-16	July 22-26	June 19-22	
Altitude	1310	20	5	12	10	
Mean Annual Temperature (ºC)	16.9	25.0	27.2	25.8	24.5	
Mean Annual Precipitation (mm)	641.9	769.5	919.7	1222.5	3880.7	
Functional traits						
Sugar content in extrafloral nectar (mg/µL)	0.003 ± 0.005[b] (178.3)	0.104 ± 0.114[ab] (109.0)	0.177 ± 0.209[a] (118.3)	0.053 ± 0.087[b] (165.4)	0.021 ± 0.034[b] (165.3)	8.599
Hydrogen cyanide content (µg/g)	15355.3 ± 11230.0[bc] (73.1)	2799.1 ± 1724.2[c] (61.6)	33228.2 ± 13225.0[a] (39.8)	4834.5 ± 3285.4[c] (68.0)	11309.9 ± 7700.2[b] (68.1)	21.62
Trichome density (trichomes/mm2)	14.1 ± 2.7[b] (19.0)	15.9 ± 3.6[ab] (22.4)	3.4 ± 0.6[c] (17.2)	15.4 ± 1.9[b] (12.4)	12.6 ± 3.0[a] (23.3)	260
LMA (g/m2)	42.5 ± 13.4[a] (31.5)	55.5 ± 6.8[b] (12.2)	42.2 ± 5.4[c] (12.8)	50.4 ± 9.9[b] (19.6)	31.4 ± 5.7[d] (18.2)	68.26
δ^{13}C (‰)	-27.6 ± 1.4[a] (4.9)	-28.5 ± 0.4[bc] (1.3)	-29.0 ±0.7[c] (2.3)	-28.4 ± 0.8[b] (2.9)	-28.9 ± 1.1[bc] (3.7)	19.9
Chlorophyll content (µg/cm2)	10.0 ± 1.2[a] (12.0)	8.9 ± 1.4[b] (15.3)	8.5 ± 1.4[b] (16.5)	7.2 ±1.3[c] (17.6)	6.5 ± 0.7[c] (10.4)	40.95
Foliar N content (µg/ mg dry mass)	0.09 ± 0.01[b] (11.5)	0.07 ± 0.01[c] (11.2)	0.08 ± 0.01[ab] (10.3)	0.07 ± 0.01[c] (13.5)	0.08 ±0.01[a] (9.7)	22.55
C:N	6.6 ± 0.8[b] (11.4)	8.8 ± 0.8[a] (9.5)	7.7 ± 0.8[b] (9.7)	9.25 ± 1.2[a] (13.3)	7.4 ± 1.0[b] (13.3)	18.05

Table 2. Results from correlation analysis between INT magnitude and climatic variables for populations of *T. velutina*. BIO12-BIO19 were obtained from worldclim database.

Variable	ρ	P
BIO12: Mean Annual precipitation	0.632	0.108
BIO13: Precipitation of the wettest moth	0.645	0.102
BIO14: Precipitation of driest month	**0.769**	**0.051**
BIO15: Precipitation seasonality (Coefficient of Variation)	0.636	0.106
BIO16: Precipitation of wettest quarter	0.653	0.098
BIO17: Precipitation of driest quarter	0.688	0.082
BIO18: Precipitation of warmest quarter	**0.939**	**0.007**
BIO19: Precipitation of coldest quarter	0.600	0.124

Table 3. Results from Principal Component Analysis performed on the functional traits of the five populations of *T. velutina* (*N* = 121 plants).

Traits	PC1	PC2	PC3	PC4
Eigenvalue	1.760	1.306	1.089	0.9205
% of Variance explained	0.387	0.213	0.148	0.106
% of Accumulated variance	0.387	0.600	0.748	0.854
Sugar content in extrafloral nectar (mg μL^{-1})	0.176	0.359	**0.527**	-0.505
Hydrogen cyanide content ($\mu g\ g^{-1}$)	**0.386**	0.352	0.193	-.264
Trichome density (trichomes per mm^2)	**-0.465**	0.346	0.030	0.137
LMA (g m^{-2})	**-0.417**	-0.384	-0.062	0.199
$\delta^{13}C$ (‰)	-0.207	**0.618**	-0.230	-0.109
Chlorophyll content ($\mu g\ cm^{-2}$)	0.354	0.059	**-0.433**	-0.348
Foliar N content ($\mu g\ mg^{-1}$ dry mass)	**-0.417**	0.291	-0.274	-0.139
C:N	-0.296	-0.100	**0.613**	-0.682

Table 4. Partial correlation coefficients between the foliar traits in plants of *Turnera velutina* and the five populations sampled. Bold values denote statistically significant correlations at *P* = 0.05. Populations appeared ordered from the driest to the wettest site.

Population†	Traits‡	EFN	HCN	LMA	TRIC	CHL	LNC	δ^{13}C
SC	HCN	**-0.484**						
	LMA	**-0.595**	0.351					
	TRIC	0.284	0.231	-0.279				
	CHL	-0.173	-0.389	0.318	-0.126			
	LNC	**0.528**	-0.272	-0.472	0.129	-0.120		
	δ^{13}C	0.134	0.061	0.180	0.254	0.252	0.069	
	C:N	-0.154	**0.502**	0.289	**0.560**	-0.122	0.038	0.572
CH	HCN	0.209						
	LMA	0.129	0.005					
	TRIC	-0.218	-0.165	0.157				
	CHL	0.304	0.289	0.012	0.061			
	LNC	-0.344	-0.064	0.052	0.258	0.007		
	δ^{13}C	0.084	-0.180	0.337	0.346	0.179	-0.221	
	C:N	**0.408**	0.086	-0.163	0.183	0.225	0.009	0.277
TR	HCN	0.058						
	LMA	-0.355	0.016					
	TRIC	-0.307	-0.176	0.086				
	CHL	-0.015	-0.239	**0.422**	-0.023			
	LNC	**0.539**	**-0.411**	-0.324	-0.123	0.328		
	δ^{13}C	-0.207	-0.304	**0.655**	0.138	0.380	0.105	
	C:N	0.369	0.084	-0.126	-0.026	-0.229	0.156	-0.106
LM	HCN	-0.189						
	LMA	0.107	0.020					
	TRIC	-0.082	-0.266	**0.489**				
	CHL	**0.717**	**-0.422**	0.242	0.017			
	LNC	0.060	-0.182	**-0.692**	-0.246	0.147		
	δ^{13}C	0.336	-0.155	**0.629**	0.350	**0.515**	**-0.402**	
	C:N	-0.057	-0.273	0.190	0.190	0.110	0.100	0.138
SN	HCN	-0.127						
	LMA	-0.198	-0.205					
	TRIC	**-0.492**	0.212	-0.111				
	CHL	**-0.475**	-0.140	0.172	0.109			
	LNC	0.103	0.318	0.055	-0.304	0.106		
	δ^{13}C	-0.280	0.033	-0.166	**0.432**	0.301	**-0.567**	
	C:N	-0.266	-0.010	-0.162	-0.053	0.010	**-0.552**	0.295

† Populations: SC: Santa Catarina, CH: Chamela, TR: Troncones, LM: La Mancha, SN: Sontecomapan.

‡ Abbreviations for traits. EFN: Sugar content in extrafloral nectar, HCN: Hydrogen cyanide content, LMA: Leaf mass per area, TRIC: Trichome density, CHL: Chlorophyll content, LNC: Leaf N content, $\delta^{13}C$: Carbon isotopes, C:N: carbon-to-nitrogen ratio.

FIGURE CAPTIONS

Figure 1. Location of the five *Turnera velutina* sampled populations in Mexico and values of Mean Annual Precipitation (MAP) according to CONAGUA data (see Table 1 for more details). SC, Santa Catarina Tlaxila; CH, Chamela; TR, Troncones; LM, La Mancha; and SN, Sontecomapan.

Figure 2. Variance partition of leaf functional traits explained by the different hierarchical levels between *T. velutina* populations across a precipitation gradient. TRIC = trichome density, LMA = leaf mass per area, CHL = Chlorophyll content, d13C = Carbon 13 isotope content, LNC = leaf nitrogen content, C:N = carbon to nitrogen ratio, EFN = sugar content in extrafloral nectar, HCN = hydrogen cyanide content. Data for EFN, HCN and TRIC were log transformed prior to analysis.

Figure 3. Variation in functional traits across a precipitation gradient. Points represents individual plants and side bars the mean values ± 1 sd. The populations are arranged in increasing Mean Annual Precipitation (MAP) values. TRIC = trichome density, LMA = leaf mass per area, CHL = Chlorophyll content, d13C = Carbon 13 isotope content, LNC = leaf nitrogen content, C:N = carbon to nitrogen ratio, EFN = sugar content in extrafloral nectar, HCN = hydrogen cyanide content

Figure 4. a. Changes in phenotypic integration magnitude (INT ± sd) of *T. velutina* populations along to precipitation gradient. b. Phenotypic space occupied by *T. velutina* populations resulting from PCA analysis. Ellipses show the confidence interval at 95%.
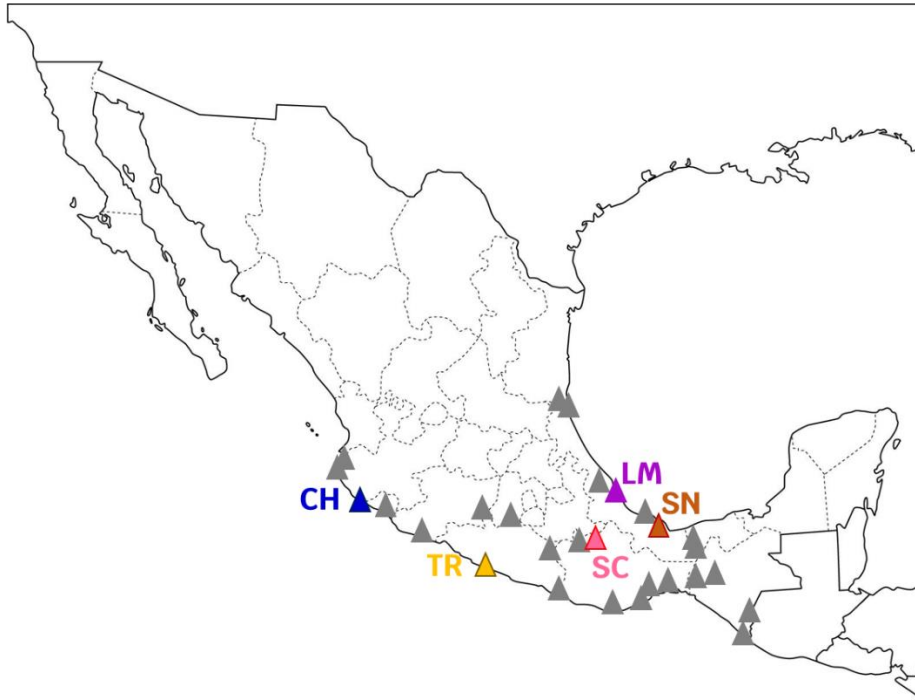
Figure 1.
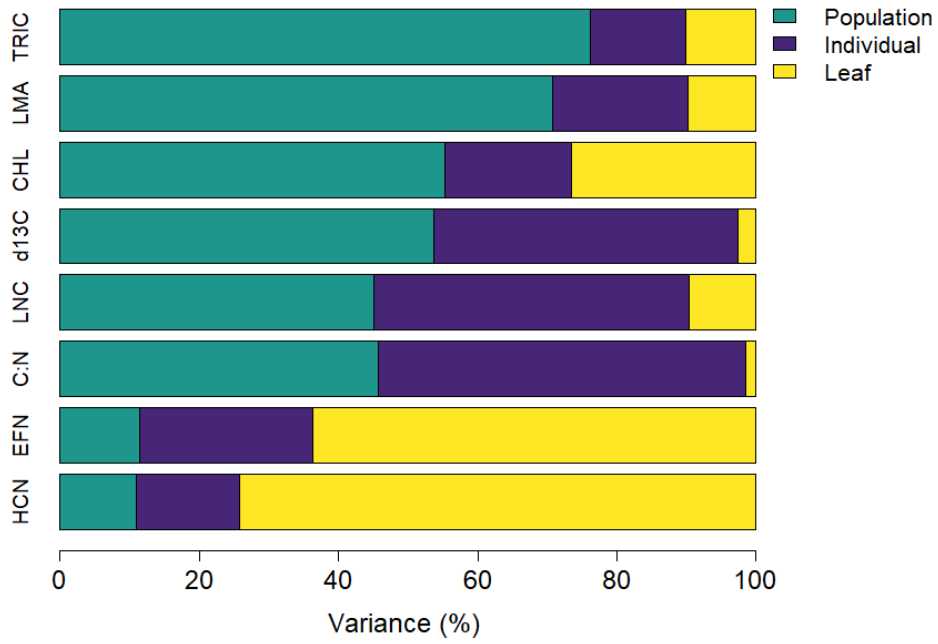
Figure 2.
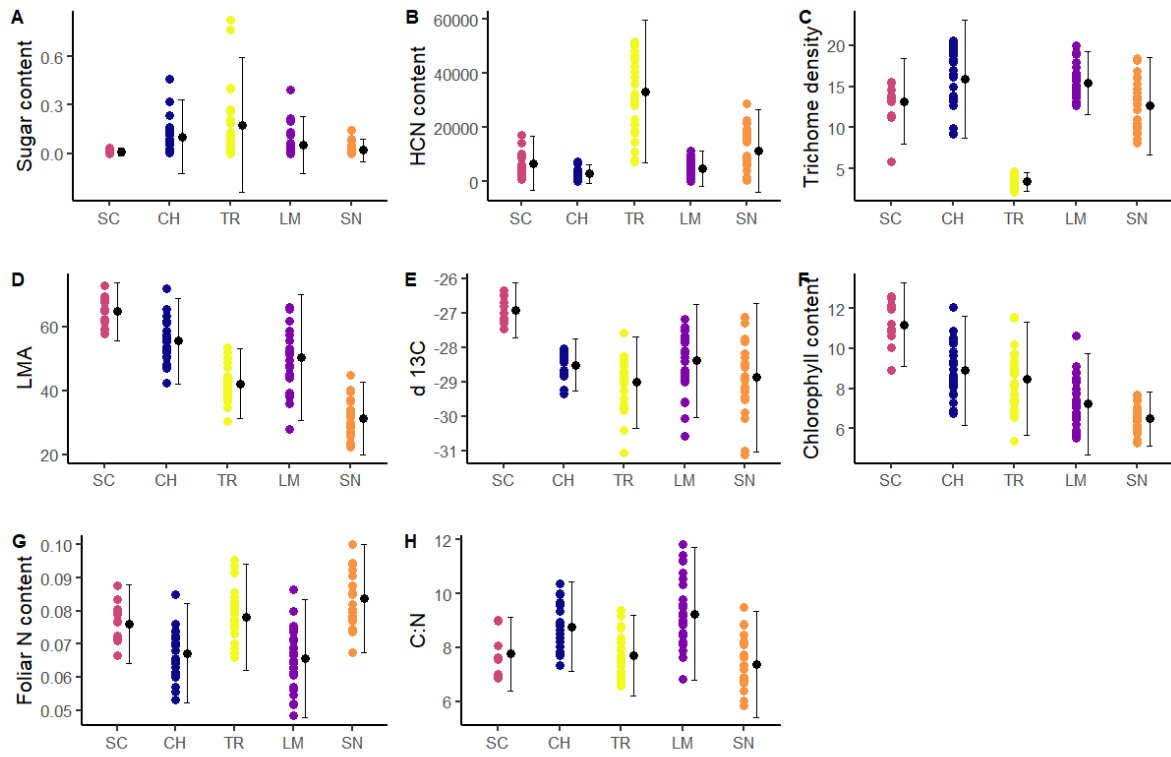
Figure 3.
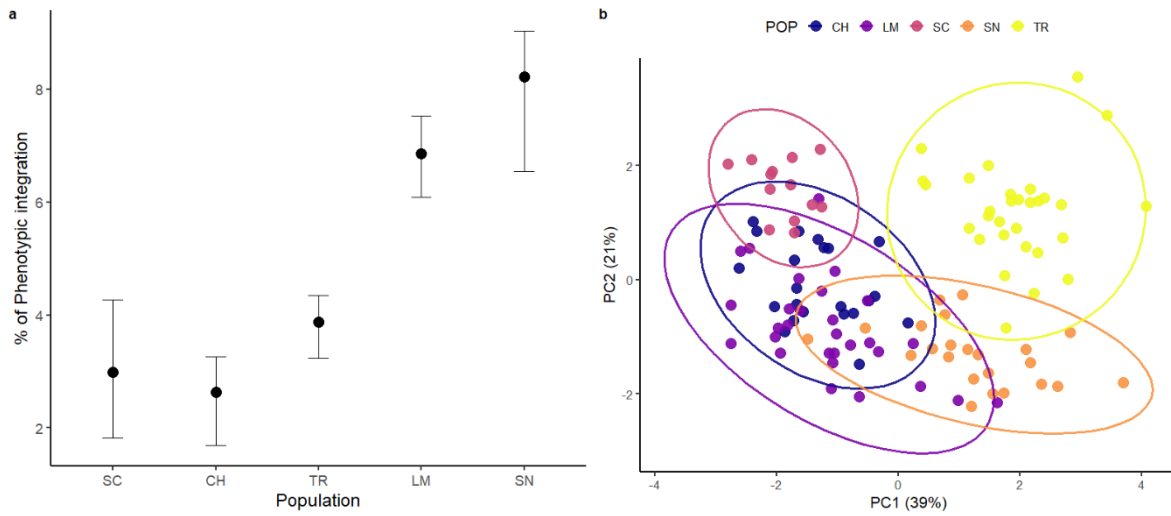
Figure 4.

Appendix S1

Table 1. Results from linear regressions between precipitation indicators and individual functional traits of *T. velutina* across populations.

Trait	MAP	
	R	*P*
Sugar content in extrafloral nectar (mg/µL)	0.067	0.004
Hydrogen cyanide content (µg/g)	0.003	0.561
Trichome density (trichomes/mm^2)	0.403	< 0.001
LMA (g/m^2)	0.126	< 0.001
δ^{13}C (‰)	0.214	< 0.001
Chlorophyll content (µg/cm^2)	0.220	< 0.001
Foliar N content (µg/mg dry mass)	0.032	0.054
C:N	0.123	0.0001

Capítulo 3. An empirical test of plasticity of phenotypic integration: the reaction norm of leaf functional strategies

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An empirical test of plasticity of phenotypic integration: the reaction norm of leaf functional strategies

SUMMARY

Recognized by its key role on phenotypic evolution and adaptation of organisms to environmental change, the concept of phenotypic plasticity has been extensively used in the study of functional traits. However, studies of plasticity have focused on single traits rather than on the network of traits representing the multivariate nature of the organisms. Here, we used estimations of the magnitude of phenotypic integration of leaf traits to determine the plasticity this complex functional trait and to analyse the foliar morsphospace occupied by *Turnera velutina* plants growing under two light environments. Plants that grew under reduced light environments showed an increase in the magnitude of integration and converged in a reduced area in the phenotypic morphospace, compared with those maternal families growing with greater light availability that showed less integrated phenotypes and had a wider distribution in the phenotypic morphospace. Interestingy, the plasticity of the magnitude of foliar integration was greater compared with that of individual functional traits. These results showed the plasticity of sets of functional traits and the multivariate nature of strategies displayed by plants under selective conditions and shows the potential of phenotypic integration as a key attribute in the expression of functional strategies according to conditions faced by plants.

INTRODUCTION

A central concept for understanding how organisms interact with their environment is phenotypic plasticity; the capacity of a given genotype to exhibit different phenotypic values for a given trait under dissimilar environmental conditions (Pigliucci 2005, Bradshaw 2006). Yet, the study of plasticity has been centered on individual traits rather than multiple traits (Forsman 2015), mainly because measuring changes in isolated phenotypic values is easier than estimating the coordinated changes among several attributes. However, adaptations are often complex or integrated phenotypes resulting from the

coordinated expression of different traits, linked by genetic, developmental, functional, and/or physiological associations (Schlichting 1989, Herrera et al. 2002, Pigliucci & Preston 2004). Hence, overlooking correlations among traits can reveal some limitations in the conclusions obtained from univariate studies. For example, correlated traits could set limits to the full expression of plasticity for individual attributes (Gianoli 2001), which can be an explanation of the observed reduced plasticity under stressful conditions (Valladares et al. 2007). Second, the apparently ubiquitous cost of plasticity has been attributed to the association among traits, considering that the cost of one trait can be influenced by its interrelationship with other traits (Ellers & Lifting 2015). Third, the conceptual framework of phenotypic integration has been focused on the variation of integration among species and not in the variation on such emergent property among genotypes or individuals within a population (Plaistow & Collin 2014, Damián et al. 2020, Matesanz et al. 2021). Finally, phenotypic integration, or suite of characters, can vary within (Ordano et al. 2008) and among populations (Herrera et al. 2002; Boucher et al. 2013) and can respond as a complex character to natural selection (Damián et al. 2020), thus opening the question if the same genotypes can express different magnitudes of phenotypic integration when exposed to different environments, through plastic multivariate responses.

Character correlations are labile not only through evolutionary time or development, but also in response to some changes in environmental conditions. Other works have previously described how correlations among multiple traits respond under different environmental conditions. For example, the plasticity of trait correlations in *Phlox drummondii, P. cuspidata* and *P. roemeriana* under experimental conditions of nutrient availability and water limitation in pots depicted the heterogeneous change in the magnitude and sign of correlations (Schlichting 1989). In addition, studies with *Arabidopsis thaliana* have revealed functional relationships between traits associated with wind stimulation (Pigliucci, 2002). In this case, plants with shorter, branched, and compact phenotypes were characteristic in conditions of sustained wind, reducing the costs of flower production (Pigliucci 2002). Furthermore, evidence of phenotypic plasticity of trait correlations includes changes not only in morphological traits, but also in behavioral

characters. For example, under selective conditions with a diversity of predators, larval anurans produce specific coordinated defenses that include the development of deeper tails and muscles with shorter and narrower bodies, or phenotypes with deep and long tails with small bodies (Relyea 2001). Although these studies have reported changes among correlations in pairs of traits, none of them had estimated the changes in the multivariate phenotype.

The conceptual shift from studying phenotypic plasticity of individual traits to that of associations among multiple characters can be visualized through the concept of phenotypic integration, with the same principles used in the study of phenotypic plasticity of individual traits as suggested by Pigliucci & Preston (2004). These authors suggested that the trajectory of mean values for a trait representing the genotype-specific environment-phenotype interaction can be replaced with some estimate of the magnitude of phenotypic integration (Pigliucci & Preston 2004), depicting the reaction norm of this complex trait. For example, in *Daphnia magna*, phenotypic integration of life history characters showed a plastic response under two food treatments (Plaistow & Collin 2014). This evidence suggests that environment can induce plasticity in a multitude of different traits at the same time. In fact, the extent of phenotypic plasticity can be evaluated as the slope of the reaction norm of the magnitude of phenotypic integration, making possible to quantify the multivariate phenotypic distances between individuals of a given species exposed to different environments (Valladares et al. 2006).

Although the effect of the environment on the covariance structure among multiple traits has been previously documented, evidence on the plasticity of phenotypic integration as an attribute is scarce (Waitt & Levin 1993, Matesanz et al. 2021). Shifts in phenotypic integration across environments has been attributed mainly to the role of stress conditioning the expression of correlated traits. It has been proposed that resource limitation can lead to more integrated phenotypes, as the result of the optimization of individual traits for the acquisition and use of scarce resources (Schlichting 1986, Gianoli 2004, Gianoli & Palacio-López 2009). An alternative explanation on the changes in phenotypic integration has focused on the plastic responses of related traits that covary

coordinately among environments, or, in other words, the integration of plasticity of individual traits (Schlichting & Pigliucci 1998, Matesanz 2021).

In previous studies we have reported the degree of variation in the magnitude and patterns of foliar phenotypic integration among genotypes of *Turnera velutina* (Damián et al 2018, Damián et al. 2020). In this tropical shrub, functional strategies change across ontogeny favoring the expression of resource acquisition mechanisms in younger plants and the transition to conservative strategies in reproductive stages (Damián et al. 2018). In addition, we have found that strongly integrated phenotypes produce more flowers and grow faster than less integrated phenotypes (Damián et al. 2020). Moreover, variation in foliar phenotypic integration in *T. velutina* plants seems to define their functional strategies in response to precipitation. In a previous study, we have described that across its geographic range, *T. velutina* plants switch from conservative strategies when growing in dry sites, to resource acquisition strategies in sites with greater water availability, simultaneously increasing the values of magnitude of integration (Damian et al. unpublished manuscript.). These different levels of variation inspired the present study, in which we asked if changes in phenotypic integration of leaf functional traits result from phenotypic plasticity of this complex trait, or if these changes are rather the consequence of plastic responses of individual traits. With this purpose, we assessed if the magnitude and patterns of phenotypic integration of leaf functional traits changed between contrasting environmental conditions to depict the reaction norm of this complex attribute and also evaluated the phenotypic plasticity of individual traits. Because *T. velutina* is a species mostly found in forest clearings and gaps on sand dunes and seems to produce fewer flowers than in sunnier than in shaded sites (pers. obs.), we hypothesize that plants have optimized their strategies to heliophilous conditions and hence face more stressful conditions in shaded conditions. Hence, we predicted an inverse relationship between light availability and the magnitude of phenotypic integration, resulting from the multivariate optimization strategies under high light conditions and trade-offs of simultaneously expressing different traits under low light availability. Last, we compared the degree of plasticity between individual leaf traits and the magnitude of their integration using a

simplified Relative Distance Plasticity Index (RDPIs, Valladares et al. 2006) and assessed the differences in the covariance morphospace that genotypes occupied under the different light environments.

MATERIAL AND METHODS

Plant materials

Turnera velutina Presl. is a self-compatible (Sosenski et al. 2016), perennial shrub endemic to Mexico (Arbo 2005). It is distributed mainly in coastal dunes and tropical dry forests. The reproductive season of the species is associated with the rainy season (Torres Hernández et al. 2000), which occurs between June to August in the study site, located in La Mancha, Veracruz, Mexico (19º35'40" W, 96º22'51" N, elevation 12 m). In this population the mean annual temperature ranges between 21°- 23°C and the mean annual precipitation is around ca. 1220 pp (smn.conagua.gob.mx/es/climatologia/informacion-climatologica/informacion-estadistica-climatologica). *T. velutina* plants grow under the canopy of trees, in forest gaps and mainly at the edge of the tropical dry forest along the coastal dune, with an average of 35% sunlight (Damián et al. 2018). Plants bearing extrafloral nectaries in their leaves maintain mutualistic interactions with several ant species that protect them from foliar damage by the Nymphalid caterpillar *Eutoiepta hegesia* (Cuautle & Rico-Gray 2003).

Genotype selection and growth conditions

To determine the extent of phenotypic plasticity of the magnitude and patterns of phenotypic integration of leaf functional traits, we introduced plants from several genotypes to experimental plots in the field and measured their phenotypic integration under two light conditions. We selected plants from *the T. velutina* experimental population established in 2014 (see Ochoa-López et al. 2018 for details) and self-pollinated flowers during August and September 2017. Mature seeds were collected in September and sown in a greenhouse at the research field station Centro de Investigaciones Costeras La Mancha. Seeds were sown in trays with a mixture of vermiculite and sand (1:1 proportion) from adjacent fields. Seeds germinated after 3 weeks and were transplanted to

individual pots (150 ml) to avoid competition for space and secure full root development. Seedlings were transplanted to field conditions in October 2017, when they had the first two leaves completely developed and their third leaf primordia was visible. Plants were transplanted with their soil mixture to best ensure transplant success and avoid desiccation.

We established ten experimental plots at an interdune area (Fig. 1a). The plots were positioned in five pairs, no farther than 2 m apart, leaving sufficient room for data collection and plot upkeep. Each plot was surrounded by an enclosure (120 cm L × 120 cm W × 60 cm H) made with a PVC frame and shade mesh (PEAD polyethylene) with either 35% (T35 hereafter), or 50% light reduction (T50 hereafter), to produce two light treatments (Fig. 1b). Within each enclosure, 50 - 60 plants from all genetic families were randomly planted in a grid with 8 × 8 rows, leaving 10 cm between each plant (Fig. 1c). Before seedling transplant, the area was partially cleared shortening grass height. Each light treatment had between 20 to 18 individuals of each genetic family, overly distributed among the five plots (12 genetic families, 4-8 individuals per family, 2 treatments, N= 587 seedlings in total). The experiment lasted from October 13th 2017 to September 2018. During this period, water was supplied three times per week, applying 1.5 liters per exclusion to maximize seedling survival.

Measurement of functional traits

In September 2018 we measured leaf functional traits on all plants. After eleven months, plants grew on average 10 cm and showed characteristics from juvenile stage (Ochoa et al. 2015). We selected the three most apical and fully developed leaves of each plant to estimate the chlorophyll content (Chl content hereafter). We used a CCH-200 chlorophyll content meter (Opti-Sciences, Tyngsboro, MA, USA) to record the average value index of chlorophyll content (ICC) from the readings in four spots at each side of the main vein. Chl content (μg cm^2) was then estimated using the formula: Chl content = -0.093 + 1.36 ×√(ICC units) (R^2 = 0.79, P < 0.001) (Damián et al. 2018). The same leaves used for the estimation of chlorophyll content were collected to estimate the leaf mass area (LMA) following Pérez-

Harguindeguy et al. (2013). Leaves were wrapped in moist paper and stored in sealed plastic bags. After 24h of rehydration each leaf was patted dry before measurement. Images of leaf projected area were captured with a digital camera (SONY Cyber-shot DSC-W570) pressed under a glass Petri plate. Each image included a 1 cm^2 square for size calibration. Images were processed with Image J software (NIH, USA) to obtain the foliar area. Afterwards, leaves were processed to measure the HCN content as described in Ochoa-López et al. (2015). Briefly, three leaf discs (0.6 cm^2), were crushed in an Eppendorf tube with 7 µl of chloroform. A 0.5 × 2 cm strip of filter paper soaked in a 5% NaCO2 and 0.5% of picric acid solution was then suspended inside the tube, avoiding direct contact with plant material. We left the tubes in darkness for 24 h at room temperature. The picrate paper reacts with the HCN released by the leaf disc producing a color change from yellow to orange. A single disc of the filter paper was eluted in 1 ml of 50% ethanol and stored in the fridge at 4ºC. Three samples of 250 µl of the eluted ethanol were placed in microplates to measure absorbance at 950 nm using a microplate reads (ELx808, BioTek Instruments Inc., Winooski, VT, USA). The absorbance readings of HCN were transformed to HCN content (µg HCN/ g dry weight) with the formula HCN (µg) = (optical density − 0.0478965)/ 0.000652 ($R2$ = 0.91, $P < 0.0001$), obtained from a standard curve using sodium cyanide (Code 7660-1 Caledon Laboratories Ltd, Canada) as a source of HCN (Ochoa-López et al. 2015). Dry mass values were obtained from three additional leaf disc cut which were dried at room temperature to later record their dry weight.

After the HCN procedure, the remaining leaf sample was dried in the oven at 70ºC for 72 h; to then determine the dry mass with an analytical scale (Acculab VIC 303, Sartorius Group, MA, USA). Trichome density was estimated in one leaf of each plant, after taking pictures of the upper and lower side of each disc using a stereoscopic microscope (Discovery V8, Zeizz, x1.5). We quantified the number of trichomes in each field (14 mm^2) processing the pictures with the software Image J 1.48v (NIH, USA). To measure leaf nitrogen content (LNC), carbon to nitrogen ratio (C:N) and δ13C isotope as a proxy of water use efficiency (Farquhar et al. 1989), we ground the dry material in a TissueLyser II (Qiagen) and prepared a compound sample with all the leaves from each plants. Two samples were

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analyzed by mass spectrometry (Thermo Scientific Delta V Advantage IRMS with EA-2000Flash Elemental Analyzer. Analyses were performed at the Laboratory of Biogeochemistry and Applied Stable Isotopes (LABASI, PUC), Chile. We calculated C:N by dividing the carbon content over the nitrogen content in 1g of dry leaf mass.

Reaction norms of individual traits and their phenotypic integration

Phenotypic plasticity was estimated for the functional traits LMA, Chl content, trichome density, HCN content, LNC, C:N and δ^{13}C. To determine whether leaf traits were phenotypically plastic, and whether the maternal families differed in plasticity, we used a two-way analysis of variance (ANOVA) with maternal families and light treatment as fixed factors. The significance of the interaction between these two variables was considered as evidence of phenotypic plasticity. To compare the level of plasticity among individual functional traits and that of their phenotypic integration, we estimated the Simplified Relative Distance Plasticity Index (RDPIs). This index was obtained from the absolute difference between mean phenotypic values per family of each trait (or the magnitude of phenotypic integration) between light treatments. This value was then divided by the sum of the mean trait value (or the magnitude of phenotypic integration) in each environment (Valladares et al. 2006). This estimation allowed us to obtain RDPIs ranging from 0 to 1, which represents the relative plasticity of the traits measured, where values near 1 represent the maximal plasticity of a given trait. Later we tested through a one-way ANOVA and post hoc Tukey mean comparisons the differences of plasticity among traits and the magnitude of phenotypic integration (see below).

Estimation of the magnitude of phenotypic integration (INT) for each family was assessed using a PCA to estimate the variance among the eigenvalues (λ_i) of the correlation matrix of leaf functional traits (Wagner 1984, Cheverud et al. 1989). High variance among eigenvalues means that traits are highly correlated and the first principal component (PC) accounts for most of the variation, resulting in a high magnitude of phenotypic integration. If variation is proportionally distributed among all the PCs, a low phenotypic integration magnitude can be inferred. We conducted the analysis using the PHENIX package on R v.

3.6.1 (R Core Team 2014; Torices & Muñoz-Pajares 2015) to calculate the INT for each family on each light treatment. Because plant size can modify the correlation structure among traits (Torices & Muñoz-Pajares 2015), INT values were obtained with the partial correlation matrices after controlling for plant height. INT values were additionally corrected as INT = (Var (λi) – (number of traits -1) / number of individuals per genetic family), due to the variation in the number of plants sampled from each family. The INT values were transformed to the percentage of the total maximum integration. Total maximum integration is the number of traits in the correlation matrix (in our case, it is 7) and was significant if the 95% confidence interval did not include zero (Herrera et al. 2002). We compared the magnitude of phenotypic integration of maternal families between each treatment using a paired two-tailed t-test. Additionally, we obtained the correlation patterns between treatments that allowed us to identify the changes in the functional strategy of plants.

Differences in the morphospace between light treatments

We used the approach of morphospaces to describe the phenotypic configuration of maternal families on each light treatment. This approach allowed us to analyze the portion of morphospace occupied by families in each treatment and to assess changes in the functional strategies displayed under different light environments (Mitteroecker & Huttegger 2009, Benitez-Vieyra et al. 2019). First, we performed a PCA to visualize the differences in the magnitude and direction of variation in the multivariate space in each light treatment. The first three PCs explained 70% of the variation and were used to build the convex hull of each light treatment within the morphospace of all possible combinations of trait values (Chartier et al. 2014). Then, we estimated the variance-covariance matrices for each maternal family in each light treatment. We estimated a new matrix called C, which allows to transform one matrix into the other and represents the differences of family matrices between treatments. Eigenvalues from C matrix are used to derive a measure of matrix dissimilarity represented by the square root of the summed squared logarithms of the eigenvalues between two matrices, called Riemannian distance

(Melo et al. 2016). Such distance is the shortest distance between two matrices in the space of all possible variance-covariance matrices (Benitez-Vieyra et al. 2019). This process was repeated to obtain the Riemannian distances for the seven families with the function MatrixDistance of the evolqg package (Melo et al. 2016). To represent each of the matrices in a bidimensional space, A Principal Coordinate Analysis (PCoA) was performed (Machado et al. 2018, Mitteroecker & Bookstein, 2009; Bookstein & Mitteroecker, 2014). As each matrix corresponds to a single point, the Riemannian distances represent the similarity among points. Within this covariance space, complex hulls were constructed to examine the portion of space occupied by families from each light treatment. Significant differences were tested through a Permutational Multivariate Analyses of Variance (PERMANOVA) with 1000 bootstrap to obtain an estimation of uncertainty of the multivariate combination of traits (Benitez-Vieyra et al. 2019).

RESULTS

Reaction norms of functional traits and phenotypic integration

The two-way ANOVA detected a significant effect of the light treatments on the mean values of LMA, trichome density, $\delta 13C$ and C:N ratio (Table 1, Fig. 2). While plants in the T50 environment had greater LMA, plants growing in T35 showed greater values for trichome density, C:N and $\delta 13C$. This changes in the expression of functional traits resulted in a shift from thin leaves with high trichome density and water-use efficiency in T35 to thick leaves and higher leaf nitrogen content in the shaded environment. Except for HCN, all measured leaf traits showed genetic variance. However, we did not find genetic variation for plasticity for any functional trait, as none of the interactions between factors Family × Treatment was significant (Table 1). In contrast, the magnitude of phenotypic integration showed a clear and significant pattern in which plants growing at T35 had lower values (mean value of integration =3.8% (sd 0.012)) than plants growing at T50, which increased their phenotypic integration in two-fold (with an average of integration = 7.1% (sd 0.017); t = -6.572, P = 0.0006, Fig. 3). Two families at T35 did not have an INT statistically different from zero, although they duplicated their INT values at T50. Changes

in foliar integration between treatments were detected also in the correlations among functional leaf traits. Whereas in both light treatments we detected a strong negative correlation between LNC and C:N, in T50 we found a positive correlation between chlorophyll content and $\delta^{13}C$ content (Fig. 3). Differences in RDPIs showed that plasticity in individual traits was surprisingly low in comparison with the plastic responses of INT and HCN content (F = 6.123, P < 0.001).

The results of PCA indicated that variation of functional traits was organized in three main axes, explaining around 70% of the variation in all traits (Table 2, Fig. S1). The PC1 explained 28% of this variance and was considered the axis of nitrogen economy mainly due to the combination of negative loadings of LNC and positive loadings of C:N. PC2 explained 24% of the variance and was defined by water economy related traits, primarily by positive values of LMA and $\delta13C$ content, and negative loadings of C:N. Finally, PC3 was related with positive loadings of HCN and chlorophyll contents, and negative values for trichome density, explaining 18% of the total variance.

Differences on the structure of variance-covariance matrices between light treatments

The principal coordinate analysis using the Riemannian distance matrix, which represents pairwise distances between maternal families' matrices, indicated that the first three principal coordinate axes (PCo) accounted for 49% of the total variation. Convex hulls indicated a small overlap between light treatments as supported by PERMANOVA (F = 1.837, P = 0.002). Maternal families at T50 were located on a narrow portion of the morphospace, while *T. velutina* families at T35 occupied an extended area along PCo2 and PCo3 (Fig. 5).

DISCUSSION

Light affected the covariance structure among leaf functional traits resulting in an increase in the magnitude of phenotypic integration as light availability increased. Growing under higher light availability, plants produced leaves with low integration, with reduced LMA, high trichome density, higher C:N, overall increasing their water use efficiency. Consequently, the area occupied by phenotypes in the multivariate morphospace was

narrower for plants in the shaded treatment whereas plants with greater light availability occupied a greater space. Plasticity in the magnitude of phenotypic integration was greater than that observed for most individual leaf traits, suggesting that leaf traits respond jointly to environmental challenges to optimize functional strategies.

Plasticity of individual traits

The expression of plasticity of foliar traits under light environments has ecological and evolutionary implications (Sultan & Bazzaz 1993, Sultan 1995; Nicotra et al. 1997; van Kleunen & Fischer 2005). Functional strategies associated with heliophilous environments include a set of traits resulting in a distinctive phenotype: smaller and thicker leaves with less chlorophyll per unit leaf mass (Chazdon & Fetcher 1984, Gratani et al. 2006) to minimize carboxylation limitations. In contrast, leaves from plants growing in shaded conditions are thinner, larger and with greater chlorophyll and nitrogen contents. Together, these traits increase the relative biomass allocation to leaf tissue (Evans 1972) to maximize light capture and optimize the interception of diffuse light (Delagrange et al. 2006, Lida et al. 2014), so that leaves reach similar rates of photosynthetic activity compared to sunny leaves (Hikosaka & Terashima 1996). In general, *T. velutina* phenotypes follow the patterns expected for plants growing in both conditions. Higher trichome density has been reported previously in plants growing in dry environments that are frequently associated with high levels of radiation (Pérez-Estrada 2000). Under this condition, higher trichome density reduces water loss (Woodman & Fernandes 1991). Unexpectedly, chlorophyll, foliar N and HCN contents did not show a change in the mean value between treatments. Although chlorophyll and foliar N content showed variability among maternal families, the variation displayed in both traits was high, constraining the identification of a tendency of change. It is possible that light was not a selective factor for the expression of HCN and foliar N content, even though this is a chemical compound based on nitrogen that can be mobilized or stored as a reserve for photosynthetic activity (Gleadow & Møller 2014). For example, in *Helianthus annus* soil moisture has a direct effect on the use of N and on plant growth (Dijkstra & Cheng 2007). Another explanation can be that the treatments were not

different enough to show a response in the expression of these traits (Pigliucci & Kolodysnka 2002), or that plasticity was not expressed at the juvenile ontogenetic stage, as leaf traits can change over plant development, as has been previously reported for *T. velutina* (Ochoa-López et al. 2018), Eucalyptus (Goodger et al. 2007) and lima bean (Balhorn et al. 2008).

Individual traits were less plastic compared to the plasticity observed for of the whole leaf phenotype, even for those traits showing some change between treatments (Fig. 1). The only trait showing a high RDPIs value was HCN content. This result can be explained by changes in the expression of HCN through plant ontogeny. In *T. velutina*, the expression of this trait decreases at the onset of the juvenile stage (Ochoa-López et al. 2005); therefore, it is likely that the absence or presence of this trait, due to variation in the specific moment when plants stop producing this defence, could have influenced the values of RDPIs. The highest values of plasticity observed for the magnitude of phenotypic integration relative to the reaction norms of individual traits highlights the relevance of considering phenotypic integration as an intrinsic characteristic of organisms, influencing species evolvability resulting from correlated responses in functional traits (Hallgrimson et al. 2009; Wagner et al. 2007).

The plasticity of integration

As plant phenotypes changed in a coordinated way, the magnitude and pattern of phenotypic integration were significantly different between light treatments. We found low integrated phenotypes in the treatment simulating the typical conditions of plants on coastal dunes (T35), and plants with lower light availability had more integrated phenotypes. This pattern coincides with the hypothesis considering canalization as a mechanism to acquire limited resources, favouring the coordinated expression of several traits (Schlichting 1986, Gianoli & Palacio-López 2009). For example, *Pontamogeton pectinatus* plants growing in sandy substrates under nutrient-limiting conditions had higher phenotypic integration (Hangelbroek et al. 2003). In this species, biomass accumulation was positively correlated with rhizome thickness and tuber number, traits mediating

carbohydrate storage on stressful conditions (Hangelbroek et al. 2003). In the case of *Pinus sylvestris*, populations on the colder edge of the distribution showed higher trait covariation among functional traits (Benavides et al. 2021). This evidence, together with the results reported in this study, differ from previous patterns exhibited along environmental gradients in which integrated phenotypes were located at the core of the distribution, associated with large population sizes, fewer abiotic constraints, and environmental filters (Boucher et al. 2003, Damián et al., unpublished). The comparison of correlation patterns between environments provides come insights about the flexibility of phenotypic integration. Concerning to the pattern of integration, regardless of the light environment, plants showed a negative correlation among foliar N and C:N, while a positive correlation between chlorophyll and δ13C was observed. The former has been previously reported as water use efficiency trait of plants on shaded, cooler, and humid conditions (Rozendaal et al. 2006). Although this change is the general pattern of differences in the correlation structure of functional traits between environments, the patterns of correlation were highly variable among maternal families (Table S1); revealing intrapopulation ranges of variation comparable to those showed among populations of *Pinus sylvestris* across its distribution in Europe (Benavides et al. 2021).

Environmental conditions impose limits to the expression of complex phenotypes, and particular combinations of traits. For this reason, phenotypes are expected to change accordingly with environmental conditions, exploring the multivariate morphospace and displaying strategies that better fit environmental pressures. Previous studies have reported that plants face strong selection pressures when resources are limiting, which promote greater trait covariances as filters of unsuitable combinations of functional traits and functional convergence among coexisting species in ecological communities (Dwyer & Laughlin 2017, Westoby & Wright 2006). In this context, in environments with reduced light availability we found similar functional responses of all maternal families, high covariance among traits, and the occupation of a reduced area of the phenotypic space. In contrast, under average light availability, the covariances among traits were more flexible, allowing to explore a greater area in the morphospace than under reduced light availability (Fig. 4).

These results showed that within populations, multivariate patterns of phenotypic variation represent the co-existence of several functional strategies with a potential adaptive value (Bontemps et al. 2017, Damián et al. 2020), and support the hypothesis of integration of phenotypes under high selection pressures (Schlichting 1986, Gianoli & Palacio López 2009) promoted by environmental filters.

Despite the small number of maternal families and the two light environments examined, the results of this study illustrate some clear patterns regarding the plasticity of phenotypic integration. The reaction norm of integration showed more integrated phenotypes under shaded conditions than in the greater light availability. This suggests strongest correlations in the multivariate phenotype when plants face limiting resources. In addition, the results show that plasticity of the multivariate phenotype is greater than that of individual functional traits, highlighting the value of analysing complex traits versus independent traits when assessing the functional strategies in plants. Thus, future studies measuring plasticity of functional traits can provide a broader view of the range of change in this complex trait.

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LEGENDS TO FIGURES

Figure 1. Experimental plots at CICOLMA dunes, Veracruz Mexico. a) Exclosures were placed on interdune areas to avoid shading from crest dunes. b) Dimensions of exclosures and shade control shelters used in this study. c) Aspect of *T. velutina* plants transplanted in October 2017, before the exclosure installation.

Figure 2. Reaction norms of individual functional traits measured on maternal families of *T. velutina* plants growing in T35 and T50 light environments. Reaction norms for individual traits were estimated on 12 families. Significant factors after ANOVA are indicated in each graph.

Figure 3. a) Reaction norm of INT in maternal genotypes of *T. velutina*. b) Relative plasticity in response to light environment among individual functional traits and the phenotype represented by the INT magnitude of maternal families. LMA, leaf mass per area; HCN, hydrogen cyanide content, TRIC, trichome density; CHL, chlorophyll content; LNC, leaf N content, C:N, carbon to nitrogen ratio; δ13C, carbon isotope content.

Figure 4. Covariance space of leaf functional traits in *T. velutina* along the first three Principal coordinates (PCo) of the morphospace. Violet points represent covariance matrices of maternal plants that received a treatment of greater light availability (T35) while green points correspond to maternal families that were exposed to limited light availability simulating shaded environments (T50).
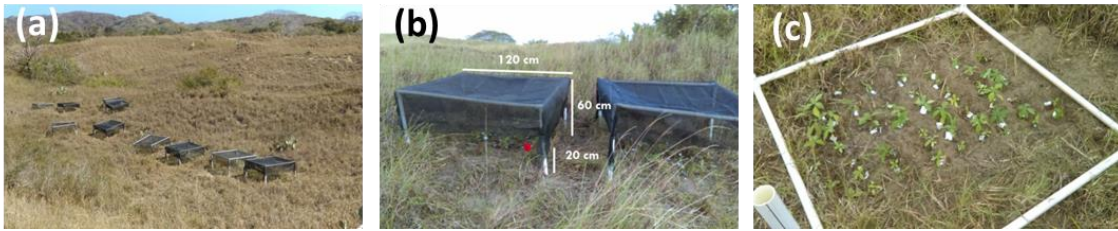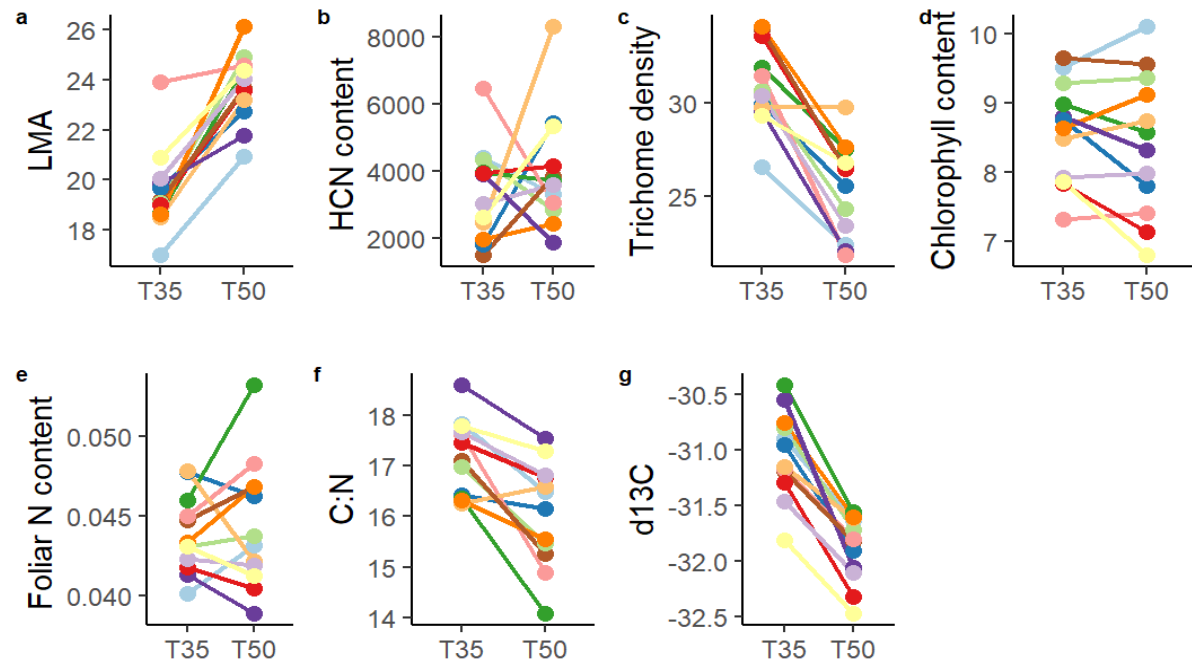
Figure 1.
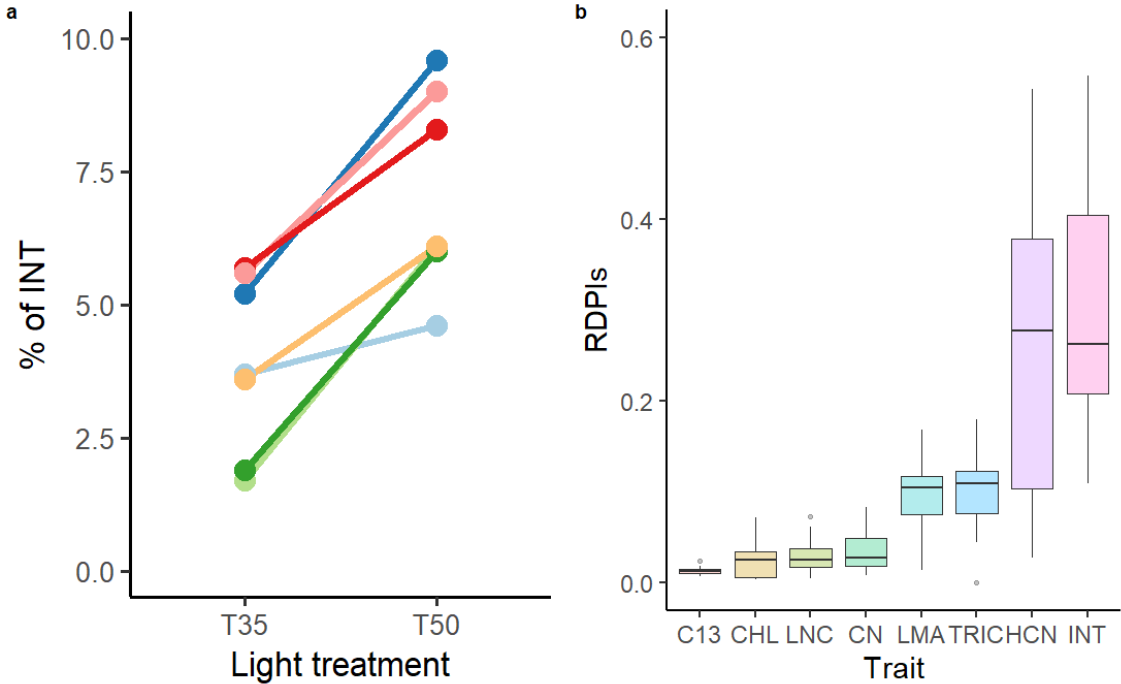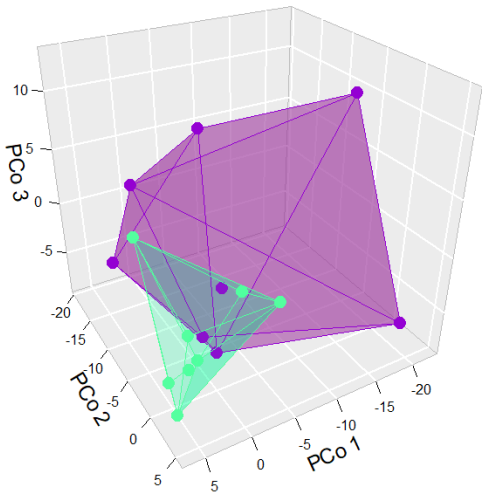

Figure 2.

Figure 3.

Figure 4.

SUPPLEMENTARY MATERIAL

Table S1. Correlation matrices among foliar traits used in the estimations of phenotypic integration magnitude in the seven *T. velutina* maternal families exposed to reduction of 35% (below the diagonal, T35 treatment) and 50% (above the diagonal, T50 treatment) of light conditions in the La Mancha population. Bold values represent significant correlations at P < 0.05. LMA, leaf mass per area; HCN, hydrogen cyanide content, TRIC, trichome density; CHL, chlorophyll content; LNC, leaf N content, C:N, carbon to nitrogen ratio; δ13C, carbon isotope content.

Correlation matrix between treatments

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		-0.124	0.422	-0.248	0.481	-0.556	0.019
HCN	0.316		0.639	-0.212	-0.108	0.192	-0.048
TRIC	0.171	-0.239		-0.141	0.228	-0.174	0.093
CHL	-0.749	-0.290	-0.146		0.093	-0.191	0.736
LNC	0.163	-0.359	0.281	-0.075		-0.947	0.556
CN	0.297	0.480	-0.449	-0.242	-0.753		-0.610
C13	-0.460	0.170	0.045	0.586	0.025	-0.224	

Family 1

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		0.143	0.082	-0.154	0.276	-0.480	0.455
HCN	0.449		0.091	0.554	0.439	-0.138	-0.306
TRIC	0.320	-0.048		-0.586	-0.150	0.479	0.159
CHL	-0.615	-0.321	-0.731		0.337	-0.350	-0.358
LNC	0.356	-0.166	0.228	-0.261		-0.197	-0.271
CN	-0.164	0.192	0.259	-0.032	-0.819		-0.413
C13	0.469	0.237	-0.148	-0.360	0.012	-0.108	

Family 2

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		0.314	-0.200	-0.293	0.117	-0.696	0.568
HCN	-0.124		0.215	-0.266	-0.030	-0.159	0.120
TRIC	-0.275	0.458		0.216	-0.113	0.436	-0.588
CHL	-0.190	0.332	-0.292		0.222	0.323	-0.655
LNC	0.208	-0.055	-0.573	0.229		-0.312	0.232
CN	-0.185	0.094	0.511	-0.396	-0.920		-0.806
C13	0.436	-0.383	0.002	-0.659	-0.508	0.653	

Family 3

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		-0.193	-0.722	-0.377	-0.480	0.360	0.672
HCN	0.307		0.202	0.640	-0.479	0.623	0.112
TRIC	0.155	0.342		0.260	0.402	-0.214	-0.481
CHL	-0.417	-0.155	-0.062		-0.084	0.162	-0.292
LNC	-0.169	0.502	-0.164	-0.252		-0.905	-0.815
CN	0.455	-0.143	0.465	0.277	-0.746		0.685
C13	0.588	0.133	0.131	-0.543	0.022	0.298	

Family 4

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		0.125	-0.098	-0.664	-0.220	-0.176	0.439
HCN	0.518		-0.273	-0.033	0.755	-0.469	0.103
TRIC	-0.340	0.263		0.326	0.061	-0.038	-0.479
CHL	0.005	0.422	0.201		0.432	0.019	-0.704
LNC	0.527	0.106	-0.141	0.076		-0.428	-0.517
CN	-0.101	0.433	0.556	-0.073	-0.687		0.106
C13	0.400	0.047	-0.337	-0.383	-0.251	0.167	

Family 5

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		-0.052	-0.355	0.208	-0.536	0.373	0.089
HCN	-0.424		-0.161	0.127	0.405	-0.396	-0.332
TRIC	-0.657	-0.039		-0.582	0.094	-0.035	0.241
CHL	0.150	0.418	-0.679		0.334	-0.188	-0.578
LNC	0.787	-0.635	-0.511	-0.226		-0.740	-0.685
CN	-0.753	0.336	0.593	-0.255	-0.629		0.640
C13	0.432	-0.024	-0.206	-0.166	0.368	0.116	

Family 6

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		-0.111	0.189	-0.418	0.261	-0.366	0.073
HCN	0.170		-0.635	0.668	0.526	-0.425	0.291
TRIC	0.107	-0.314		-0.882	-0.595	0.489	0.158
CHL	-0.269	0.237	-0.468		0.295	-0.303	-0.071
LNC	0.289	0.168	-0.287	0.506		-0.779	-0.302
CN	-0.357	-0.237	0.431	-0.151	-0.733		0.120
C13	0.359	0.059	-0.017	-0.454	0.093	-0.255	

Family 7

Trait	LMA	HCN	TRIC	CHL	LNC	CN	C13
LMA		-0.451	-0.157	-0.802	0.216	-0.067	0.030
HCN	0.088		0.263	0.458	0.141	0.203	0.138
TRIC	-0.090	-0.539		0.035	0.532	-0.470	-0.136
CHL	-0.242	0.316	-0.064		-0.046	0.023	-0.199
LNC	0.176	-0.045	-0.318	0.600		-0.669	-0.622
CN	0.223	0.261	0.509	-0.184	-0.555		0.551
C13	0.029	0.259	-0.250	-0.476	-0.574	0.110	
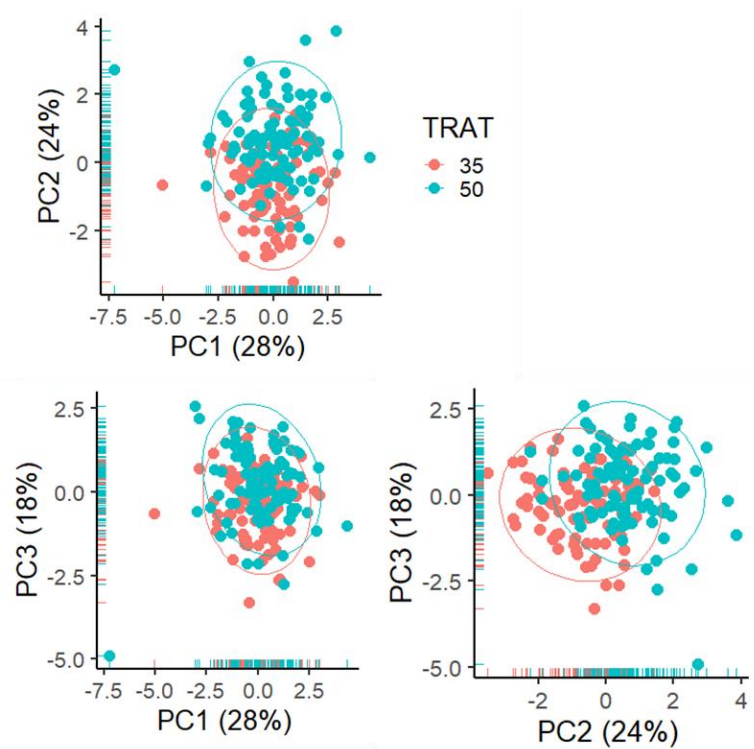
Figure S1. Results of PCA on the correlation matrix of leaf functional traits of *T. velutina* plants growing under two light treatments. T35 represents the mean conditions of plants experienced in the coastal dunes (red dots), while T50 are a shaded environment with 50% of radiation available to plants (blue dots). PC1 was considered the axis of nitrogen economy, PC2 is influenced by water economy-related traits, and PC3 was a combination of HCN and chlorophyll content.

DISCUSIÓN GENERAL

Los organismos están constituidos por la expresión integrada de múltiples rasgos, resultado de efectos ontogenéticos, genéticos, funcionales y ambientales sobre el fenotipo (Merilá y Björkland 2004). La expresión de fenotipos multivariados puede ser analizada a diferentes escalas; ontogenéticamente dentro de los individuos, entre individuos que pertenecen a la misma población, entre poblaciones de la misma especie o entre especies (Messier et al. 2018). Aunque las determinantes macroevolutivas sobre los patrones de covarianza han sido analizadas empleando el enfoque de la integración fenotípica (Ordano et al. 2008, Marroig y Cheverud 2001, Adams y Felice 2014, Goswami et al. 2014, Benítez-Vieyra et al. 2018), las causas y consecuencias de la variabilidad de la integración entre poblaciones, individuos o estadios ontogenéticos ha sido menos explorada. Usando enfoques descriptivos y experimentales, este trabajo evaluó a la integración fenotípica como un rasgo con valor adaptativo y que varía dentro y entre poblaciones. Evidencia previa describe la variación de la magnitud y el patrón de integración foliar en dos estadios ontogenéticos en el arbusto tropical *Turnera velutina* (Damián et al. 2018), donde estadios juveniles mantienen una estrategia de ganancia de recursos con fuertes correlaciones entre el contenido de nitrógeno, de carbono y la masa foliar específica. En contraste, en plantas de etapa reproductiva la estrategia se modifica y se asocia con la conservación de recursos, donde la estructura de correlaciones se modifica fuertemente alrededor de la masa foliar específica, la densidad de tricomas y el contenido de agua de las hojas (Damián et al. 2018, Anexo 1). Si la integración de las hojas muestra niveles variables dentro de los individuos, entonces es razonable analizar si esos cambios también confieren ventajas en la adecuación y cómo cambian en diferentes escenarios selectivos.

La integración fenotípica varía entre individuos de la misma población y tiene efectos positivos sobre la adecuación

El método tradicional para analizar el significado adaptativo de un rasgo consiste en determinar una relación entre la variación de dicho rasgo y alguno de los componentes de

adecuación en individuos que pertenecen a la misma población. En un modelo de selección jerárquica, el análisis de selección clásico se aplica no sólo en los rasgos individuales, sino también en grupos de rasgos o en la magnitud de la integración fenotípica (Ordano et al. 2008). Dentro de esta jerarquía de asociación de atributos, la magnitud de la integración refleja los efectos combinados de la asociación funcional, genética y/o del desarrollo de los rasgos que describen al fenotipo. En el capítulo uno dichas asociaciones se interpretaron como las estrategias funcionales desplegadas por diferentes familias maternas en la población de *T. velutina* establecida en La Mancha, Veracruz. Por un lado, se determinó que la estrategia funcional de estas plantas gira en torno al uso de agua y al uso del nitrógeno. Las familias con fuertes correlaciones entre el contenido de $\delta^{13}C$, la masa foliar específica y la densidad de tricomas, y que tienen mayores valores en la magnitud de la integración también crecen más rápido y producen más flores (Damián et al. 2020). Por otro lado, las evidencias apuntan a que además de la magnitud de la integración, también hay grupos de rasgos que confieren ventajas en los componentes de adecuación.

Trabajos previos en otros sistemas ya habían documentado el papel adaptativo de grupos de rasgos (Ordano et al. 2008, Johnson et al. 2009) e incluso la variabilidad de estrategias funcionales entre individuos de una población (Bontemps et al. 2017, Messier et al. 2018), sin embargo, no cuantificaron los efectos sobre la adecuación de la covarianza entre todos los rasgos a través de la estimación de la integración fenotípica (Lázaro y Santamaría 2016). Este capítulo mostró por primera ocasión evidencia de que los fenotipos integrados de las hojas tienen una ventaja sobre otros fenotipos donde ciertos rasgos no están fuertemente asociados entre sí. Suponemos que las ventajas de los fenotipos integrados se obtienen a través de la optimización de un grupo de rasgos que confiere mayor ventaja al reducir los costos de mantener la estrategia funcional. Por ejemplo, en *T. velutina*, los fenotipos más integrados no sólo podrían aumentar la eficiencia en el uso de agua (medido a través del contenido de $\delta^{13}C$), sino también disminuir la pérdida de agua a través de los tricomas y mantener las reservas de nitrógeno. Dichos rasgos, actuando en conjunto contribuyen a una estrategia de ganancia de recursos, incrementando la biomasa y la velocidad de crecimiento, y almacenando suficientes recursos para producir flores (Damián

et al. 2020). Por otro lado, la variación tanto en la magnitud como en el patrón de integración foliar podría mantener respuestas alternativas en la población que permitirían explorar el espacio fenotípico y resultar ventajosas en función de los escenarios selectivos a los que los individuos se enfrentan dentro de la población (Ramírez-Valiente et al. 2011, Laforest-Lapointe et al. 2014, Bontemps et al. 2017).

Este capítulo aporta la única evidencia sobre el valor adaptativo de la integración fenotípica empleando los rasgos funcionales de las hojas, y es uno de los pocos trabajos que ha intentado establecer un mecanismo funcional detrás de los cambios de la integración en órganos diferentes a las flores, sobre los que se ha enfocado gran parte del esfuerzo teórico y empírico sobre la integración fenotípica en plantas. Estos resultados resaltan la importancia de incluir el análisis de las presiones selectivas actuando en un conjunto de rasgos y no únicamente en rasgos funcionales individuales. Aunque esta no es una idea nueva para los estudios de ecología evolutiva, el enfoque de la integración fenotípica nos permite realizar estimaciones para cuantificar la intensidad de la selección en el fenotipo como un todo, incluso cuando los rasgos individuales no muestran un valor adaptativo siguiendo el método univariado tradicional.

Las respuestas funcionales de los fenotipos integrados cambian a través de un gradiente de precipitación

Al analizar la expresión de los rasgos funcionales de las plantas a lo largo de gradientes ambientales se esperan ciertos patrones de cambio asociados a disyuntivas y limitaciones energéticas (Bonser 2006, Maire et al. 2013). Sin embargo, uno o varios rasgos estudiados de forma independiente adquieren mayor relevancia si se entienden en el contexto del cambio simultáneo con otros rasgos del fenotipo (Yang et al. 2019). Entonces, analizar cómo una especie modifica la expresión de la covarianza entre sus rasgos a través de un gradiente ambiental provee información para entender cómo el contexto ambiental modula los cambios en el fenotipo multivariado (Boucher et al. 2013, Salgado-Negret et al. 2015, Benavides et al. 2021).

En el capítulo dos se analizaron los cambios en los componentes de la integración bajo el marco de la diferenciación ecotípica, partiendo de que la integración, como atributo, podría diferenciarse a través de presiones de selección divergente en poblaciones bajo diferentes condiciones ambientales (McKay y Latta 2002, Lucek et al. 2014). Dado que *T. velutina* se distribuye a lo largo de un gradiente de precipitación y que el agua es un recurso que influye en la expresión de la estrategia funcional de las plantas (Meng et al. 2015, Siepielski et al. 2017), se analizó si los patrones de integración están asociados con la canalización de la estrategia funcional para la explotación de este recurso. Evidencias previas han documentado que en ambientes áridos o semiáridos la eficiencia del uso de agua tiene fuertes correlaciones con el contenido de nitrógeno y la masa foliar específica, por lo que mayor integración de los fenotipos constituiría un mecanismo adaptativo en condiciones de baja disponibilidad de agua (Schlichting y Pigliucci 1998). Por otro lado, en poblaciones donde el agua no es un recurso limitante, se esperaba que el fenotipo respondiera ante otras presiones selectivas, resultando en un desacople de los rasgos funcionales, menor magnitud de la integración y un patrón diferente de la asociación entre rasgos.

Contrario a nuestras expectativas y, a pesar del número limitado de poblaciones muestreadas, la integración fue más alta en las poblaciones en el extremo más húmedo del gradiente. Los fenotipos con baja integración mostraron fuertes correlaciones entre rasgos con valor adaptativo bajo condiciones de limitación de agua (Dudley 1996, Donovan et al. 2007, Kooyers et al. 2014), como el contenido de cianogénicos y el contenido de nitrógeno. Por esta razón, consideramos que en el extremo más seco del gradiente, las plantas de *T. velutina* tienen un filtro ambiental más intenso que incrementa la selección en unos pocos rasgos funcionales clave para la supervivencia, especialmente en los meses más secos. Alternativamente, la baja magnitud de integración podría explicarse por medio de alta plasticidad en los rasgos individuales, lo cual disminuye la existencia de correlaciones entre los rasgos que describen al fenotipo (Gianoli y Palacio-López 2009). Una tercera posibilidad consiste en que las poblaciones situadas en el extremo más seco del gradiente tengan mayor plasticidad en la integración como rasgo, mostrando flexibilidad en la matriz de covarianza de los rasgos funcionales al enfrentarse a condiciones heterogéneas como la estacionalidad

de la precipitación, tal y como se ha reportado para los rasgos individuales (Sultan 2000). Esta alternativa abre la posibilidad de que la baja magnitud de la integración también sea un rasgo adaptativo, pregunta que aún queda por resolver.

Además de identificar el patrón positivo entre la integración y rasgos relacionados con la precipitación, los patrones de correlación entre los rasgos funcionales, especialmente los de las poblaciones en el extremo más húmedo del gradiente; confirmaron asociaciones intensas entre los rasgos que previamente se identificaron con la estrategia funcional de esta especie, LMA, δ^{13}C y contenido de nitrógeno (Damián et al. 2020). Este capítulo aporta información valiosa que contribuye a conceptualizar el espacio multivariado en el que una especie puede desplegar las estrategias funcionales mediadas por factores ambientales, y amplía la información disponible al cambio de la integración a través de gradientes ambientales que se han reportado para otras especies.

Integración fenotípica en diferentes escenarios selectivos: plasticidad de las estrategias funcionales

Después de mostrar evidencias de la variación de la magnitud y patrones de la integración dentro de una población y de cómo los factores ambientales influyen en el cambio de dichas propiedades, resulta razonable preguntarnos si la integración fenotípica, como un carácter complejo, es susceptible de cambiar bajo diferentes condiciones en una mismoa familia genética; es decir, si es un rasgo plástico. Se ha propuesto que altos niveles de integración están asociados a la optimización de un fenotipo que responde a altos niveles de estrés (Schlichting 1989, Waitt y Levin 1993) lo cual ha sido confirmando analizado el número de correlaciones significativas en matrices de covarianza en plantas de *Arabidopsis thaliana* en diferentes intensidades de viento (Pigliucci 2002). Sin embargo, en plantas de *Thellungiella salsaginea* bajo estrés salino se ha reportado el patrón opuesto (Yao et al. 2013), mientras que en *Lepidium bonariense* en tratamientos que combinan disponibilidad de luz y agua los niveles de integración no muestran cambios significativos (Mallit et al. 2010). Además de no tener un patrón general que relacione la integración y diferentes condiciones selectivas, estos trabajos no han evaluado la magnitud de la integración como un carácter en sí mismo,

sino a través de estimadores indirectos y sin considerar la variación en diferentes genotipos. Teniendo como antecedentes los resultados previos en la población de *T. velutina* en la población de La Mancha, Veracruz; en el tercer capítulo se adoptó un enfoque experimental para someter a plantas de familias maternas con niveles contrastantes de la magnitud de integración reportado en el capítulo uno (Damián et al. 2020) y exponerlas a dos tratamientos lumínicos. Con ello, se analizó la norma de reacción de la magnitud de la integración probando la hipótesis de que condiciones limitantes en un recurso producen fenotipos más integrados (Gianoli y Palacio-López 2009).

Los resultados mostraron que la magnitud de la integración foliar no sólo se incrementa en ambientes con menor disponibilidad de luz, sino que, en una comparación entre la plasticidad de la integración y la plasticidad de los rasgos funcionales individuales, la primera resultó de mayor magnitud. Adicionalmente, cuando visualizamos las regiones del morfespacio de covarianzas que ocupan las plantas en diferentes ambientes, el área de distribución de las plantas en ambientes sombreados tiene una reducción en comparación con el área de distribución en el ambiente que representa las condiciones lumínicas típicas de *T. velutina*. Tomados en conjunto, estos resultados muestran que la plasticidad desplegada en la estrategia funcional de las plantas es alta y que genera la convergencia de los patrones de covarianza en el morfoespacio funcional de las familias maternas de *T. velutina*, lo cual respalda la idea de combinaciones de rasgos optimizadas en un ambiente limitado por un recurso.

Este capítulo presenta evidencias a favor de la hipótesis que propone que los ambientes estresantes producen fenotipos más integrados (Schlichting 1986, Gianoli y Palacio-López 2009), mostrando la flexibilidad de la matriz de correlación en el despliegue de estrategias funcionales de las plantas. Nuestros resultados indican que un enfoque univariado tiene limitaciones en detectar plasticidad en los rasgos funcionales foliares de forma independiente y, sorprendentemente, incluso evaluando la plasticidad relativa de los rasgos que muestran plasticidad entre ambientes, la plasticidad en un grupo de rasgos fue mayor. Sin embargo, el valor adaptativo de la plasticidad desplegada en la magnitud de la integración aún queda por determinar.

Conclusiones generales

Los resultados de este trabajo demuestran que el estudio de la integración fenotípica nos permite incluir una perspectiva multivariada a los estudios de ecología evolutiva. En este sentido, no se trata sólo de incluir más rasgos en los estudios; sino de analizar sus patrones de asociación, de interpretar los mecanismos subyacentes a las asociaciones entre rasgos y determinar si estos patrones resultan en ventajas para la supervivencia y reproducción de los organismos.

Aunque este enfoque no es nuevo, sí lo es el considerar de forma explícita que la estructura de covarianza es variable entre los genotipos que componen una población y entre poblaciones de la misma especie donde cada nivel de organización nos permite obtener información sobre diferentes fenómenos: la ontogenia generando diferencias en las estrategias funcionales asociadas a los requerimientos de cada estado de desarrollo, la selección natural optimizando una estructura de covarianza en una población, o los factores ambientales influyendo en la flexibilidad de las estrategias funcionales de acuerdo a los ambientes selectivos.

Parafraseando a Aristóteles, los organismos son más que la suma de sus rasgos individuales.

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Anexo 1. Ontogenetic changes in the phenotypic integration and modularity of leaf functional traits

RESEARCH ARTICLE

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Ontogenetic changes in the phenotypic integration and modularity of leaf functional traits

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Abstract

1. Changes in resource availability, functional demands, hormonal regulation and developmental constraints can promote differences in the expression of leaf traits during plant development and foster changes in the targets of natural selection. As a consequence, the pattern and magnitude of covariation among traits, and therefore their phenotypic integration and modularity are equally expected to change throughout ontogeny. However, these changes have not been described yet.

2. We measured leaf economic, defensive and morphological traits in plants of *Turnera velutina* and estimated the magnitude and pattern of foliar integration and modularity for juvenile and reproductive individuals. In addition, we assessed the relationship between plant biomass and foliar integration within and among ontogenetic stages.

3. Both the pattern and magnitude of foliar integration changed across plant ontogeny. Foliar integration was lower in juvenile than in reproductive plants, and the pattern of phenotypic integration and modularity was different between ontogenetic stages, whereas leaves from juvenile plants showed two functional modules related to plant defence and leaf economy, traits from reproductive plants had greater interconnectivity and hence lower modularity.

4. The relationship between plant biomass and foliar integration was negative within each ontogenetic stage but positive between ontogenetic stages, suggesting that processes intrinsic to plant development influenced the magnitude of foliar integration to a greater extent than plant size.

5. Our findings indicate that plants can change the patterns of covariation among leaf traits during their development. However, a lower foliar integration in juvenile plants could allow for greater lability to explore a multi-trait phenotypic space, canalisation of leaf attributes along ontogeny should promote greater phenotypic integration, constraining the number of multi-trait combinations that plants can express. Hence, we suggest that ontogenetic changes in foliar integration allow plants to deal with changing selective dynamics and physiological priorities along their development.

KEYWORDS
defence, functional modules, leaf economy, leaves, modularity, ontogeny, phenotypic integration, *Turnera velutina*

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1 | INTRODUCTION

The concepts of phenotypic integration and modularity are quite valuable to study the non-independent expression and evolution of multiple traits. Phenotypic integration is defined as the correlation structure of multiple characters (Klingenberg, 2009; Schlichting & Pigliucci, 1998; Zelditch, 1988), and can be estimated as the pattern and magnitude of correlations among traits (Herrera et al., 2002). Modularity, in turn, identifies sets of tightly correlated traits (usually identified as part of a functional module) and relatively independent from other traits or modules (Klingenberg, 2009; Murren, 2012; Wagner, Pavlicev, & Cheverud, 2007). Although the evolution of phenotypic integration and modularity has been described for flowers (Fornoni, Ordano, Pérez-Ishiwara, Boege, & Domínguez, 2016; Herrera et al., 2002; Ordano, Fornoni, Boege, & Domínguez, 2008), little attention has been paid to the concerted evolution of functional traits comprising the prime structural and metabolic unit of plants, the leaf. One of the main functions of leaves is carbon assimilation into photosynthates, which in turn allow plant growth, but are also the main resource for animals, fungi and bacteria feeding on plants. Hence, leaves must also defend against their consumers. Because the priority and trade-offs between these functions can change during plant ontogeny (Mason & Donovan, 2014), the expression of carbon assimilation and defensive traits often varies as plants develop, which could result in ontogenetic changes in both foliar phenotypic integration and modularity (Mason & Donovan, 2014). These changes, however, have been not described yet.

General patterns of variation in the simultaneous expression of leaf economic traits have been described at both interspecific (Baraloto et al., 2010; Carlson & Holsinger, 2012; Dunbar-Co, Sporck, & Sack, 2009; Forrestel, Ackerly, & Emery, 2015; Pérez-Ramos et al., 2012; Wright et al., 2004) and intraspecific (Brouillette, Mason, Shrik, & Donovan, 2014; Grady et al., 2013) levels. However, the expression of leaf-economic traits can also vary across whole-plant ontogeny. For example, leaf area, stomatal size, conductance and photosynthetic rate have been found to increase during plant development in different *Protea* species (Carlson & Holsinger, 2012). Similarly, lamina thickness, leaf mass per area and phosphorous concentration have been reported to increase in leaves during plant development of nine *Inga* species in Costa Rica (Palow, Nolting, & Kitajima, 2012); and photosynthetic rate, nitrogen content, leaf mass per area, water content, pH and vein density have been reported to vary across whole-plant ontogeny in three *Helianthus* species (Mason & Donovan, 2014; Mason, McGaughey, & Donovan, 2013). Ontogenetic changes in laminar area:perimeter ratio, specific leaf area, percent of parenchyma and stomatal length and density have been also reported in different habitats, as is the case for *Lasthenia* species associated with terrestrial or vernal pool habitats (Forrestel et al., 2015). Because the expression of multiple plant defences can also vary throughout plant ontogeny (see Barton, 2014; Ochoa-López, Villamil, Zedillo-Avelleyra, & Boege, 2015; Quintero, Lampert, & Bowers, 2014), changes in trade-offs and correlations between

leaf economic and defensive traits during plant development are expected (Arntz & Delph, 2001). For example, Mason and Donovan (2014) demonstrated that ontogenetic changes in leaf economic traits are conditioned by resource trade-offs with plant defence in three species of *Helianthus*.

Overall, natural selection is expected to optimise the most efficient combination of leaf traits allowed by trade-offs (Pearse, 2011), developmental constraints (Arnold, 1992) and environmental filters (Palow et al., 2012) acting on functional traits at each ontogenetic stage (Mason et al., 2013; Palow et al., 2012). We first predicted that two functional modules should be distinguished in leaves, one related to the joint expression of carbon assimilation related traits (i.e. leaf economy module), and one associated with defensive traits (i.e. defensive module). Second, we expected that young plants, with high growth demands, should invest more resources in the leaf economy than in the defensive module, as do fast-growing species at one extreme of the interspecific leaf economic spectrum (i.e. with high concentration of nutrients, high photosynthetic rates, short life span and low dry mass investment per unit of area; Wright et al., 2004). In contrast, leaves from reproductive individuals should express lower values in the leaf economic module, and a greater investment in the defensive module, as do slow-growing species at the other leaf economic spectrum extreme (i.e. with long leaf lifetimes, high leaf mass area, low nutrient concentrations and low rates of photosynthesis and respiration; Mason et al., 2013; Mason & Donovan, 2014; Stamp, 2003; Wright et al., 2004). According to these expectations, Mason and Donovan (2014) reported greater resource-acquisition strategies and reduced defensive attributes for young than for older plants of three *Helianthus* species. An unexplored question, however, is if the correlation patterns among functional leaf traits can also change throughout plant ontogeny.

Because size influences plant vigour (Evans, 1972; Price, 1991; Thomas & Winner, 2002), architecture, and sectoriality (Marquis, 1996; Poorter, Niinemets, Poorter, Wright, & Villar, 2009; Watson & Casper, 1984), variation in leaf economic and defensive traits, their correlations, and consequently their phenotypic integration could be a by-product of increases in plant biomass across plant development. For morphological traits, the concepts of static and ontogenetic allometries have been useful to understand the patterns of variation and covariation in traits within or among ontogenetic stages, respectively (Klingenberg & Zimmermann, 1992). Borrowing these concepts could be useful to disentangle the effects of plant size from the influence of plant development on the simultaneous expression of leaf functional traits and their phenotypic integration.

Accordingly, the aim of this study was to (1) assess if the expression of leaf traits in juvenile and reproductive plants can be explained by the leaf economics spectrum predictions, (2) describe ontogenetic changes in the magnitude and pattern of foliar integration, (3) identify the presence of functional modules associated with leaf economic and defensive functions and, (4) evaluate the influence of plant biomass on foliar integration within and between ontogenetic stages.

2 | MATERIALS AND METHODS

2.1 | Study site and study system

This study was conducted at the Centro de Investigaciones Costeras La Mancha (CICOLMA) located in Veracruz, Mexico (19°35′ N, 96°22′ W, <100 m a.s.l.). Climate in this area is warm and sub-humid with a mean annual precipitation of 1,286 mm, which occurs mostly between June and September. Annual average temperature ranges between 24 and 26°C (Moreno-Casasola, van der Maarel, Castillo, Huesca, & Pisanty, 1982). *Turnera velutina* Presl. (Passifloraceae, Figure 1) is an endemic Mexican shrub that grows in tropical dry forests and in sand-dune vegetation under the canopy of trees. Flowering occurs mostly during the rainy season (Torres-Hernández, Rico-Gray, Castillo-Guevara, & Vergara, 2000) and seeds are dispersed by ants (Cuautle, Rico-Gray, & Díaz-Castelazo, 2005). Leaves display defensive characters such as trichomes, toughness and extrafloral nectaries (EFNs) located near the petiole (Cuautle & Rico-Gray, 2003; Ochoa-López et al., 2015). Secreted nectar in EFNs is harvested by several patrolling ant species, consequently reducing foliar damage caused by the most frequent herbivore, *Euptoieta hegesia* Cramer (Lepidoptera: Nymphalidae; Cuautle & Rico-Gray, 2003). Previous studies have demonstrated that juvenile plants have higher foliar damage than reproductive plants, the latter with a higher expression of mechanical (trichomes, toughness) and biotic (extrafloral nectar and number of EFNs) defences (Ochoa-López et al., 2015; Villamil, Márquez-Guzmán, & Boege, 2013). Because ontogenetic changes in plant defence can drive changes in leaf economic traits (Mason & Donovan, 2014), *T. velutina* was considered a suitable system to investigate changes in phenotypic integration of multiple foliar functional traits.

Plants of 24 genetic families of *T. velutina* were self-pollinated to produce full sib seeds within a shade house, *c.* 1 km from a natural population of *T. velutina* in the forest edges close to an established coastal dune. Canopy cover where *T. velutina* plants grow ranges from 22% to 59%. Hence, we used a black mesh to reduce direct sunlight 35%, as a mid-point value of the conditions observed in the field. Two batches of seedlings were generated at two different times: in August 2011 we grew 168 plants (*N* = 7 plants per genetic family) for 4 months until they reached their reproductive stage (after blooming, with 18–20 fully expanded leaves). In September 2011 we grew the same number of plants during 2 months until they reached their juvenile stage (plants with six fully expanded leaves). Temperature and precipitation was quite stable between August and September (http://smn.cna.gob.mx/es/informacion-climatologica-ver-estado?estado=ver), hence, growing conditions were similar for both batches. This protocol allowed simultaneous measurements of leaf traits in juvenile and reproductive plants in November 2011 (Figure 1), reducing variation associated with differences in environmental conditions when measures are taken at different moments (Mason et al., 2013). In addition, this experimental design allowed independent assessments of the relationship between plant biomass and foliar integration at two different plant ontogenetic stages (i.e. batches were analysed separately and were not considered "temporal blocks"). For both batches, seeds were sown in germination trays after manually removing their elaiosomes. Once cotyledons were visible, seven plants per genetic family and batch were transplanted into individual 2L pots with a mix of soil, sand and vermiculite. In November 2011, once plants reached their corresponding ontogenetic stage, six leaf economic and five defensive traits were measured in the four most apical and fully expanded leaves of each plant (Figure 1). In juvenile plants these corresponded to leaves in nodes 3, 4, 5, and 6, while in reproductive plants corresponded to the four most apical, fully expanded leaves bearing reproductive structures (floral buds, flowers, or fruits). Variation associated with leaf development was controlled by only choosing fully expanded leaves. Plants were not exposed to natural enemies or mutualists, as they were inside the shade house.

2.2 | Selection of leaf traits

We chose leaf traits known to affect the interaction between plants and herbivores, as well as ecophysiological and morphological characters related to carbon assimilation and biomass production (i.e. leaf economics). Nitrogen content is directly associated with photosynthetic proteins (Field & Money, 1986), but also with leaf palatability (Kursar & Coley, 1991); carbon content is related to biomass gain (Lambers, 2008). Chlorophyll content was selected as a proxy of photosynthetic capacity (Jifon, Sylversten, & Whaley, 2005). Morphological traits are often related to the physiological performance of leaves, hence we choose petiole length because it influences light harvesting and mechanical support (Niinemets, Al Afas, Cescatti, Pellis, & Ceulemans, 2004). Dissection index, an estimate of leaf shape and serration (see below), was measured because its association with photosynthetic capacity and convective heat exchange (Lynn & Waldren, 2001; Schuepp, 1993). Leaf mass per area (LMA) is a measure of the cost of light interception per leaf tissue produced (Gutschick & Wiegel, 1988) and is a good estimator of leaf toughness
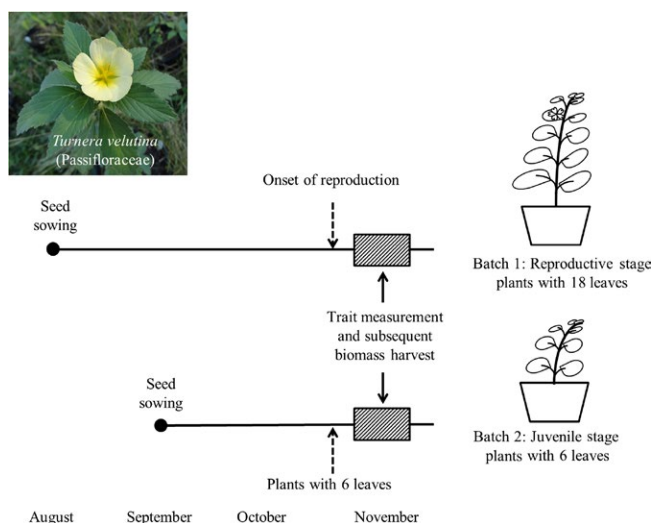

FIGURE 1 Timeline for the production of two batches of *Turnera velutina* plants at two ontogenetic stages (juvenile and reproductive). Indications of when plants were measured and harvested are provided by the arrows

influencing herbivore preference (Clissold, Sanson, Read, & Simpson, 2009). We included trichome density given its role as a physical barrier against herbivores. We also quantified the number of extrafloral nectaries and sugar content in extrafloral nectar, as a measure of the quantity and quality of rewards for ants (Villamil et al., 2013). Finally, water content is the inverse of leaf dry mass content, frequently used in ecophysiological studies (Shipley, Lechowicz, Wright, & Reich, 2006) but also associated to plant palatability for insects (Read & Sanson, 2003).

2.3 | Leaf economic and morphological traits

For each selected leaf, we measured chlorophyll content index (CCI), using a CCM-200 chlorophyll content meter (Opti-Sciences, Tyngsboro, MA, USA), while the leaf was still attached to the plant. CCI was converted to total chlorophyll content after a calibration curve, using the N, N-dimethlyformamide chlorophyll extraction procedure (Porra, Thompson, & Kriedemann, 1989) and using the conversion equation: μg chlorophyll cm^{-2} = −0.093 + 1.36 × √(ICC units); R^2 = 0.79, p < .001). After CCI readings, between 1300 and 1400 hr leaves were collected and stored in sealed plastic bags to avoid dehydration. Using an analytical scale (Acculab VIC 303, Sartorius Group, MA, USA), fresh mass was measured within the first 2 hr after leaves were cut to avoid variance due to moisture loss. Right after leaf collection, we also measured petiole length with a digital calliper (Mitutoyo, resolution 0.01 mm) and foliar area and perimeter using a digital portable scanner (CI-202 CID Inc, Cama, Washington, USA). The latter measurements were used to calculate the dissection index (d) as d = leaf perimeter/[2√leaf area × π] (Lynn & Waldren, 2001). Leaves were then stored in glassine paper bags and air-dried at room temperature until no changes in mass were recorded to quantify dry mass. The accuracy of this procedure was further verified by comparing an independent set of air-dried and oven dried leaves, which were found to have the same final weight (χ^2 = 0.76; df = 45; p = .38). Carbon (C) and nitrogen (N) concentrations were assessed by microcombustion with a Perkin Elmer 2400 Series II CHNS/O Elemental Analyser (Waltham, MA, USA). We used concentration values (grams of N or C per 100 g of dry leaf tissue) to avoid autocorrelations with total plant biomass.

2.4 | Defensive traits

Before leaf collection and during the peak of extrafloral nectar secretion (between 1100 and 1300 hr, Villamil et al., 2013), we quantified nectar produced by extrafloral nectaries of each leaf using 1 μl microcapillary pipettes. Volume (V) was estimated from the height of the nectar column and sugar concentration (C) was determined with a hand-held refractometer (0–50° Brix, Leica Buffalo, New York, USA). We calculated the total amount of sugar (S) in EFN (μg/μl) as S = (V × C)/100). In addition, we counted the number of EFNs in each leaf. Trichome density was measured on each side of all collected leaves in four 14 mm^2 fields, using a stereoscopic microscope (Carl Zeiss Stemi SV6). We used the average trichome density of all eight fields for data analysis. Water content was calculated as the

difference between fresh and dry weight expressed as a percentage. Leaf toughness was estimated through LMA, which was calculated as the dry mass divided by leaf area.

2.5 | Plant biomass

After all leaf traits were measured in plants of both ontogenetic stages, we harvested each plant, including leaves, stems and roots (Figure 1). Fresh material was stored in paper bags and air-dried until no changes in mass were recorded using an analytical scale (Acculab VIC 303).

2.6 | Statistical analyses

2.6.1 | Ontogenetic differences among traits

Differences in the expression of leaf traits between ontogenetic stages were assessed using a multivariate analysis of variance with data for 168 juvenile and 164 reproductive plants (four plants died before they bloomed), after verifying that assumptions of normality and homogeneity of variances were met. Water and chlorophyll content was log transformed to meet these assumptions. Mean values of all measured traits were calculated for each plant and included as response variables whereas genetic family and ontogenetic stage were considered explanatory variables in the model. Subsequent post hoc univariate ANOVAs were used to determine ontogenetic differences and genotypic variation for each foliar trait, adjusting p-values with a Bonferroni correction to account for Type I errors.

2.6.2 | Differences in foliar integration and modularity between ontogenetic stages

We used three complementary matrix-based approaches to describe and compare three properties of the genetic variance–covariance matrices (**G**-matrices) of leaf traits: the magnitude and pattern of their association and the degree of modularity. First, to estimate and compare the magnitude of phenotypic integration between ontogenetic stages we applied an ordination method based on principal component analysis (PCA) (Ordano et al., 2008; Phillips & Arnold, 1999; Steppan, Phillips, & Houle, 2002). PCA transforms a set of correlated characters into a new set of orthogonal uncorrelated variables known as principal components. Results from a PCA can be graphically visualised as an ellipsoid in a multivariate space (Manly, 1994). The length of each of the major orthogonal axis (principal components) of the ellipsoid indicates the amount of variance (i.e. eigenvalue) that is explained by the additive combination of multiple traits. PCA transforms a matrix of correlated traits (variance–covariance matrices), providing information about the magnitude and pattern of their association (i.e. integration; Manly, 1994). In particular, the variance of the eigenvalues (Var [λ_i]) of a matrix is a measure of the magnitude of phenotypic integration (hereafter INT) (Cheverud, Wagner, & Dow, 1989; Pavlicev, Cheverud, & Wagner, 2009; Wagner, 1984). High variance among eigenvalues indicates that most of the variation is organised along few

major axes in the multivariate space, reflecting high mean correlations within a matrix. In contrast, low variance indicates that the variation is evenly distributed among most axes of the ellipsoid indicating low mean correlations within the matrix. Armbruster, Pélabon, Bolstad, and Hansen (2014) stated that the variance of the eigenvalues is appropriate as an estimator of phenotypic integration when connected to a priori functional hypotheses. In our case, we hypothesised that the leaf constitutes a functional unit, in which the expression of both defence and leaf-economy traits is shaped by natural selection to optimise physiological priorities at each ontogenetic stage. In addition, because this index is commonly used to report floral integration (Fornoni et al., 2016; Herrera et al., 2002; Ordano et al., 2008), it allowed the comparison between foliar and floral integration within *T. velutina* and relative to other species.

To obtain one genetic variance–covariance matrix (**G**-matrix) for each stage, we first calculated mean leaf trait values for each individual plant and used them to obtain mean values for each genetic family (seven full-sibs). Genotypic means for each leaf trait were used to construct a square matrix at each ontogenetic stage composed of genetic variances and covariances. To assess significance of foliar integration, we used 95% confidence intervals for INT calculated with a jackknife resampling procedure in the PHENIX package in R (Torices & Muñoz-Pajares, 2015). Because the number of plants/genotype was between 6 and 7, integration values were corrected using the formula INT = Var [λ_i] – [(number of traits – 1)/(number of individuals/genotype)] (Wagner, 1984). We considered significant foliar integration if the 95% confidence interval did not include 0 (Torices & Méndez, 2014). Non-overlapping confidence intervals between ontogenetic stages indicated significant differences in their foliar integration. Because INT is sensitive to the number of traits used in the matrix it is recommended to transform INT values into the percentage of the maximum possible value of INT, considering that the maximum value equals the number of traits (Herrera et al., 2002). Hence, in the case of this study %INT = (INT/11) × 100.

A second approach to analyse the **G**-matrices allowed us to describe the ontogenetic differences in the pattern of covariation among foliar traits. With this purpose, we used the common principal components analysis (CPCA), also known as the Flury hierarchical analysis (Phillips & Arnold, 1999). This test is an extension of PCA that performs a hierarchical comparison of differences among matrices in terms of their size, shape and orientation using their eigenvectors and eigenvalues. Besides testing whether two or more matrices differ or not, which most alternative methods do, CPCA can also test for differences in several descriptors of the internal structure of the matrices. For this reason, this approximation has been preferentially applied during the last decade (Charmantier, Garant, & Kruuk, 2014; Phillips & McGuigan, 2006; Preston & Pigliucci, 2004). The series of analyses of CPCA start by testing the hypothesis that **G**-matrices are equal (identical size, shape and orientation, indicated by equal eigenvectors and eigenvalues). The second hypothesis tests for proportionality (similar shape and orientation, but different proportional size; when matrices share eigenvectors, but differ in eigenvalues by a proportional constant). If **G**-matrices are not proportional, then the procedure

evaluates if they have all components in common (same shape and orientation but non-proportional differences in size, interpreted from equal eigenvectors but different eigenvalues). Finally, if the **G**-matrices have dissimilar eigenvector and eigenvalues, they are considered to have a completely unrelated structure (differences in size, shape and orientation). Hence, this approach allowed to test whether **G**-matrices of foliar traits in juvenile and reproductive plants had the same size, shape, and/or orientation, or did not share any of these attributes. The best model was chosen using the Akaike Information Criteria (AIC) following the "jump up" approach as suggested by Phillips and Arnold (1999). All CPCA were performed with the CPC software available at http://pages.uoregon.edu/pphil/software.html.

The third approach was applied to describe modularity of **G**-matrices at each ontogenetic stage. Based on our selection of foliar traits, we a priori defined two functional modules within our matrix: one related to defensive functions (leaf toughness, trichome density, water content, EFN and sugar in extrafloral nectar), and another related to carbon assimilation functions (carbon, nitrogen and chlorophyll contents, dissection index, foliar area and petiole length). Using the RV coefficient as a scalar measure of the strength in the association between modules within matrices (Klingenberg, 2009), we tested the delimitation of these two modules for each ontogenetic stage. RV coefficient represents a ratio between the magnitude of covariation between modules and the magnitude of the covariation within modules. Hence, values of RV coefficient are proportional to the interdependence between sets of traits (i.e. values close to 0 indicate complete independence and denote greater modularity than values closer to 1, which correspond to total interdependence between sets of traits; Klingenberg & Marugán-Lobón, 2013). This coefficient was calculated as

$$RV = \frac{trace(S_{ij}S_{ji})}{\sqrt{trace(S_iS_i)trace(S_jS_j)}}$$

where S_i and S_j represent the variance–covariance matrices of the i and j sets of traits, S_{ij} is the covariance matrix between these two set of traits, and S_{ji} represents its transpose. The trace of the matrices is calculated as the sum of the diagonal elements. The significance of RV coefficients was established using a permutation test, specifically testing the null hypothesis that subsets of traits are completely independent (Klingenberg, 2009), hence denoting a high modularity. RV coefficient and its significance were calculated for each ontogenetic stage using the FactoMineR package in R (R Development Core Team, 2012).

2.7 | Relationship between plant biomass and phenotypic integration

To discard the influence of plant size in the ontogenetic differences in foliar integration, we assessed the relationship between plant biomass and foliar integration (%INT) within each ontogenetic stage. Similar patterns within and between ontogenetic stages would reveal the influence of size in foliar integration (i.e. ontogenetic drift, sensu Evans, 1972). Opposite patterns would indicate the influence of

ontogeny-related factors, different from plant size, in the expression of foliar integration (see Klingenberg & Zimmermann, 1992).

Using a jackknife procedure, the magnitude of foliar integration was estimated at the genotypic level, because we had insufficient degrees of freedom to calculate INT values for each individual plant. To produce one INT value for each genotype, we used the four leaves from the seven plants of each genotype (up to 28 leaves in total). However, to account for the non-independence of leaves belonging to the same plant, foliar integration was calculated seven times per genotype, each time excluding all leaves from one particular individual. Because the final number of leaves/genotype was variable for each run (N = 16–24), integration values were corrected, using the formula INT = Var [λ_i] − [(number of traits − 1)/number of leaves)] (Wagner, 1984), and foliar integration was expressed as %INT. We then used a series of regression models with replicates (regressions with more than one y value for each x value; Sokal & Rohlf, 1995) to assess the relationship between the average biomass per genotype (x variable) and the multiple jackknifed INT values/genotype (y variables). This method avoids overestimation of degrees of freedom due to multiple values of INT/genotype. All statistical analyses were performed using R software (R Development Core Team, 2012).

TABLE 1 Phenotypic values ($M \pm SE$ [coefficient of variation]) and results from two-way ANOVAs of foliar traits in *Turnera velutina* at two ontogenetic stages. Bold values are statistically significant at *p < .002 after Bonferroni adjustment

Trait	Juvenile	Reproductive	Source	F
Defence traits				
Trichome density	2.05 ± 0.05	13.54 ± 0.55	Ontogeny (O)	**896.285***
(number per mm^2)	(12.46)	(20.75)	Genotype (G)	**2.163***
			O × G	2.026
Water content	88.13 ± 0.11	78.71 ± 0.23	Ontogeny (O)	**1571.291***
(%)	(0.62)	(1.57)	Genotype (G)	1.184
			O × G	1.183
EFNs	1.88 ± 0.04	2.64 ± 0.08	Ontogeny (O)	**262.351***
(number per leaf)	(10.21)	(15.63)	Genotype (G)	**3.876***
			O × G	**3.556***
Sugar content	0.001 ± 0.0002	0.04 ± 0.01	Ontogeny (O)	**96.793***
(µg/µl)	(184.62)	(67.46)	Genotype (G)	1.977
			O × G	2.023
LMA	12.80 ± 0.21	34.40 ± 1.11	Ontogeny (O)	**322.842***
(g/m^2)	(7.97)	(15.50)	Genotype (G)	0.873
			O × G	0.855
Leaf economy traits				
Carbon content	37.59 ± 0.23	40.55 ± 0.15	Ontogeny (O)	**200.311***
(g/g dry mass)	(2.68)	(1.99)	Genotype (G)	2.141
			O × G	1.068
Nitrogen content	4.90 ± 0.05	3.88 ± 0.08	Ontogeny (O)	**210.590***
(g/g dry mass)	(4.93)	(10.30)	Genotype (G)	**2.663***
			O × G	1.200
Chlorophyll content	3.28 ± 0.05	6.51 ± 0.09	Ontogeny (O)	**3,227.148***
(µg/cm^2)	(7.06)	(7.15)	Genotype (G)	**3.145***
			O × G	**3.038***
Dissection index	5.19 ± 0.02	4.96 ± 0.03	Ontogeny (O)	**33.870***
	(2.49)	(3.20)	Genotype (G)	1.197
			O × G	1.186
Foliar area	9.58 ± 0.23	24.93 ± 0.84	Ontogeny (O)	**806.857***
(cm^2)	(11.79)	(16.42)	Genotype (G)	**2.501***
			O × G	**2.600***
Petiole length	5.22 ± 0.09	7.79 ± 0.19	Ontogeny (O)	**297.905***
(mm)	(8.22)	(11.59)	Genotype (G)	**2.407***
			O × G	1.575

3 | RESULTS

3.1 | Ontogenetic changes in foliar traits

We found significant ontogenetic (Wilks' $\lambda = 0.02$, $F_{1,11} = 1227.22$, $p < .0001$) and genetic (Wilks' $\lambda = 0.15$, $F_{23,253} = 2.23$, $p < .0001$) differences in the expression of all leaf traits. Leaves from juvenile plants produced EFNs, but only 7% secreted extrafloral nectar. As a consequence, average production of sugar in EFNs in juvenile plants was only 2.5% of that observed in reproductive plants (Table 1). Leaves from reproductive plants were 2.6 times larger, had longer petioles and were less serrated than leaves from juvenile plants. In addition, reproductive plants had leaves with 10% more carbon, double chlorophyll content, and 6.6 times greater trichome density than leaves of juvenile plants (Table 1). Leaves of young plants, in contrast, had 11% and 25% more water and nitrogen content than leaves of reproductive plants, respectively. Significant genetic variation was found for most traits (Table 1).

The magnitude and sign of genotypic correlations among foliar traits were different between ontogenetic stages. In particular, we found positive correlations among leaf economic traits at the juvenile stage, and negative correlations between such traits and defensive attributes at the reproductive stage (Table 2).

3.2 | Ontogenetic changes in the magnitude and pattern of phenotypic integration

Foliar integration was significantly lower in juvenile (INT = 2.12, 95% CI = 1.79–3.12) than in reproductive plants (INT = 5.89, 95% CI = 4.76–6.67, Figure 2). Moreover, Flury hierarchical analysis revealed that genotypic variance–covariance matrices of both ontogenetic stages were unrelated, as they did not share any principal component (see Appendix S1).

The principal component analysis on the **G**-matrix for each ontogenetic stage revealed that the major axis of genetic variation (PC1)
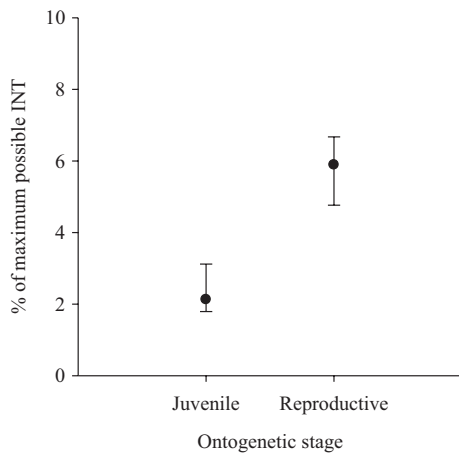

FIGURE 2 Percentage of the maximum possible integration of foliar integration and 95% confidence intervals (error bars) estimated from genotypic variance–covariance matrices of foliar traits for juvenile and reproductive stages of *Turnera velutina*

TABLE 2 Pairwise genetic correlations between foliar traits of *Turnera velutina* in juvenile (values below the diagonal) and reproductive (values above the diagonal) plants. Bold values highlight significant correlations ($p < .05$) among traits in the defence (light grey) and economy (dark gray) a priori expected modules

	Defence				Leaf economy							
Trait	Trichome density	Water content	EFNs	Sugar content	LMA	Carbon content	Nitrogen content	Chlorophyll content	Dissection index	Foliar area	Petiole length
Trichome density		**−0.52**	−0.08	−0.12	0.24	−0.44	−0.56	−0.28	−0.39	−0.66	−0.42
Water content	−0.32		0.08	0.37	−0.45	0.49	0.69	0.15	0.00	0.73	0.70
EFNs	−0.08	0.03		−0.09	−0.05	0.19	−0.07	−0.34	0.02	0.11	0.18
Sugar content	−0.12	−0.17	0.15		0.57	0.29	0.14	0.02	0.02	0.41	0.34
LMA	0.11	−0.32	0.17	−0.29		−0.16	−0.17	−0.13	−0.40	−0.17	−0.30
Carbon content	0.05	0.07	0.06	−0.17	−0.18		0.64	0.29	0.30	0.33	0.30
Nitrogen content	−0.31	0.12	0.25	−0.29	**−0.41**	0.72		**0.64**	0.03	**0.42**	0.40
Chlorophyll content	0.23	−0.30	0.35	0.23	0.30	−0.07	0.24		−0.10	−0.01	0.02
Dissection index	−0.08	0.30	0.34	0.12	0.19	−0.13	−0.36	−0.30		0.03	0.06
Foliar area	0.32	−0.15	0.26	0.37	0.41	0.05	0.15	0.76	−0.24		0.75
Petiole length	0.16	−0.09	0.30	0.45	0.50	−0.47	**−0.64**	0.08	**0.50**	0.28	

explained 29% and 37% of the multiple-trait variation in juvenile and reproductive plants, respectively, and was influenced by defensive and leaf economic traits at both ontogenetic stages (Table 3). Nevertheless, in the case of juvenile plants, PC2 was influenced mostly by leaf economic traits (nitrogen and chlorophyll content, dissection index and foliar area), and PC3 by defensive traits (trichome density, water content and sugar concentration in EFN; Table 3). Hence, the multivariate phenotypic space suggested the distinction of two functional modules (i.e. sets of correlated traits sharing a common function) in juvenile but not in reproductive plants. RV coefficients corroborated this conclusion. The RV coefficient for juvenile plants was lower and non-significant (RV = 0.226, p = .178), indicating a complete independence of both sets of traits (i.e. high modularity). This modularity was mainly influenced by the significant correlations observed in the leaf economy module, as defensive traits were not significantly correlated (Table 2). In contrast, an almost two-fold value RV coefficient was found to be significant for reproductive plants (RV = 0.412, p = .0002), indicating lower modularity and greater co-variation among all leaf traits at the reproductive stage as observed in the pairwise correlations (Table 2).

3.3 | Relationship between phenotypic integration and plant biomass

We found significant negative relationships between plant biomass and foliar integration within both juvenile and reproductive stages (significant effect of linear regressions, Table 4, Figure 3). Interestingly, the opposite trend was observed between ontogenetic stages (Figure 3), as juvenile plants (small) had lower foliar integration than reproductive plants (large).

4 | DISCUSSION

The main contribution of this study is the finding that both foliar integration and modularity changed across plant ontogeny, and that ontogenetic changes in foliar integration were beyond the effects of plant size on phenotypic integration. Differences in the optimisation of leaf phenotypes as a function of plant age and size could be a consequence of ontogenetic variation in resource acquisition abilities, sectoriality, different selective pressures, physiological priorities, hormonal regulation and/or developmental constraints on the expression of foliar traits (Schlichting, 1989).

Leaf investment strategies related to plant growth have been generalised along a single spectrum, with trait correlations persistent across the globe and among species (Wright et al., 2004). In addition, interspecific general patterns of plant defence have been proposed across a continuum of plant growth strategies (Stamp, 2003). As expected, we found that the expression of traits in young plants was at one extreme of that leaf economic spectrum equivalent to fast-growing species, with high nitrogen concentrations and low dry mass. In contrast, reproductive plants expressed leaf traits at the other extreme, with high LMA, lower nitrogen contents and high defence investments as has been demonstrated for slower growing plants species in interspecific comparisons. This suggests that the functional value of growth and its trade-offs with defence across plant development reflects the interspecific patterns observed for fast and slow growing species.

Maximum foliar integration was rather low at both stages (between 2% and 6%) in comparison to levels of phenotypic integration reported for flowers, which in general have a maximum possible integration ranging between 10% and 20% (Ordano et al., 2008). In the case of *T. velutina*, floral integration has been reported to

TABLE 3 Principal component analysis on the genotypic values of 11 foliar traits of *Turnera velutina* at two ontogenetic stages. Loadings >0.30, highlighted in bold, were considered as the threshold to define the relative importance of traits for each PC

		Juvenile stage					Reproductive stage				
		PC1	PC2	PC3	PC4	PC5	PC1	PC2	PC3	PC4	PC5
	Eigenvalue	3.211	2.402	1.658	1.064	0.920	4.147	1.658	1.367	1.269	0.8311
	Variance %	29.190	21.836	15.076	9.672	8.364	37.696	15.077	12.415	11.541	7.555
	Cumulative variance %	29.190	51.026	66.102	75.775	84.139	37.696	52.773	65.188	76.729	84.284
Defence	Trichome density	0.194	0.152	**−0.349**	**0.714**	0.059	**−0.365**	0.024	0.014	**−0.317**	**0.441**
	Water content	−0.224	−0.228	**0.379**	0.047	−0.225	**0.427**	0.100	0.132	−0.140	−0.008
	EFNs	0.113	0.133	**0.621**	0.250	−0.067	0.031	**0.469**	0.221	**0.477**	**0.359**
	Sugar content	**0.371**	0.025	0.160	**−0.509**	0.265	0.223	0.143	−0.163	**−0.472**	**0.492**
	LMA	**0.447**	0.053	−0.125	−0.154	**0.400**	−0.191	0.045	**0.607**	0.249	−0.068
Leaf economy	Carbon content	−0.270	0.280	0.118	0.183	**0.703**	**0.342**	−0.141	−0.012	0.332	**0.536**
	Nitrogen content	**−0.345**	**0.411**	0.275	−0.095	0.103	**0.391**	**−0.362**	0.199	0.122	0.105
	Chlorophyll content	0.235	**0.493**	0.113	0.001	**−0.327**	0.168	**−0.649**	0.130	−0.039	−0.013
	Dissection index	0.149	**−0.406**	**0.397**	0.283	0.278	0.113	0.035	**−0.678**	**0.435**	−0.110
	Foliar area	0.296	**0.449**	0.130	0.049	−0.133	**0.391**	0.289	0.129	−0.143	−0.304
	Petiole length	**0.452**	−0.219	0.180	0.135	−0.068	**0.366**	0.299	0.069	−0.179	−0.164

TABLE 4 Regression analyses with replicates (ANOVAs) showing the influence of plant biomass on the magnitude of foliar integration in *Turnera velutina*. Separate analyses were performed for each ontogenetic stage

Source	Juvenile				Reproductive			
	df	SS	MS	*F*	*df*	SS	MS	*F*
Among genotypes	23	8.17	0.355	17.008	23	14.86	0.646	20.761
Linear regression	1	2.014	2.014	7.193**	1	3.120	3.120	5.843**
Deviations from regression	22	6.153	0.280	13.333**	22	11.738	0.534	17.226**
Within genotypes	144	3.01	0.021		144	4.48	0.031	
Total	167	11.17			167	19.34		

*p < .05, **p < .01 and ***p < .001.
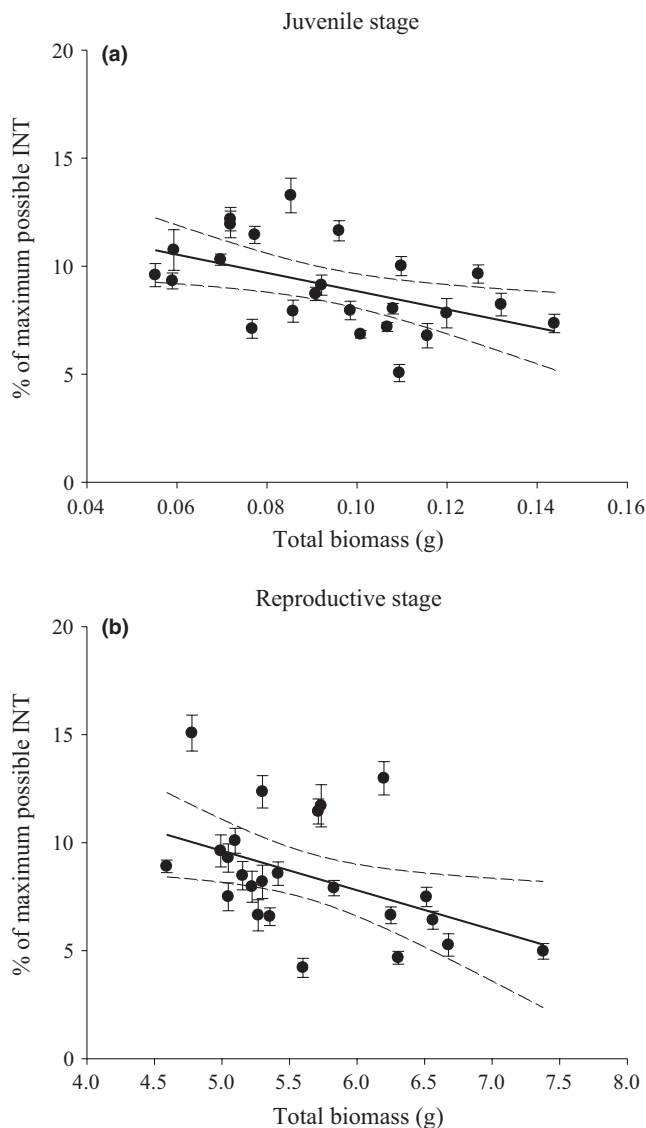
FIGURE 3 Relationship between total biomass and values of %INT of foliar traits for 24 genotypes in plants at the (a) juvenile and (b) reproductive stage. Dots and error bars represent genotypic jackknifed *M* and associated *SE* for each genotype

be, on average, between 24% and 30% (Ochoa, 2016). This difference between floral and foliar integration coincides with the original hypothesis predicted by Berg (1960), in which she stated that trait correlations should be less canalised and more plastic in vegetative than in floral modules, as the latter are subject to strong pollinator-mediated selection. Although other works have reported independence in the patterns of correlation among reproductive and vegetative plant parts (Conner & Sterling, 1996; Hansen, Pélabon, & Armbruster, 2007; Pélabon, Armbruster, & Hansen, 2011), they usually include traits from different vegetative modules (e.g. stems and leaves) involving only morphological, rather than functional vegetative traits. In this context, this work offers the first report on phenotypic integration of leaves considered as a functional unit, including both functional and morphological traits.

In addition, we report for the first time ontogenetic changes in both the magnitude and pattern of foliar integration, which was significantly lower (by almost three-fold) in juvenile plants compared to reproductive individuals. Because highly integrated phenotypes have fewer possibilities to express multi-trait plastic responses to different environments (Pigliucci, 2001; Schlichting, 1989), a functional interpretation of our findings is that weaker correlations among traits could allow juvenile plants to quickly fine-tune responses to the environment where they germinate and develop. Later during plant development, compromises with other functions such as maintenance, defence or reproduction can produce trade-offs driven by resource limitation, resulting in greater phenotypic integration (see Mason & Donovan, 2014). In particular, ontogenetic changes in foliar integration could be associated with developmental genetic programmes that trigger the transition between vegetative and reproductive phase changes in plants. For example, heteroblasty (i.e. changes in leaf size, shape and trichome density) in *Arabidopsis thaliana* has been associated with specific genetic networks and the expression of microRNAs that delay or promote flowering, but also affect different leaf traits (Huijser & Schmid, 2011). In addition, hormonal regulation has been reported to have an important role in the expression of growth and defensive traits (Brütting et al., 2017).

There was a discrepancy in the relationship between plant biomass and foliar integration within and between ontogenetic stages. Specifically, we found a negative relationship between plant biomass and foliar integration within each ontogenetic stage. This relationship suggests that

plant size *per se* can influence the expression and correlation among leaf traits. Larger plants experience greater micro-environmental heterogeneity and greater sectoriality, which in turn can promote greater variance of some leaf traits, and hence a decrease in their correlations and phenotypic integration. Interestingly, small juvenile plants expressed lower foliar integration than larger reproductive plants. This contrasting results suggests that processes intrinsic to the ontogeny of plants influence the magnitude of foliar integration, to a greater extent than processes associated with individual variation in plant size.

Two functional modules could be identified in leaves of juvenile plants: PC2 was influenced mostly by assimilation traits, which suggest the relevance of allocating more nitrogen and chlorophyll to larger and dissected leaves at this ontogenetic stage. The delimitation of a defensive module in the third PC could be explained by the generalised low values of physical and biotic defences of *T. velutina* at this stage (Ochoa-López et al., 2015). The fuzzy delimitation between functional modules of defence or assimilation traits in the main axis of variation (PC1), and in the rest of PCs particularly at the reproductive stage, could be promoted by the multi-functionality of the studied leaf traits (Ackerly et al., 2000; Geber & Griffen, 2003). For example, trichomes and LMA can act as barriers to herbivores but also participate in leaf thermoregulation (Hanley, Lamont, Fairbanks, & Rafferty, 2007), gas exchange (Wright et al., 2004) and water absorption (Papini, Tani, Di Falco, & Brighigna, 2010). Water is a key molecule for biochemical reactions of physiological processes, but can also influence herbivore preference and tissue damage (Agrawal & Fishbein, 2006). Finally, EFNs and nectar secretion have been related to ant defence, they also have a key role in seed dispersal by ants in *T. velutina* (Cuautle et al., 2005). Hence, these traits could belong to both defensive and leaf economic modules, or change their main role during plant development (Barton & Boege, 2017), restricting the distinction of discrete functional modules. Hence, we acknowledge the limitation of our conclusions regarding the functional modularity, particularly due to an oversimplification of trait function and/or the nature of the selected traits. Certainly, the leaf economic spectrum includes other traits that directly quantify gas exchange or metabolic rates (Wright et al., 2004), for example, photosynthetic efficiency and leaf longevity that influence the fitness value of leaves (Caruso, Maherall, Mikulyuk, Carlson, & Jackson, 2005; Saldaña, Lusk, Gonzáles, & Gianoli, 2007). In addition, further studies should assess foliar integration in plants growing directly in the field, as the presence of multiple interacting species or different abiotic conditions could certainly change the expression of inducible traits, such as trichome density, water content and even the production of extrafloral nectar.

In this paper, we have shown how leaf traits are expressed in different magnitudes and combinations as a function of plant ontogeny, producing different levels of foliar integration, which we found to be also influenced by plant biomass. We believe that studying complex phenotypes with an ontogenetic perspective is an excellent opportunity to build a bridge between developmental biology and evolutionary ecology, for a better understanding of plant ecophysiology and plant defence evolution.

AUTHORS' CONTRIBUTIONS

X.D., K.B. and J.F. planned and designed the research. X.D. performed experiments, conducted field work and analysed data. X.D. and K.B. led the writing of the manuscript and J.F. and C.A.D. significantly contributed to the drafts and approved the final version for its publication.

DATA ACCESSIBILITY

Data used in this manuscript are available from Dryad Digital Repository https://doi.org/10.5061/dryad.jd5j3 (Damián, Fornoni, Domínguez, & Boege, 2017).

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