



UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE ECOLOGÍA
BIOLOGIA EVOLUTIVA

**VALOR ADAPTATIVO DE LAS TRAYECTORIAS ONTOGENÉTICAS DE LA DEFENSA EN
PLANTAS.**

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

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P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Subcomité de Ecología y Biología Evolutiva del Posgrado en Ciencias Biológicas, celebrada el día **9 de noviembre de 2020**, aprobó el siguiente jurado para la presentación de examen para obtener el grado de **DOCTORA EN CIENCIAS**, de la estudiante **OCHOA LÓPEZ SOFÍA** con número de cuenta: **302068425**, con la tesis titulada: "**VALOR ADAPTATIVO DE LAS TRAYECTORIAS ONTOGENÉTICAS DE LA DEFENSA EN PLANTAS**", bajo la dirección de la **DRA. KARINA BOEGE PARÉ**, quedando integrado de la siguiente manera:

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Suplente: DR. JUAN SERVANDO NÚÑEZ FARFÁN

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"
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COORDINADOR DEL PROGRAMA



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“... la teoría de Darwin debe entenderse como un hipótesis que concibe la evolución como una suma de acontecimientos casuales y que en ello no hay nada finalista, ni en lo que concierne al hombre, ni en lo que atañe al mundo. Estamos aquí, pues, por casualidad a la espera de que otra casualidad menos compasiva nos arranque de este mundo.”

Circunstancias casuales – Carlo Flamigni (2016)

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Resumen

La ontogenia se ha identificado como una fuente de variación importante en la expresión de fenotipos vegetales. Sin embargo, cuando se realizan estudios sobre la evolución de atributos defensivos, comúnmente se analizan valores de algunos atributos de defensa en uno o a lo mucho en un par de estadios ontogenéticos, sin considerar el efecto que la ontogenia tiene sobre la expresión de estos atributos. Para comprender los mecanismos detrás de la evolución de las defensas de las plantas, necesitamos considerar que los patrones que observamos en la expresión de las defensas pueden ser producto de las normas de reacción ontogenéticas, en respuesta a factores intrínsecos y extrínsecos. Usando el arbusto mirmecofílico *Turnera velutina*, se exploró si el riesgo de ataque, daño por herbívoros, heredabilidad y plasticidad fenotípica pueden promover o limitar el potencial evolutivo de las trayectorias ontogenéticas en tres rasgos defensivos. Se expusieron 20 genotipos de *T. velutina* a ambientes contrastantes (invernadero y parcelas experimentales) y se midió el potencial cianogénico, la densidad de tricomas y el contenido de azúcar en el néctar extrafloral en plántulas, juveniles y plantas reproductivas. Con estos datos, se estimó la varianza genética, y la heredabilidad de los rasgos defensivos en cada etapa ontogenética y de las trayectorias ontogenéticas mismas. Además, se determinó si la selección natural sobre diferentes defensas y su valor adaptativo cambian a lo largo del desarrollo de la planta. Finalmente, se evaluó el efecto de las trayectorias ontogenéticas de las defensas sobre las tasas de crecimiento, supervivencia y fecundidad de la población de *T. velutina*.

Para las plantas bajo condiciones de invernadero, se encontró que hubo variación genética y heredabilidad para el potencial cianogénico en las plántulas y para la densidad de tricomas en todas las etapas ontogenéticas. La variación genética y la heredabilidad de las trayectorias ontogenéticas se detectaron sólo para la densidad de tricomas. Sin embargo, estos prerrequisitos genéticos para la evolución no se detectaron en parcelas experimentales en el campo, lo que sugiere que la variación ambiental y las respuestas plásticas fenotípicas enmascaran cualquier variación hereditaria. Además, se determinó que las trayectorias ontogenéticas son plásticas, ya que difirieron entre condiciones de invernadero y de campo para las mismas familias genéticas. Por otro lado, se encontró que en las plántulas la selección natural favoreció una defensa química baja, lo que parece explicarse por la eficiencia de asimilación y la capacidad del herbívoro especialista para secuestrar glucósidos cianogénicos. Mientras que la densidad de tricomas no fue favorecida en plantas juveniles, aumentó la adecuación en las plantas reproductivas. En esta etapa, también se encontró un gradiente correlativo positivo entre el potencial cianogénico y el contenido de azúcar en el néctar extrafloral. Finalmente, se encontró que las trayectorias ontogenéticas de la defensa tienen un efecto diferencial sobre las tasas vitales de la población.

La aproximación experimental utilizada permitió demostrar que existen normas de reacción ontogenéticas de la defensa y que constituyen estrategias defensivas formadas por la expresión combinada de diferentes rasgos. Sin embargo, el potencial evolutivo y constancia de estas estrategias puede verse limitado por la plasticidad fenotípica expresada en ambientes heterogéneos. Los resultados del estudio, además, aportan evidencia de que la selección natural actúa de manera diferencial sobre la defensa según la etapa de desarrollo de la planta, lo que indica que la influencia de esta fuerza evolutiva no se puede inferir sin la evaluación de estrategias ontogenéticas en la expresión de múltiples defensas.

Abstract

Ontogeny has been identified as a main source of variation in the expression of plant phenotypes. However, when conducting studies on the evolution of defensive attributes, most studies only consider a single or a couple of plant ontogenetic stages, without considering the effect of the whole plant ontogeny can have on the expression of these attributes. To fully comprehend the mechanisms behind the defense in plants, we need to consider that the patterns we observe can be influenced by ontogenetic reaction norms in response to intrinsic and extrinsic factors. Using the myrmecophytic shrub *Turnera velutina*, we explored if risk of attack, herbivore damage, heritability, and phenotypic plasticity can promote or constrain the evolutionary potential of ontogenetic trajectories in three defensive traits. Twenty genotypes of *T. velutina* were exposed to contrasting environments (shade house and field plots), and measured the cyanogenic potential, trichome density, and sugar content in extrafloral nectar in seedlings, juveniles and reproductive plants. I estimated genetic variance, broad sense heritability, and evolvability of the defensive traits at each ontogenetic stage, and of the ontogenetic trajectories themselves. Additionally, I evaluated whether the targets of natural selection on different defenses and their adaptative value changed across plant development. Finally, I assessed if the ontogenetic trajectories of defense influenced the growth, survival and fecundity rates in *T. velutina* population.

Plants growing in the shadehouse showed genetic variation and broad sense heritability for cyanogenic potential in seedlings, and for trichome density at all ontogenetic stages. Genetic variation and heritability of ontogenetic trajectories in trichome density was detected for trichome density only. These genetic prerequisites for evolution, however, were not detected in the field, suggesting that environmental variation and phenotypic plastic responses masks any heritable variation. Indeed, ontogenetic trajectories were found to be plastic, differing between shadehouse and field conditions for the same genetic families. Additionally, we found that low chemical defense was favored in seedlings, which seems to be explained by the assimilation efficiency and the ability of the specialist herbivore to sequester cyanogenic glycosides. Whereas trichome density was unfavored in juvenile plants, it increased relative plant fitness in reproductive plants. At this stage I also found a positive correlative gradient between cyanogenic potential and sugar content in extrafloral nectar. Finally, the ontogenetic trajectories of defense had a differential effect on vital rates of the population.

The used experimental approach allowed to detect the defensive ontogenetic strategies expressed through ontogenetic reactions norms of different defenses expressed in particular combinations. However, the evolutionary potential of these strategies may be limited by phenotypic plasticity expressed in heterogeneous environments. In addition, we provide evidence that natural selection acts differentially on different defenses according to the stages of plant development in which they have particular adaptive value, indicating that the influence of natural selection cannot be inferred without evaluating ontogenetic strategies in the expression of multiple defenses.

Introducción general

El desarrollo de los organismos está marcado por cambios en los atributos morfológicos, fisiológicos y conductuales, los cuales pueden ser producidos por la asignación diferencial de recursos hacia las funciones que los organismos deben realizar, como el crecimiento, el almacenamiento de recursos, la reproducción y la defensa contra enemigos naturales. Estos cambios también pueden ser producto de costos fisiológicos y ecológicos, así como de restricciones inherentes al proceso de desarrollo mismo (Herms & Mattson 1992; West *et al.* 2001; Hou *et al.* 2008; Maherali *et al.* 2009). En el caso de las plantas, el uso de recursos para cada una de estas diferentes funciones puede variar dependiendo de la etapa de su ciclo de vida (Farnsworth 2004; Weiner 2004). Por ejemplo, en una plántula, el crecimiento requiere más recursos para adquirir tejidos fotosintéticos y de almacén, para posteriormente asignar recursos para otras funciones como puede ser la defensa contra herbívoros o patógenos. En contraste, cuando la planta se encuentra en estadio reproductivo, la producción de gametos y dispersión de semillas pueden utilizar la mayor cantidad de recursos (Farnsworth 2004; Weiner 2004; Boege & Marquis 2005; Barton & Koricheva 2010).

Cuando hablamos de las historias de vida de los organismos, debemos recordar que éstos tienen una cantidad limitada de recursos durante su vida, y los recursos utilizados para una función no pueden ser utilizados en otra (Cody 1966). Por esta razón, la expresión de cualquier atributo puede reflejarse en una disyuntiva con otras funciones como el crecimiento, la supervivencia o la fecundidad de los organismos. En el caso de la producción de defensas en las plantas, se ha demostrado que ésta puede ser costosa (Herms & Mattson 1992; Purrington 2000; Walters & Heil 2007; Cipollini *et al.* 2014), ya que requiere de energía y recursos que podrían ser usadas en otras funciones. A su vez, esto puede verse reflejado en una reducción del crecimiento, o en efectos negativos con la interacción de su ambiente biótico o abiótico. De acuerdo con la teoría de historias de vida, la selección natural puede modificar la asignación de recursos a funciones que maximicen la contribución de un genotipo a las siguientes generaciones (Cody 1966; León 1976). Por esta razón, lo que los valores de los atributos defensivos de las plantas pueden cambiar durante el desarrollo en función de la contribución que estos atributos tienen a la adecuación de la planta.

En el caso de los atributos defensivos, se han propuesto dos hipótesis alternativas que explican en que momentos del desarrollo la producción de defensas puede tener mayor contribución a la adecuación de las plantas. La primera es la hipótesis de balance de crecimiento-diferenciación (HBCD), la cual propone que existe una disyuntiva fisiológica entre el crecimiento y los procesos de diferenciación. La diferenciación consiste en el cambio químico y morfológico de las células maduras, como el engrosamiento de la cutícula, la producción de metabolismo secundario y de tricomas; mientras que el crecimiento es la producción de raíces, tallos y hojas, generados a partir de división celular y elongación de las células ya existentes (Herms & Mattson 1992). Considerando esta hipótesis en un contexto ontogenético, se predice que las plantas reproductivas producirán mayores cantidades de defensas contra los herbívoros en contraste con plantas jóvenes, que asignarán los recursos principalmente en el crecimiento y tendrán niveles bajos de defensa.

La segunda propuesta es la hipótesis de defensa óptima (HDO), que predice que los factores extrínsecos, como la selección por herbívoros, e intrínsecos, como la aportación a la adecuación final de los órganos o los estadios de la planta, favorecen altos niveles de defensa en los órganos/estadios más vulnerables, que generalmente son los juveniles; seguido por un decremento conforme la planta crece (o las hojas maduran) y se vuelven menos susceptible a los ataques de los herbívoros (McKey 1974; Rhoades & Cates 1976). Estas dos hipótesis nos permiten explorar cuáles son los factores que pueden moldear la expresión de las defensas en las plantas

incluyendo la presencia de restricciones inherentes al proceso de desarrollo (Falcão *et al.* 2003; Quintero *et al.* 2013; Villamil *et al.* 2013), y los cambios en las presiones de selección de factores bióticos y abióticos (Boege & Marquis 2005; Loe *et al.* 2007; Donovan *et al.* 2009; Miranda-Pérez *et al.* 2016; Kooyers *et al.* 2018; Ochoa-López *et al.* 2018). Entonces, podríamos esperar que los patrones ontogenéticos observados sean resultado de un balance entre los beneficios de expresar las defensas y las restricciones por disyuntivas con funciones que tengan un mayor efecto sobre la adecuación de las plantas.

Se han registrado cambios ontogenéticos en los atributos de defensa contra herbívoros en numerosos estudios (revisados por Boege & Marquis 2005; Barton & Koricheva 2010; Massad 2013; Quintero *et al.* 2013). Estos cambios ocurren en distintos atributos de defensa, como la resistencia física, química o biótica, la tolerancia o incluso en el escape, y se reconocen como trayectorias ontogenéticas de la defensa. Sin embargo, la mayoría de los estudios han buscado caracterizar las trayectorias ontogenéticas de un solo tipo de defensa (Jogia *et al.* 1989; Swihart & Bryant 2001; Van Bael *et al.* 2003) o sólo en algunos casos de tres o más atributos (Quintero & Bowers 2011; Villamil 2012); en general comparando dos estadios ontogenéticos (Jogia *et al.* 1989; Swihart & Bryant 2001; Van Bael *et al.* 2003), o etapas tempranas y tardías de un mismo estadio (Hanley *et al.* 1995; Del-Val & Dirzo 2003; Holeski *et al.* 2009). Sin embargo, estos estudios no suelen explorar los mecanismos detrás de los patrones ontogenéticos propuestos, y no se conocen cuáles son los factores ambientales y genéticos que regulan estos cambios ontogenéticos (Barton & Boege 2017).

La producción de defensas implica costos fisiológicos para las plantas, lo que se refleja en la cantidad y tipo de defensas producidas en cada estadio ontogenético. La asignación de recursos a distintas estrategias defensivas puede ser redundante, por lo que se ha predicho que se favorecería una relación negativa entre estrategias defensivas (Simms & Triplett 1994; Fineblum & Rausher 1995; Roy & Kirchner. 2000). Sin embargo, existe evidencia de la producción de niveles intermedios de ambas estrategias, al considerar que los costos y beneficios son variables y no existe una relación lineal entre éstos (Fornoni *et al.* 2004), lo que propone un escenario de defensa mixta, sobre todo cuando existen varios herbívoros presentes en la interacción con la planta (Carmona & Fornoni 2013). En este contexto, durante la ontogenia también se podrían originar estrategias de este tipo de defensa expresadas mediante cambios opuestos en distintos mecanismos de defensa. Estos cambios se han propuesto con el nombre de “switches” ontogenéticos, es decir el cambio simultáneo y opuesto en distintas estrategias de defensa durante el desarrollo de la planta (Boege *et al.* 2007). Por ejemplo, en *Eucalyptus froggatti*, la producción de sesquiterpenos y monoterpenos incrementa durante la ontogenia, en contraste con los fenoles totales que disminuyen (Goodger *et al.* 2013).

La expresión de atributos defensivos podrían considerarse como un fenotipo complejo, si tomamos en cuenta que su expresión se realiza de manera conjunta y esta cambia durante el desarrollo (Atchley 1987; Quintero *et al.* 2013). Si estas estrategias ontogenéticas de defensa permiten a las plantas optimizar el uso de los recursos disponibles e incrementar su adecuación, podrían considerarse como un fenotipo complejo que tiene un valor adaptativo sujeto a selección natural. Por lo tanto, determinar si existe un valor adaptativo de las trayectorias ontogenéticas de la defensa nos permitirá comprender la evolución de las estrategias de vida de las plantas, en función del cambio ontogenético de las presiones de selección y de su capacidad de respuesta de éstas. Las defensas que las plantas han desarrollado por presión de sus herbívoros suelen expresarse simultáneamente en combinaciones particulares (Quintero *et al.* 2013; Ochoa-López *et al.* 2015; Barton *et al.* 2019). Debido a esto, para comprender su evolución debemos dejar de separarlas temporal y espacialmente, y estudiar una combinación de atributos con efectos específicos en la adecuación de las plantas en función de cuando se

expresan durante la vida de las mismas. Sin embargo, para que las trayectorias ontogenéticas puedan considerarse como un atributo adaptativo que evoluciona bajo selección natural, éstas deben tener variación genética heredable, lo cual ha sido explorado en algunos estudios (Gleadow & Woodrow 2002; Barton 2007b; Donaldson & Lindroth 2007). Algunos de los atributos defensivos con variación genética incluyen defensas químicas (van Dam & Vrieling 1994; Adler *et al.* 1995; Campbell 1997; Stowe 1998; Agrawal *et al.* 2002; Andrew *et al.* 2007; Barton 2007a), físicas (Geber & Griffen 2003; Johnson *et al.* 2009) y bióticas (Geber & Griffen 2003; Wooley *et al.* 2007). Incluso se han reportado algunos sistemas en los que se reporta variación genética en la expresión ontogenética de los atributos de defensa (Barton 2007a; Cope *et al.* 2019).

Para poder estudiar la evolución de las defensas en plantas con un enfoque multivariado y temporal, es necesario tener un sistema de estudio que permita incorporar varios tipos de defensa y cuyo desarrollo pueda ser monitoreado con facilidad. En este trabajo se investigó, con una aproximación experimental, el valor adaptativo que tienen las trayectorias ontogenéticas de la defensa de *Turnera velutina*, un arbusto mirmecofílico perene que presenta varios atributos de defensa a lo largo de su desarrollo.

En *T. velutina*, se han reportado previamente trayectorias ontogenéticas de atributos defensivos (TOD). El potencial cianogénico (defensa química) disminuye con el desarrollo, mientras que la densidad de tricomas (defensa física) y contenido de azúcar en el néctar extrafloral (defensa biótica) incrementa. Además, en la población de estudio, el daño por herbívoros es mayor en plantas juveniles que en plantas reproductivas (Ochoa-López *et al.* 2015) (Anexo). Este sistema de estudio se utilizó para evaluar si las trayectorias ontogenéticas de la defensa son un fenotipo complejo que puede evolucionar por selección natural. Para responder esta pregunta, fue necesario primero evaluar si existe un componente genético heredable detrás del cambio ontogenético de la expresión de las defensas, además de determinar si existe un efecto de la expresión de estas trayectorias sobre la adecuación de la planta. Finalmente, para no perder el contexto poblacional de la expresión de las trayectorias, se evaluó el efecto que tienen las defensas expresadas a lo largo de la vida de las plantas sobre las tasas vitales poblacionales.

El primer capítulo explora la presencia de variación genética y heredabilidad de los cambios de defensas durante la ontogenia de las plantas. Además, se reporta una relación entre el patrón ontogenético con el riesgo de las plantas de ser consumida por herbívoros. Finalmente, se presenta evidencia de que las trayectorias ontogenéticas pueden expresar normas de reacción (plasticidad fenotípica) en función del ambiente donde se expresan (campo vs invernadero).

El segundo capítulo se centra en evaluar si los blancos de selección natural cambian durante la ontogenia. Es decir, se investigó el efecto que tiene la producción de cada tipo de defensa en distintos estadios ontogenéticos sobre la adecuación de la planta. Además, se exploró la presencia de estrategias ontogenéticas de la defensa en plantas que favorezcan su adecuación.

Finalmente, en el tercer capítulo se explora si existe un efecto de los cambios ontogenéticos de las defensas de diferentes genotipos sobre las tasas vitales y la demografía de la población de estudio, ya que la expresión de las trayectorias ontogenéticas de la defensa puede reflejarse en cambios en la supervivencia, crecimiento y fecundidad de los individuos dentro de las poblaciones, lo que conlleva a cambios demográficos en poblaciones con genotipos que expresan diferentes estrategias defensivas.

Este estudio constituye la primera aproximación experimental que aborda de manera integral la descripción de las trayectorias ontogenéticas de defensa con un enfoque tanto evolutivo como demográfico, explorando si estas trayectorias cuentan con los requisitos para evolucionar por selección natural, y ser consideradas como un fenotipo complejo.

Capítulo 1:
Riesgo de ataque por herbívoros y heredabilidad de trayectorias ontogenéticas en la defensa de plantas



Risk of herbivore attack and heritability of ontogenetic trajectories in plant defense

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Abstract

Ontogeny has been identified as a main source of variation in the expression of plant phenotypes. However, there is limited information on the mechanisms behind the evolution of ontogenetic trajectories in plant defense. We explored if risk of attack, herbivore damage, heritability, and phenotypic plasticity can promote or constrain the evolutionary potential of ontogenetic trajectories in three defensive traits. We exposed 20 genotypes of *Turnera velutina* to contrasting environments (shadehouse and field plots), and measured the cyanogenic potential, trichome density, and sugar content in extrafloral nectar in seedlings, juveniles and reproductive plants. We also assessed risk of attack through oviposition preferences, and quantified herbivore damage in the field. We estimated genetic variance, broad sense heritability, and evolvability of the defensive traits at each ontogenetic stage, and of the ontogenetic trajectories themselves. For plants growing in the shadehouse, we found genetic variation and broad sense heritability for cyanogenic potential in seedlings, and for trichome density at all ontogenetic stages. Genetic variation and heritability of ontogenetic trajectories was detected for trichome density only. These genetic pre-requisites for evolution, however, were not detected in the field, suggesting that environmental variation and phenotypic plastic responses mask any heritable variation. Finally, ontogenetic trajectories were found to be plastic, differing between shadehouse and field conditions for the same genetic families. Overall, we provide support for the idea that changes in herbivore pressure can be a mechanism behind the evolution of ontogenetic trajectories. This evolutionary potential, however, can be constrained by phenotypic plasticity expressed in heterogeneous environments.

Keywords Defense · Genetic variation · Broad-sense heritability · Ontogenetic trajectories · Reaction norms

Introduction

Ontogeny has been recognized as one of the main sources of phenotypic variation in both plants and animals, as phenotypes drastically change throughout development (Pigliucci and Schlichting 1995; Sultan 2015). This variation results in

ontogenetic trajectories of the phenotype, defined as functions of phenotypic changes resulting from internal factors (genetic and developmental) and external environmental effects (physical conditions and biotic interactions). Hence, ontogenetic trajectories can be considered as a reaction norm of the genotype exposed to different environments across development (Schlichting and Pigliucci 1998; Schlichting and Smith 2002; Whitman and Agrawal 2009). Knowledge on the phenotypic and genetic aspects of this ontogenetic variation is relevant for our understanding of phenotypic evolution, and could reveal why some biotic interactions shift over time (Kirkpatrick and Lofsvold 1992; Larsson et al. 1998).

Ontogenetic trajectories in anti-herbivore defensive traits have now been described for many plant species (Boege and Marquis 2005; Barton and Koricheva 2010), and are considered a ubiquitous characteristic of plant phenotypes (Barton and Boege 2017). The direction and degree of such ontogenetic changes seem to be quite variable, although

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patterns have emerged with respect to plant life histories, and the specific developmental window or defensive trait examined (Barton and Koricheva 2010; Quintero et al. 2013). The emergence of some general patterns suggests that these ontogenetic trajectories constitute common functional adjustments to changes in resource allocation (Bryant et al. 1991), regulatory mechanisms (Campos et al. 2016), gene expression (Mauricio 2005), and/or developmental constraints (Villamil et al. 2013). Additional mechanisms driving ontogenetic trajectories could be due to the multifunctionality of traits (Barton and Boege 2017) or the variation in the selective pressures by consumers acting on plants throughout their lifetimes (Boege and Marquis 2005; Barton and Koricheva 2010). Despite the abundant evidence of ontogenetic changes in plant defense, there is currently not enough data on the mechanisms and sources of variation driving the evolution of these ontogenetic trajectories, necessary to assess whether they can be considered adaptive complex phenotypes under natural selection (Barton and Boege 2017).

The capacity of a population to undergo evolutionary changes in their ontogenetic trajectories requires a significant degree of evolvability, which is a measure of its potential to respond as a complex trait to natural selection and other evolutionary processes (Flatt 2005). One relevant factor that can influence this potential is the degree of genetic variation in plant defenses at each ontogenetic stage. In particular, the risk of herbivore attack, the cost of plant defense, and the fitness consequences of damage have been proposed as factors that can select for optimal defense allocation patterns (Coley 1983; Bryant et al. 1991). Because the probability of being damaged by herbivores (Bryant et al. 1992;

Hanley et al. 1995; Fenner et al. 1999; Maron and Crone 2006; Kant et al. 2015; Schuman and Baldwin 2016), the costs of defenses (Boege et al. 2007; Orians et al. 2010), and the impacts of damage (Boege 2005; Barton 2014; Tito et al. 2016) can change across plant development, natural selection should optimize the expression of defenses at each ontogenetic stage (Barton and Boege 2017). Following the optimal defense theory (McKey 1974; Rhoades 1979), vulnerable ontogenetic stages should be exposed to stronger selection pressures to increase their defenses against herbivores than developmental stages with lower risk of attack and/or less susceptible to herbivore damage. As a consequence, genetic variation should be differentially eroded by natural selection throughout ontogeny. In other words, when risk of herbivore attack is high, natural selection should favor those genotypes maximizing the investment in defenses, hence reducing the proportion of heritable variation in these traits. For example, when young stages have greater risk of attack, natural selection should favor genotypes with high levels of defense at this stage (Fig. 1a). In a scenario where risk of attack is greater at older ontogenetic stages, the opposite trend should be expected (Fig. 1b). Thus, genetic variation should be reduced at those ontogenetic stages under stronger selective pressure on plant defense. If the risk of herbivore damage is evenly distributed or unpredictable across plant ontogeny, genetic variation in plant defense should be expected to be equivalent among ontogenetic stages (Fig. 1c). Under this scenario, selective agents other than herbivory, such as resource allocation trade-offs due to other functional priorities, developmental constraints or phenotypic plasticity could promote variable ontogenetic patterns in plant defense.

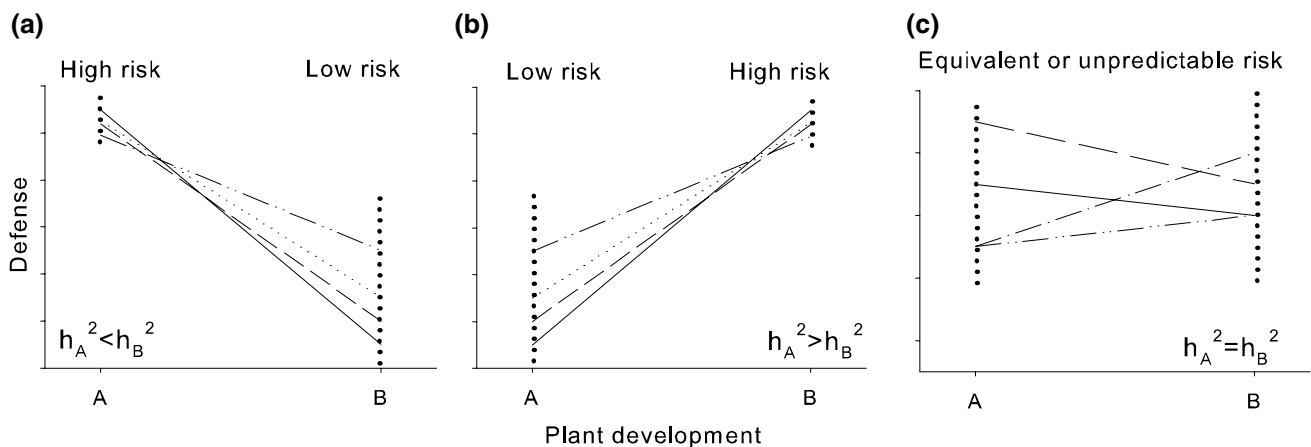


Fig. 1 Expected relationship between risk of attack and genetic variation (represented by vertical dashed lines) of plant defense across plant development. Different ontogenetic trajectories represent trends for different genotypes. **a** Younger stages have higher risk of attack, and hence natural selection favors higher levels of defense and erodes genetic variation in contrast with older stages. **b** Risk of

attack is greater in older ontogenetic stages, promoting higher defense with low genetic variation than in earlier ontogenetic stages. **c** Risk of attack is equivalent or unpredictable across plant development, hence genetic variation in plant defense remains high and similar for all ontogenetic stages, producing different ontogenetic patterns in plant defense among genotypes

The expression and evolution of ontogenetic trajectories can indeed be influenced by phenotypic plasticity at one or more ontogenetic stages (Pigliucci and Schlichting 1995). The release and/or amplification of phenotypic variation as a result of plasticity could mask heritability of plant defenses, and as a consequence of their ontogenetic trajectories. Acknowledging that both internal factors and external environments often change across plant development, one major but unanswered question is whether ontogenetic trajectories in plant defense are fixed or actually vary as a function of the environmental context in which plants develop. In other words, are the ontogenetic trajectories themselves plastic across variable environments?

A fair number of studies on single ontogenetic stages have confirmed that both constitutive and induced defenses have significant levels of heritable variation (Underwood et al. 2000; Andrew et al. 2005; Stevens and Lindroth 2005), and that the correlation among multiple traits can influence their joint evolution (Berenbaum et al. 1986; Stowe 1998). To date, what we know for a limited number of species is that genetic variation of plant defense can change across ontogeny (i.e., genotype \times age interactions). For example, in *Plantago major* and *P. lanceolata*, genetic variation was detected in the expression of iridoid glycosides throughout ontogeny (Bowers and Stamp 1993; Barton 2007). In addition, there is one report on the ontogenetic changes in heritability of trichome density (Mauricio 2005), but no information is available on the heritability of the ontogenetic trajectories themselves nor their extent of phenotypic plasticity. Hence, a first step to explore the evolutionary potential of ontogenetic trajectories is the assessment of how heritable genetic variation in plant defense changes across plant development. A second step is evaluating the evolutionary potential of the ontogenetic trajectories themselves. In this study, we: (1) assessed the genetic variation and broad sense heritability of three defensive traits at three ontogenetic stages, (2) explored the relationship between heritable variation and the risk of herbivore attack and proportion of leaf area consumed by herbivores throughout plant development, and (3) estimated if heritability of the ontogenetic trajectories in defensive traits are contingent upon the environment in which plants grow. We predicted that if ontogenetic trajectories in plant defense represent a functional adjustment tuned by the selective pressure of herbivore damage, a negative relationship between levels of defense and genetic variation should be found across plant development (Fig. 1a, b). We also predicted changes in the expression of defensive traits and their genetic variation in different environments, which would in turn promote plasticity of their ontogenetic trajectories.

Materials and methods

Study system

Turnera velutina (Passifloraceae) is a myrmecophytic shrub endemic to Mexico growing in coastal sand dunes and tropical dry forests (Arbo 2005; Villamil et al. 2013). Because we were particularly interested in assessing the evolutionary potential of the ontogenetic trajectories in plant defense within a population under field and greenhouse conditions, we focused on one population of *T. velutina* located on the coast of Veracruz, Mexico (19°36' N, 96°22' W), within the Centro de Investigaciones Costeras La Mancha (CICOLMA). The climate in this area is warm subhumid with an average annual temperature ranging between 21.1 °C in January and 27.3 °C in June. The rainy season occurs mostly from June to September, and the total annual precipitation ranges from 899 to 1829 mm (Travieso-Bello and Campos 2006). Ontogenetic trajectories in chemical (cyanogenic potential), physical (trichomes density), and biotic (sugar in extrafloral nectar as a reward for patrolling ants) defensive traits have been previously reported for *T. velutina* as follows: whereas the cyanogenic potential (HCN) decreases from the seedling to the reproductive stage, both trichome density and sugar in extrafloral nectar (EFN) increase across plant development (Ochoa-López et al. 2015). In addition, previous reports indicate that younger ontogenetic stages have greater percentages of consumed leaf area than later stages (Ochoa-López et al. 2015). The main herbivore of *T. velutina* is the specialist herbivore *Euptoieta hegesia* Cramer (Lepidoptera: Nymphalidae), which is commonly found in the studied population (Ochoa-López et al. 2015).

To reduce maternal effects, in June 2013, we produced a F1 generation ($N = 300$ plants) of *T. velutina*, using seeds from 20 maternal plants naturally growing in the field in different sites of the dry forest vegetation surrounding the established sand dunes. To obtain the seeds, multiple flowers of each plant were self-crossed and further excluded from pollinators. Fruits were collected when ripe. After removing their elaiosomes, seeds were sown in germination trays in a mixture of local soil and vermiculite (1:1). Trays were bottom watered for 3 weeks until germination. F1 plants were transplanted into 2-L pots and watered until their reproductive stage. In June 2014, between 9 and 16 F1 plants per family were self-pollinated (using several flowers of each plant) to obtain a total of 2000 (F2) full-sib seeds. We used 25 seeds/family to produce a first batch of plants that were maintained within the shadehouse in the facilities of CICOLMA under homogeneous environmental conditions, restricting their contact with herbivores, pollinators, and patrolling ants. A second batch of plants of

the same genetic families was produced using 75 seeds/family to expose them to all biotic interactions and natural environmental variation in the field. Seeds were germinated as described above. For the shadehouse batch of F2 plants, we assigned 20 seedlings per family to one of the following three ontogenetic stages: *Seedlings* (individuals with the first two true leaves fully expanded), *Juvenile* (plants with the 10th leaf fully expanded), and *Reproductive* (individuals with fully expanded leaves bearing the first flowers). We transplanted plants to individual germination trays (seedlings), 1-L pots (juvenile plants), or 2-L pots (reproductive plants), and watered them daily. We did not observe pot binding of roots at any ontogenetic stage, and due to the small root size of seedlings and juvenile plants, it is unlikely that pot size affected plant growth or other traits at any ontogenetic stage. For the field batch, 1200 seedlings were transplanted at the 1st true leaf stage directly into the soil, assigning them to one of the ontogenetic stages in which they were later measured ($N = 20$ plants/family/stage). We established twenty 1×1 m plots in the sand dune under the partial shade of vegetation canopy, where *T. velutina* plants naturally grow. Between 3 and 10 plants/family were planted in each plot using a 10×10 cm grid.

Ontogenetic trajectories in plant defense

For both batches, when plants reached their corresponding ontogenetic stage (i.e., plants were measured only once), we quantified EFN sugar and collected two fully expanded leaves, one to assess cyanogenic potential and one to quantify trichome density.

We estimated cyanogenic potential through HCN content (Ballhorn et al. 2005), which was assessed by a quantitative and colorimetric assay (Schappert and Shore 1995) using the most apical fully expanded leaf at each ontogenetic stage. In each leaf, we cut six leaf discs (0.6 cm^2) with a whole punch. Three discs were stored in a glassine paper bag and dried at room temperature, until constant dry weight was recorded. We crushed the remaining three discs in an Eppendorf tube with $7 \mu\text{L}$ of chloroform. A 0.5×1 cm strip of filter paper previously soaked in a 5% NaCO_2 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 ($25\text{--}30^\circ\text{C}$). The picrate paper changed from yellow to orange, rust, or dark brown depending on the amount of HCN released by the leaf discs. We punched a single disc from the reacted picrate-soaked filter paper and eluted it in 1 ml of 50% ethanol for 24 h in the fridge at 4°C . $250 \mu\text{L}$ of the eluted ethanol was then placed in microplates (96-well EIA/RIA plates, Corning, NY, USA) to measure absorbance at 590 nm using a microplate reader (ELx808, BioTek Instruments Inc., Winooski,

VT, USA). We transformed absorbance readings to HCN contents ($\mu\text{g HCN/g dry weight}$) using the formula $\text{HCN} (\mu\text{g}) = (\text{optical density} - 0.0478965)/0.000652$ ($r^2 = 0.91$, $P < 0.0001$), obtained from a standard curve using the same protocol as described above, but using sodium cyanide (Code 7660-1 Caledon Laboratories Ltd, Canada) as a source of HCN.

To quantify trichome density, we used the penultimate apical fully expanded leaf of each plant, after leaves were dried at room temperature, and no changes in weight were observed. Two pictures were taken of the upper and lower sides of each leaf using a stereoscopic microscope (Discovery V8, Zeiss, $\times 1.5$). We quantified the number of trichomes per area using the software Image J 1.48v (NIH, USA). We estimated the production of sugar in EFN in the three most apical leaves following the procedures described by Heil et al. (2001). Briefly, we added $2 \mu\text{L}$ of distilled water to the extrafloral nectar using a micropipette ($0.5\text{--}10 \mu\text{L}$ Nichipet Premium, Nichiro CO, Japan), without completely discharging the volume of water into the nectary. Then, the mix of water and nectar was reabsorbed and placed in a hand-held refractometer ($0\text{--}50^\circ$ Brix, Reichert 137531L0, Munich, Germany) to quantify sugar concentration. Finally, the mix of nectar and water was reabsorbed again from the refractometer using $5\text{-}\mu\text{L}$ capillary tubes (Blaubrand intra-MARK, Brand, Germany) and its volume was estimated by measuring the length of the nectar column with a caliper. Sugar content in EFN was estimated as $\text{sugar (mg)} = \text{nectar concentration (} B^\circ \text{)} \times \text{nectar mix volume} (\mu\text{l})/100$ (Heil et al. 2000, 2001).

Risk of attack and herbivore damage

To assess the selective potential of herbivores on different ontogenetic stages of *T. velutina*, we estimated butterfly oviposition preferences of adult stages of *E. hegesia*, for different *T. velutina* ontogenetic stages. In addition, we quantified the percentage of leaf area consumed by herbivores, as a measure of the potential impacts on plant fitness and hence the intensity of selection pressure on defensive traits. To assess oviposition preference of *E. hegesia* butterflies on different ontogenetic stages of *T. velutina*, we first reared caterpillars on *T. velutina* plants until pupation. Once they emerged, 11 pairs of butterflies (one female and one male) were placed into one individual cylindrical cage made of a fine mesh net and a wire circle ($N = 11$ cages of 60 cm of diameter $\times 100 \text{ cm}$ in height). Within each cage, we placed four 2-L pots, two of them containing a single reproductive plant and two containing a group of ten seedlings to match the number of leaves of reproductive plants. A cotton ball dipped in artificial nectar (Schappert and Shore 1998) was hung from the top of the cage to provide food for the butterflies, in addition to flowering *Eupatorium* sp. and *Lantana*

camara cuttings. Only five pairs of butterflies succeeded to mate. After mating, females were kept inside the cages for 48 h to allow oviposition. We counted the number of eggs on seedlings and reproductive plants. Oviposition preference was estimated as the number of eggs/total leaf area available of each ontogenetic stage within each cage (i.e., adding the leaf area of all individuals/stage). After verifying the normality of the data, differences between ontogenetic stages were assessed using a paired *t* test (R Core Team 2017).

For a subset of the F2 plants growing in the field ($N = 368$), we estimated the percentage of area consumed by herbivores establishing visual categories of damage to all leaves (1 = 0%, 2 = 0–25%, 3 = 25–75% and 4 = 75–100%) Damage was estimated in each plant at the seedling, juvenile and reproductive stage. Ontogenetic differences in the percentage of leaf area lost were assessed using a repeated-measures linear mixed model with restricted maximum likelihood (REML) estimations, considering family as a random factor and ontogenetic stage as a fixed factor. Significance of the fixed factor was estimated using ANOVA type III SS. We did not test for the significance of random factors, as we were only interested in testing the ontogenetic differences in herbivore damage.

Genetic variance, heritability, and evolvability of plant defenses and their ontogenetic trajectories

To assess if ontogenetic trajectories of defensive traits had the pre-requisites to evolve by natural selection, we assessed their genetic variance, broad sense heritability (hereafter heritability), and evolvability. As a first step and to understand the possible constraints on the evolutionary potential of these trajectories, these attributes were estimated for each trait within each ontogenetic stage and environment, using the variance components obtained through linear mixed-model ANOVAs with restricted maximum likelihood (REML) estimations. Models included each trait as the response variable and family as a random factor. Genetic variance was determined repeating the models with and without the family term and using likelihood ratio tests for significance (Littell et al. 1996; Zuur et al. 2009). Full-sib broad sense heritabilities (H^2) were obtained by dividing two times the variance component of the family term by the total variance (Roff 1997), and significance was tested using the *P* value of the genetic family term (Bingham and Agrawal 2010). For comparative purposes with other studies, evolvabilities for each defensive trait were calculated at each ontogenetic stage as $CV = (\text{variance} \times 2)^{1/2}/\text{mean trait value}$ from models including only family as a random factor (Bingham and Agrawal 2010). To explore the relationships among all defensive traits within and across ontogenetic stages, Pearson correlations were calculated from family means for each ontogenetic stage and defensive trait.

To estimate genetic variation, heritability, and evolvability of the ontogenetic trajectories of the three defensive traits, we used the same approach as in Bingham and Agrawal (2010), in which they estimated heritability and evolvability on the inducibility of defensive traits (i.e., plasticity across different herbivore-damage environments). In our case, we treat ontogenetic stages as different “environments”, and estimate variation, heritability, and evolvability of the change in defensive traits across ontogeny. Because ontogenetic trajectories among the three stages were not linear, we had to partition our data to compare different fractions of the whole ontogenetic trajectory: seedling vs. juvenile and juvenile vs. reproductive. For each of the two data subsets, we used mixed models ANOVAs including ontogenetic stage as a fixed factor, family as a random factor, and their interaction, using log-transformed data. We visually inspected the residual plots to check for deviations from homoscedasticity and normality. To test for significance of random factors, we used the likelihood ratio statistic, taking the difference between the -2REML log likelihood of the full model (including ontogenetic stage, family and their interaction) and a reduced model (without the random factor and/or the interaction; Littell et al. 1996; Zuur et al. 2009). Genetic variation in the ontogenetic trajectories was inferred from the significance of the ontogenetic stage \times family term. To assess H^2 of the trajectories between each pair of ontogenetic stages, we used two times the family \times ontogenetic stage variance component, divided by the total phenotypic variance (Relyea 2005; Bingham and Agrawal 2010). The significance of the heritability was tested using the *P* value of the interaction term.

We calculated evolvabilities of the ontogenetic trajectories between each pair of ontogenetic stages as $CV_G = (\text{variance} \times 2)^{1/2}/(\text{mean trait value of stage } i - \text{mean trait value of stage } j)$, where *i* and *j* correspond to the younger and older stage, respectively, and the *variance* term was obtained from the family \times ontogenetic stage component of the full model used to calculate H^2 of the trajectories. Absolute values of evolvability were used to avoid negative values of decreasing ontogenetic trajectories, and were included for comparative purposes with other studies. We interpret all these genetic effects with caution because plant material was obtained from a single population and may not reflect broad patterns for *T. velutina*.

Developmental reaction norms

To estimate plasticity of the ontogenetic trajectories of each defensive trait, we performed full factorial linear mixed ANOVA models with REML, using ontogenetic stage and environment as fixed factors, and family with all possible interactions as random factors. Significance of ontogenetic stage \times environment term was considered as evidence of

developmental plasticity (Pigliucci and Schlichting 1995). Significance of random factors was tested as described above. All statistical analyses were performed in R 3.3.0 (R Development Core Team 2017), using lme4 package (Bates et al. 2015).

Results

Ontogenetic patterns in plant defense and risk of herbivore attack

As previously reported, we found non-linear ontogenetic changes in the three focal defensive traits of *T. velutina* (Ochoa-López et al. 2015), showing that the cyanogenic potential decreased and trichome density and sugar in EFN increased during plant development (Table 1, Fig. 2, suppl. material S1). Nevertheless, mean values of these traits and their degree of variation among families were contingent upon the ontogenetic stage and the environment: whereas trichome density and cyanogenic potential had greater expression in the field, plants produced more sugar in EFN within the shadehouse. In addition, greater variation among genetic families in the three defensive traits was observed in the field than within the shadehouse, but as a function of plant ontogeny: seedlings showed greater variation in cyanide potential; juvenile plants had more phenotypic variation in the expression of trichome density; and reproductive plants had greater variation in the production of extrafloral nectar than in the other defensive traits (Fig. 2). Interestingly, we found only one significant genetic correlation between trichome density and HCN at the juvenile stage ($r^2 = -0.57$, $P < 0.05$), and only for plants growing in the field. This suggests a possible trade-off between trichomes and HCN at this stage.

We found that *E. hegesia* butterflies preferred to oviposit more frequently on seedlings than on adult plants ($t = 3.308$, $d.f. = 4$, $P = 0.0297$; Fig. 3a). In addition, the percentage of leaf damaged by herbivores was greater for seedlings and decreased across plant ontogeny ($F_{2,736} = 24.42$, $P < 0.001$) such that seedlings had 1.5 and 2 times more damage than juvenile and reproductive plants, respectively (Fig. 3b).

Genetic variation, heritabilities, and evolvabilities of defensive traits and their ontogenetic trajectories

We detected evidence for genetic variation, heritability, and evolvability for trichome density and cyanogenic potential, but only when plants grew in the shadehouse. Moreover, whereas trichome density showed significant heritability and evolvability at all ontogenetic stages, cyanogenic potential was heritable only at the seedling stage (Table 2). In addition, we found that the ontogenetic trajectories of trichome density from seedlings to juvenile and from juvenile to

Table 1 Factorial mixed-model ANOVA testing for the effects of ontogenetic stage and full-sib family on cyanogenesis, trichome density and extrafloral nectar

Environment	Defense attribute	df	F or χ^2	P value
Field plots	Cyanogenic potential			
	Ontogenetic stage	2433	65.15	< 0.0001
	Family	1	1.94	0.1632
	Stage \times family	1	< 0.0001	0.9999
	Trichomes density			
	Ontogenetic stage	2433	101.27	< 0.0001
	Family	1	< 0.0001	0.9999
	Stage \times family	1	< 0.0001	0.9999
	Sugar in extrafloral nectar			
Ontogenetic stage	1433	60.13	< 0.0001	
Family	1	< 0.0001	0.9999	
Stage \times family	1	< 0.0001	0.9999	
Shadehouse	Cyanogenic potential			
	Ontogenetic stage	2962	75.11	< 0.0001
	Family	1	2.34	0.126
	Stage \times family	1	0.82	0.3634
	Trichomes density			
	Ontogenetic stage	2962	245.91	< 0.0001
	Family	1	69.05	< 0.0001
	Stage \times family	1	33.96	< 0.0001
	Extrafloral nectar production			
	Ontogenetic stage	1681	91.06	< 0.0001
	Family	1	< 0.0001	0.9999
	Stage \times family	1	< 0.0001	0.9999

The interaction term shows significant genetic variation of the ontogenetic trajectories and was used to assess the significance of their heritability (Bold values)

*For random effects, the *F*-ratio is replaced with Chi-square values obtained from the difference between the $-2 \log$ likelihoods with that factor included vs. excluded from the model (Littell et al. 1996; Bingham and Agrawal 2010)

reproductive stages showed significant genetic variation (i.e., ontogenetic stage \times family interaction) and significant, although rather low, heritability and evolvability values (Table 3).

Developmental reaction norms

We found significant phenotypic plasticity of ontogenetic trajectories for the three defensive traits, with different trajectories observed in the field vs. shadehouse (ontogenetic stage \times environment interaction, Table 4). However, the developmental reaction norms were different for each defensive trait. For example, cyanogenic potential had contrasting ontogenetic trajectories, with field plants showing significant declines across ontogeny while shadehouse plants tended to show no ontogenetic shifts or only a slight decrease

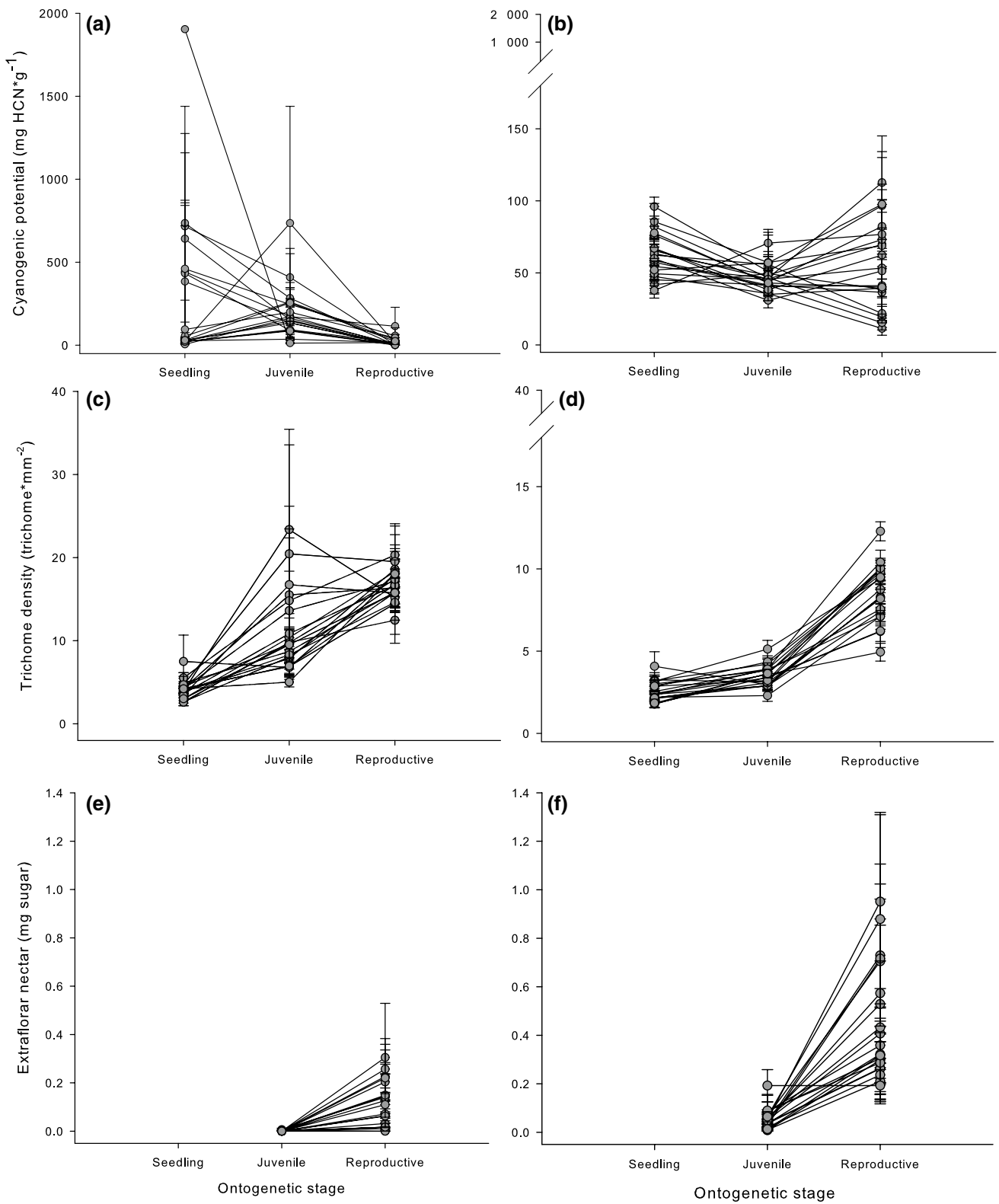


Fig. 2 Ontogenetic trajectories of defensive attributes in 20 genetic families of *Turnera velutina* plants growing in field plots (left side) or within a shadehouse (right side). Lines represent family means with standard error bars. Note the differences in scale

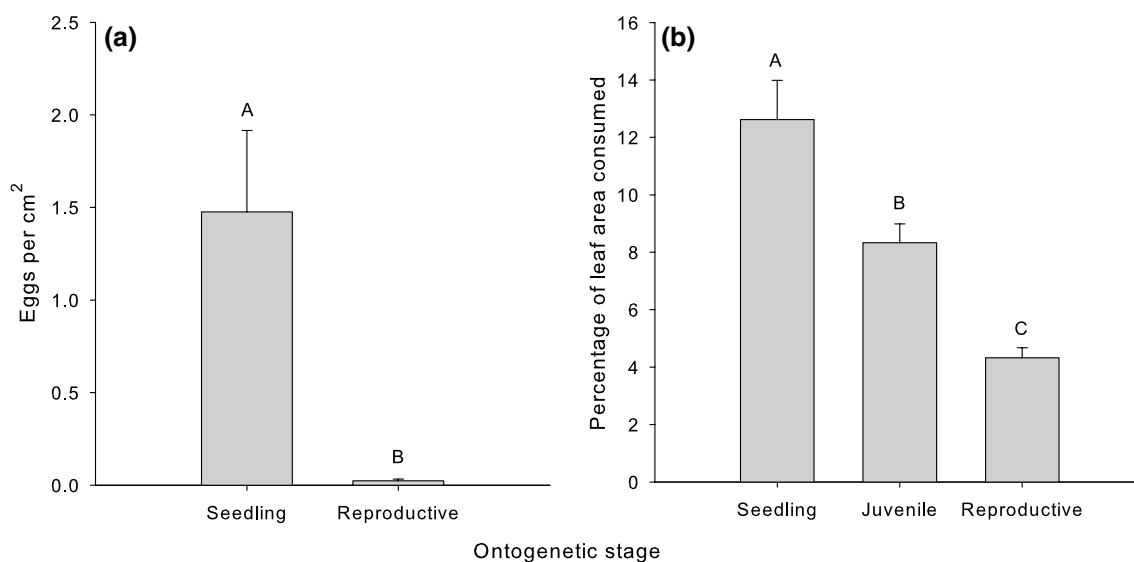


Fig. 3 Risk of attack of *T. velutina* across ontogenetic stages. **a** Oviposition preference of *E. hegesia* butterflies for seedlings or reproductive plants. Different letters represent significant difference among ontogenetic stages. **b** Percentage of leaf area consumed by herbivores

in seedlings, juvenile and reproductive plants of *Turnera velutina*. Different letters represent significant difference among ontogenetic stages ($P < 0.001$). Error bars indicate the standard error of the mean value

(Fig. 4a). In contrast, trajectories in trichome density and EFN sugar were consistent in directionality for field and shadehouse plants, but differed in the magnitude of change. Ontogenetic increases in trichome density were of greater magnitude in the field (Fig. 4b), while ontogenetic increases in EFN sugar were of greater magnitude in the shadehouse (Fig. 4c).

Discussion

A recent call has been made to move forward from the description of ontogenetic trajectories in plant defense towards the assessment of the mechanisms behind their evolution and/or regulation (Barton and Boege 2017). A first step to better understand the potential of ontogenetic trajectories to evolve through natural selection is to identify the different sources of their variation. In this study, we provide a first report on the genetic and environmental variation of three defensive traits (cyanogenic potential, trichome density and sugar in EFN), assessed at each ontogenetic stage. Our key findings were that the heritable component of variation increased during plant development for trichome density, but decreased during plant development for cyanogenic potential. In the case of trichomes, the amount of heritable variation increased as the risk of attack and herbivore damage intensity decreased, supporting the assumption that natural selection on this defensive trait is likely to be more intense at early ontogenetic stages. In addition, we detected significant heritability for ontogenetic trajectories, but only for trichome

density. However, this potential to evolve was contingent upon the environment in which plants grew, as heritabilities were not detected when plants faced different challenges in the field. These findings suggest that ontogenetic trajectories for trichome density in the studied population of *Turnera velutina* show plastic responses and that environmental heterogeneity can actually constrain their evolution as a complex trait.

Genetic variance and heritability of defensive traits in shadehouse plants

We found that the proportion of genetic variation in defensive traits changed as a function of plant age and type of defense. In the case of biotic defenses, only a couple of studies have reported a significant genetic component in the variance for the number (Wooley et al. 2007) and morphology (Rudgers 2004) of extrafloral nectaries, but not on the actual rewards offered to ants (i.e., sugar in EFN), and thus there are no available studies to which we can compare our results. The quality of EFN has been reported to be strongly affected by both the presence and identity of patrolling ants (Heil et al. 2009; Bixenmann et al. 2011), so it is not surprising that we did not find genetic variation in EFN sugar content in the absence of ants within the shadehouse. This lack of significant genetic variation suggests that in the studied population, EFN sugar is a highly inducible trait influenced by environmental variables. Inclusion of larger populations with different ant densities and greater genetic

Table 2 Genetic (V_{Fam}) and environmental (V_{Env}) components of phenotypic variance, coefficients of genetic (CV_G), and environmental (CV_E) variation, and broad sense heritabilities (H^2) for defensive traits within each ontogenetic stage of *Turnera velutina*

Defense attribute	V_{Fam} (%)	V_{Env} (%)	CV_G	CV_E	$H^2 \pm \text{SEM}$
Cyanogenesis					
Field plots					
Seedling	$2.09e^{-14}$ (7.76e ⁻¹³)	2.69 (100)	$5.78e^{-10}$	0.0117	0.0009 ± 0.1121
Juvenile	0.0996 (2.7)	3.570 (97.3)	0.0027	0.0167	0.05451 ± 0.0289
Reproductive	0.0834 (3.5)	2.266 (96.4)	0.022	0.1149	0.0711 ± 0.0492
Shadehouse					
Seedling	0.062 (8.8)	0.641 (91.2)	0.0055	0.0179	0.1766 ± 0.0473
Juvenile	0 (0)	0.957 (100)	0	0.0297	0.00023 ± 0.0011
Reproductive	0.0741 (2.1)	3.396 (97.9)	0.0069	0.0467	0.0426 ± 0.0215
Trichomes density					
Field plots					
Seedling	0.0079 (9)	0.0795 (90)	0.03	0.0953	0.1806 ± 0.0093
Juvenile	$7.40e^{-19}$ (1.12e ⁻¹⁶)	0.659 (100)	$7.79e^{-11}$	0.0735	$1.41e^{-15} \pm 4.94e^{-15}$
Reproductive	0 (0)	0.109 (100)	0	0.0278	$2.78e^{-16} \pm 2.78e^{-16}$
Shadehouse					
Seedling	0.0132 (10.9)	0.1076 (89.1)	0.0617	0.1763	0.2186 ± 0.0431
Juvenile	0.0155 (14.9)	0.0884 (85.1)	0.0501	0.1197	0.2973 ± 0.0571
Reproductive	0.0393 (28.4)	0.0990 (71.6)	0.0329	0.0522	0.5682 ± 0.0854
Sugar in Extrafloral nectar					
Field plots					
Seedling	NA*	NA*	NA*	NA*	NA*
Juvenile	$3.22e^{-19}$ (1.79e ⁻¹²)	$1.79e^{-5}$ (100)	$2.37e^{-6}$	10.071	$1.27e^{-14} \pm 4.37e^{-14}$
Reproductive	$1.81e^{-16}$ (5.51e ⁻¹³)	$3.28e^{-2}$ (100)	$1.49e^{-7}$	2.0152	$3.56e^{-15} \pm 3.56e^{-15}$
Shadehouse					
Seedling	NA*	NA*	NA*	NA*	NA*
Juvenile	0.0004 (4.3)	0.0100 (95.7)	0.5535	2.5923	0.08668 ± 0.0476
Reproductive	0 (0)	0.162 (100)	0	1.2754	$3.045e^{-15} \pm 1.18e^{-14}$

Values in parentheses represent the percentage of total phenotypic variation. Significant H^2 values are shown in bold and were inferred from the significance of the family term

*These values are not available because seedlings do not produce extrafloral nectaries at all

variation could shed light on whether this is a general trend for *T. velutina*.

In the case of cyanogenic potential, genetic variation was detected only early in the ontogeny of plants, which does not support our original hypothesis of a negative relationship between the expression of defense, and the amount of heritable variation. The fact that the main herbivore of *T. velutina*, *E. egesia* is a specialist herbivore capable of sequestering cyanogenic compounds (Schappert and Shore 1999), can help to explain oviposition preferences and why this trait has not been fixed as an effective defense at this vulnerable ontogenetic stage. Tolerance, an alternative mechanism to deal with herbivore damage, actually has been reported to be greater for seedlings than in older stages (Ochoa-López et al. 2015), and is likely to represent an alternative mechanism of *T. velutina* to deal with herbivore damage, given the apparent low effectiveness of cyanogenesis. Previous work with *Eucalyptus polyanthemus* has reported that cyanogenic potential is largely genetically determined with little environmental

effects (particularly of nitrogen availability; Goodger et al. 2004). What is novel from our results is the finding that the genetic component of the variance in cyanogenic potential varies across plant development.

For trichome density, we found that heritability increased as plants developed, which actually supports our hypothesis of lower heritability at the most vulnerable stages. However, the fact that this defensive trait also had their lowest levels of expression at this ontogenetic stage suggests that seedlings either rely on other defensive mechanisms (i.e., cyanogenesis or tolerance; Ochoa-López et al. 2015) or have developmental constraints or trade-offs limiting the amount of trichomes they can produce at this stage.

One study has previously reported a decrease in heritability of trichome density during plant development in *Arabidopsis thaliana* (Mauricio 2005). However, in that study, leaf age was not controlled across plant development, and hence it is difficult to disentangle shifts due to leaf ontogeny from those of whole-plant ontogeny. For *T. velutina* in contrast,

Table 3 Variance of genetic and environment interaction ($V_{\text{Fam} \times \text{Stage}}$) and environmental (V_{Env}) components, coefficients of genetic (CV_G) and environmental (CV_E) variation and broad sense heritability (H^2) of two ontogenetic transitions of defensive traits in *Turnera velutina*

Defense attribute	$V_{\text{fam} \times \text{stage}}$ (%)	V_{Env} (%)	CV_G	CV_E	$H^2 \pm \text{SEM}$
Cyanogenesis					
Field plots					
Seedling–Juvenile	0.0763 (2.2)	3.3063 (97.7)	0.0029	0.0193	0.0451 \pm 0.0250
Juvenile–Reproductive	0 (0)	3.052 (95.7)	0	0.0187	1.01e ⁻¹⁶ \pm 4.78e ⁻¹⁶
Shadehouse					
Seedling–Juvenile	0.0248 (2.9)	0.8216 (97.1)	0.0138	0.0795	0.0569 \pm 0.0305
Juvenile–Reproductive	0.0170 (0.79)	2.1020 (98.4)	0.0200	0.2225	0.0164 \pm 0.0158
Trichomes density					
Field plots					
Seedling–Juvenile	0 (0)	0.49 (100)	0	0.2567	0.00005 \pm 0.0004
Juvenile–Reproductive	0 (0)	0.443 (100)	0	0.0071	9.32e ⁻¹⁶ \pm 3.72e ⁻¹⁵
Shadehouse					
Seedling–Juvenile	0.0091 (8.2)	0.0970 (87.1)	0.1449	0.4714	0.1647 \pm 0.0455
Juvenile–Reproductive	0.0175 (14.5)	0.0935 (77.5)	0.0203	0.0469	0.2938 \pm 0.0465
Extrafloral nectar production					
Field plots					
Seedling–Juvenile	NA*	NA*	NA*	NA*	NA*
Juvenile–Reproductive	1.18e ⁻¹⁷ (9.2e ⁻¹⁴)	0.0128 (100)	3.68e ⁻¹¹	0.0012	4.84e ⁻¹⁵ \pm 2.32e ⁻¹⁴
Shadehouse					
Seedling–Juvenile	NA*	NA*	NA*	NA*	NA*
Juvenile–Reproductive	0 (0)	0.0835 (100)	0	0.0443	7.91 e ⁻⁵ \pm 0.0007

Values in parentheses represent the percentage of total phenotypic variation. Significant heritability values of the ontogenetic trajectories are shown in bold and were inferred from the significance of the family \times stage interaction term

*These values are not available because seedlings do not produce extrafloral nectaries at all

we controlled for leaf age and show that heritability and evolvability of trichome density increased as plants developed from seedlings to reproductive plants. In addition, the existence of significant heritability at the three ontogenetic stages represents an opportunity for ontogenetic trajectories to evolve (see below). In general, both the heritability and evolvability of trichomes and HCN were low compared to what has been previously reported for these traits (Schappert and Shore 2000; Geber and Griffen 2003; Johnson et al. 2009). This might be explained by the fact that we used plant material from a single population. Further investigations should assess if this is the case in different populations of *T. velutina*, which have a non-continuous distribution of along the coastline (Arbo 2005), with probable reduced gene flow among them.

Sources of variation and evolutionary potential of ontogenetic trajectories in defensive traits within the shadehouse

Interestingly, in the absence of the interaction with herbivores and ants under a homogeneous environment in the shadehouse, the ontogenetic trajectories of each defensive trait included different primary sources of variation. In the

case of cyanogenic potential and EFN sugar, ontogenetic trajectories a large proportion of the variation (between 95 and 100%) was unexplained by plant genotypes. In contrast, for trichome density we found significant evolutionary potential of their ontogenetic trajectories at the two developmental transitions, even though a greater heritability was found for the transition from juvenile to reproductive stages than for the transition from seedling to juvenile stages. Hence, if trichome density represents an effective barrier against herbivory at least in one developmental stage, the ontogenetic trajectories could have the potential to evolve through natural selection. However, as discussed below, environmental effects reduced this evolutionary potential under field conditions.

Influence of the environment on the heritable component of defensive traits and their ontogenetic trajectories

Environmental stress has been predicted to either increase or decrease heritable variation, as a consequence of the release and amplification of phenotypic variation (reviewed by Hoffmann and Merilä 1999; Badyaev 2005). In our study, when the same genotypes used in

Table 4 Factorial mixed-model ANOVA for the effects of ontogenetic stage, environment, and full-sib family on cyanogenesis, trichome density and extrafloral nectar*

Defense attribute	df	F or χ^2	P value
Cyanogenesis			
Ontogenetic stage	2,1396	130.34	< 0.0001
Environment	1,1396	24.32	< 0.0001
Family	1	0.84	0.3592
Stage \times environment	2,1396	15.63	< 0.0001
Stage \times family	1	1.41	0.2349
Environment \times family	1	3.87	0.049
Stage \times environment \times family	1	< 0.00001	0.99999
Trichome density			
Ontogenetic stage	2,1396	367.4	< 0.0001
Environment	1,1396	350.69	< 0.0001
Family	1	34.63	< 0.0001
Stage \times environment	2,1396	12.85	< 0.0001
Stage \times family	1	0.8299	0.3623
Environment \times family	1	0.11	0.7352
Stage \times environment \times family	1	8.15	0.004
Extrafloral nectar			
Ontogenetic stage	1,1025	86.84	< 0.0001
Environment	1,1025	38.01	< 0.0001
Family	1	< 0.00001	1
Stage \times environment	1,1025	11.65	0.0006
Stage \times family	1	< 0.00001	0.99999
Environment \times family	1	< 0.00001	0.99999
Stage \times environment \times family	1	0.01	0.9182

Stage \times environment \times family interaction term shows significant genetic variation of the ontogenetic reaction norms of defensive attributes. Bold values indicate significant terms

*For random effects, the *F*-ratio is replaced with Chi-square values obtained from the difference between the $-2 \log$ likelihoods with that factor included vs. excluded from the model (Littell et al. 1996; Bingham and Agrawal 2010)

the shadehouse were exposed to field conditions, the heritable component of cyanogenic potential, trichome density, and the ontogenetic trajectory of the latter were minimized. We suggest that stressful conditions in the field (i.e., herbivore attack, high light intensity and water stress) triggered plastic responses, thereby masking the heritable variation detected in shadehouse plants growing under more homogeneous conditions and in the absence of multiple biotic interactions. Hence, the evolutionary potential of these defensive traits and their ontogenetic trajectories (in the case of trichome density) are likely to be constrained by phenotypic plasticity (Blum 1988; Gebhardt-Henrich and Van Noordwijk 1991).

Developmental reaction norms or plasticity in the ontogenetic trajectories

More than 20 years ago, Pigliucci and Schlichting (1995) proposed the concept of developmental reaction norms to address changes in phenotypic plasticity during ontogeny, and highlighted the importance to merge the study of environmental effects and developmental changes on the expression of phenotypes to understand the mechanisms behind phenotypic plasticity. Ontogenetic reaction norms have been previously reported in plants for phenological timing, plant height, and leaf number (Pigliucci and Schlichting 1995), leaf area and biomass (Turkington 1983; Roach 1986), and also in animals for head morphology in cichlid fishes (Meyer 1987), body length in *Daphnia* (Ebert et al. 1993), and the induction of spines in dragonfly larvae (Arnqvist and Johansson 1998). In this study, we report for the first time developmental reaction norms, or ontogenetic changes in the phenotypic plasticity of three plant resistance traits. In the case of cyanogenic potential and trichome density, both mean values and phenotypic variation were greater in the field than within shadehouse conditions, and the opposite pattern was observed for sugar in extrafloral nectar. This plasticity was probably triggered by the presence and variation of different environmental challenges, such as light exposure, water stress, herbivory, and ant patrolling. For example, the absence of ants within the shadehouse likely triggered an decrease in the rewards to recruit potential defenders.

We also report that ontogenetic reaction norms of trichome density had a genetic component (i.e., significant ontogenetic stage \times environment \times family), indicating that genotypes differed in their ability to modify their ontogenetic trajectories as a function of the environmental challenges. This variation in the ontogenetic trajectories across environments offers the opportunity to further investigate the conditions under which particular trajectories are adaptive, and further identify likely agents of selection and/or developmental constrains. The understanding of the regulatory genes underlying the described ontogenetic changes in these traits would be particularly revealing and enhance our understanding of the mechanisms driving adaptive plasticity (Arnqvist and Johansson 1998; Mauricio 2005). Overall, we provide evidence that the ontogenetic trajectories of some defensive traits are heritable, but contingent upon the environment in which plants grow. From this, we can now evaluate the effect of selective pressures and demographic consequences of these complex phenotypes, to comprehend the mechanisms behind the drivers of evolution in plant defense. Nevertheless, because heritability values reported in this work represent the variation from a single population of *T. velutina*, we recommend further studies to assess genetic variation in ontogenetic

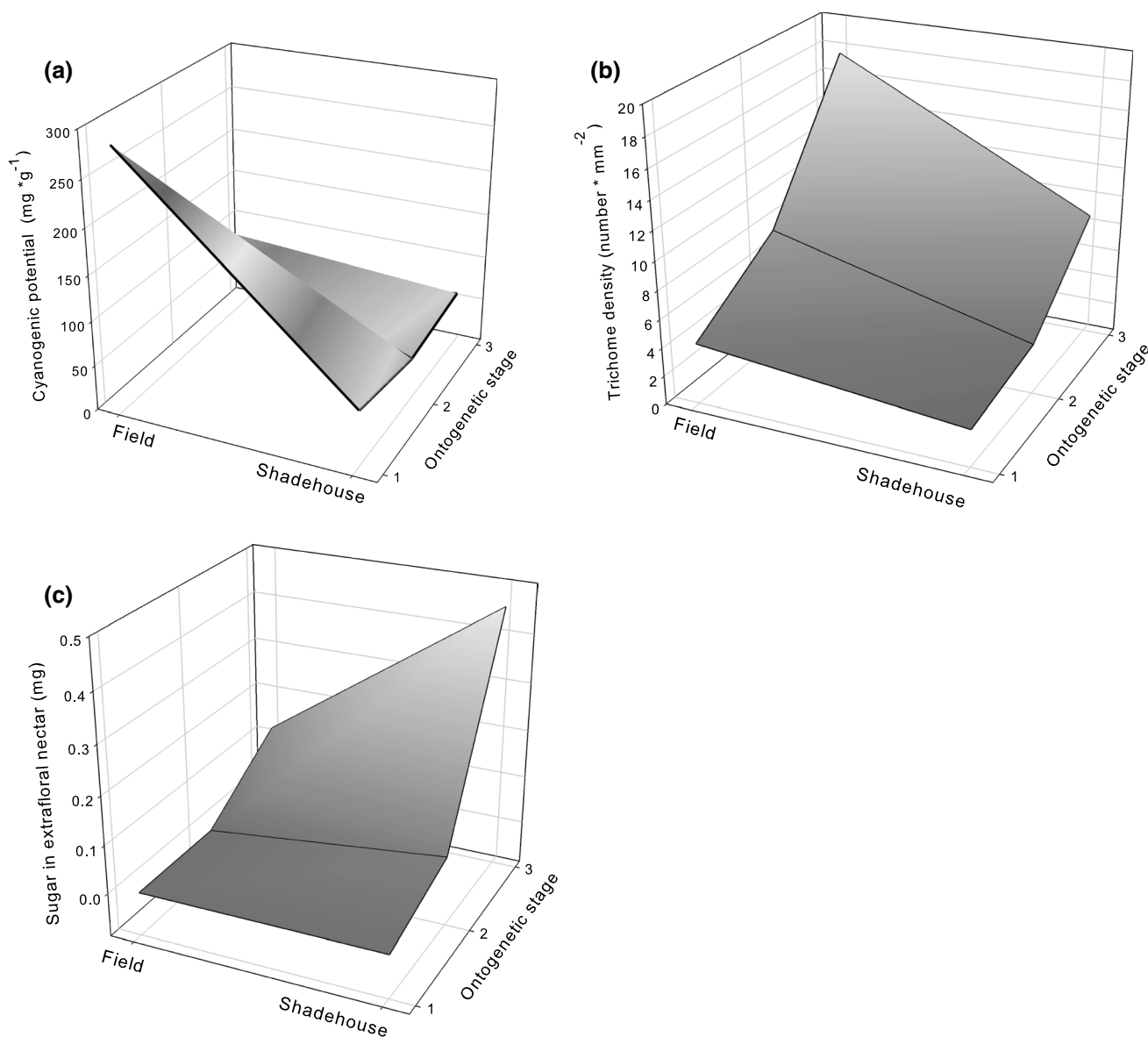


Fig. 4 Ontogenetic reaction norms of *Turnera velutina* of: **a** cyanogenic potential, **b** trichome density and **c** extrafloral nectar expressed during three ontogenetic stages: (1) seedlings, (2) juvenile and (3) reproductive under shadehouse and field conditions

trajectories across populations, to confirm that the trends found for this population represent broad patterns for the species.

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Capítulo 2:
Cambios ontogenéticos en los objetivos de selección natural en las defensas de plantas

Ontogenetic changes in the targets of natural selection in three plant defenses

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Summary

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- The evolution of plant defenses has traditionally been studied at single plant ontogenetic stages, overlooking the fact that natural selection acts continuously on organisms along their development, and that the adaptive value of phenotypes can change along ontogeny.
- We exposed 20 replicated genotypes of *Turnera velutina* to field conditions to evaluate whether the targets of natural selection on different defenses and their adaptive value change across plant development.
- We found that low chemical defense was favored in seedlings, which seems to be explained by the assimilation efficiency and the ability of the specialist herbivore to sequester cyanogenic glycosides. Whereas trichome density was unfavored in juvenile plants, it increased relative plant fitness in reproductive plants. At this stage we also found a positive correlative gradient between cyanogenic potential and sugar content in extrafloral nectar.
- We visualize this complex multi-trait combination as an ontogenetic defensive strategy. The inclusion of whole-plant ontogeny as a key source of variation in plant defense revealed that the targets and intensity of selection change along the development of plants, indicating that the influence of natural selection cannot be inferred without the assessment of ontogenetic strategies in the expression of multiple defenses.

Introduction

Both plants and animals display considerable ontogenetic variation in the expression of phenotypic traits, allowing them to deal with stage-specific selective pressures and different environmental challenges and ecological contexts as they develop. This variation can be promoted by resource allocation needs to different functions (e.g. growth, reproduction, defense), physiological and ecological costs, and/or restrictions inherent to developmental processes (Herms & Mattson, 1992; West *et al.*, 2001; Hou *et al.*, 2008; Maherali *et al.*, 2009). Furthermore, in many cases, individuals may change their habit or resource use as they develop (i.e. ontogenetic niche shifts; Wilbur, 1980). In this context, morphological, physiological and/or behavioral traits expressed at particular ontogenetic stages can determine survival rates to the following stages, ultimately influencing their reproductive success (Gagliano *et al.*, 2007). Hence, natural selection is expected to adjust the phenotypic expression of multiple traits involved in antagonistic and mutualistic interactions across the lifetime of individuals (Perez & Munch, 2010). For example, life-history studies have revealed that ontogenetic loss in decoy tail coloration in skink lizards can increase survival against predators when individuals are young, but this selective force is relaxed when they are older (Watson *et al.*, 2019).

Similarly, changes in the direction and strength of natural selection have been reported for the reef fish *Pomacentrus amboinensis*, with shifts from favoring small sizes early in their ontogeny to prevent starving, to faster growth in older individuals to reduce predation risk (Gagliano *et al.*, 2007). In the case of plants, despite the accumulated evidence of changes in plant defensive traits (Senner *et al.*, 2015) during the development of leaves (Coley & Barone, 1996; Koricheva & Barton, 2012; Wiggins *et al.*, 2016; Barton *et al.*, 2019) and during the whole ontogeny of individuals (Boege & Marquis, 2005; Barton & Koricheva, 2010), the study of natural selection on such traits has mostly focused on single points across the lifetime of individuals (Barton & Boege, 2017; but see Cope *et al.*, 2019), usually controlling for leaf age (Mauricio & Rausher, 1997; Tiffin & Rausher, 1999; Agrawal *et al.*, 2008a). A few reports suggest, however, that the intensity of natural selection can change across plant ontogeny (Tiffin, 2002; Gómez, 2008), promoting ontogenetic trajectories of ecophysiological (Maherali *et al.*, 2009) and defensive traits (Cope *et al.*, 2019). Whereas the degree to which natural selection can produce evolutionary responses and shape ontogenetic trajectories in plant defense depends on the degree of heritability and the influence of environmental variation, which can vary for different defensive traits. What remains to be explored is how natural selection acts

on combinations of multiple defenses across plant development, promoting ontogenetic defensive strategies.

Here, we used a comprehensive approach focusing on the whole-plant ontogeny level, defined as the development of plants through different discrete ontogenetic stages (i.e. from seedling to juvenile/sapling, mature and finally senescent stages). Changes in the expression of defensive traits over the lifetime of plants (hereafter ontogenetic trajectories, *sensu* Boege & Marquis, 2005) have been reported for many plant species (Boege & Marquis, 2005; Barton & Koricheva, 2010; Quintero *et al.*, 2013), but it remains unclear which are the drivers or mechanisms behind such ontogenetic variation. Because herbivore pressure can be variable along plant development (Hanley *et al.*, 1995; Fenner *et al.*, 1999; Warner & Cushman, 2002; Quintero *et al.*, 2013), it can act as a selective force favoring the expression of greater values of defensive traits at the most vulnerable plant ontogenetic stages (Agrawal *et al.*, 2012; Barton & Boege, 2017). For example, a recent study shows that the adaptive value of salicinoid phenolic glycosides changes during the ontogeny of *Populus tremuloides* (Cope *et al.*, 2019). The benefit of particular defensive traits is likely to change during plant development as a function of the physiological priorities of different plant stages (Herms & Mattson, 1992), the fitness value of different organs (Boege & Marquis, 2005; Barton & Koricheva, 2010; Villamil, 2017), their efficiency deterring specific herbivores (Van Bael *et al.*, 2003; Boege, 2005; Boege & Marquis, 2006) and/or as a result of ontogenetic niche changes derived from the interaction with different species (Perez & Munch, 2010; Fonseca-Romero *et al.*, 2019; Villamil *et al.*, 2019).

Myrmecophytic plant species represent an especially useful system to examine ontogenetic changes in the adaptive value of defensive traits, because developmental and architectural limitations constrain the expression of rewards for mutualistic ants in young plant stages (Quintero *et al.*, 2013). Hence, their ecological niche changes from being nonmyrmecophytic to the interaction with their ant partners. We hypothesize that myrmecophytic plants should benefit from expressing high values of direct defenses in younger stages, when damage by herbivores and fitness costs are usually high, but they do not yet have the protection by ants (Fig. 1). By contrast, if ant defense is either more effective or less costly for the plants than the production of direct defenses, then natural selection should favor a reduction in the latter and greater production of rewards for ants (Fig. 1), according to the fitness benefits promoted by the presence of their mutualistic partners (Palmer *et al.*, 2010; Stanton & Palmer, 2011; but see Fonseca-Romero *et al.*, 2019).

Several studies have reported direct selection on individual or multiple defense traits such as trichomes (Valverde *et al.*, 2001), secondary metabolites (Mauricio & Rausher, 1997; Shonle & Bergelson, 2000; Agrawal *et al.*, 2008b; Agrawal, 2011) and even biotic defenses (Rudgers, 2004; Rutter & Rausher, 2004; Kessler & Heil, 2011; Quintero *et al.*, 2013). However, plant age, size or ontogenetic stage are usually controlled to assess the direct and indirect impacts of these traits on relative fitness. In this context, the aim of this study was to quantify changes in the intensity of natural selection on different defense traits and assess their

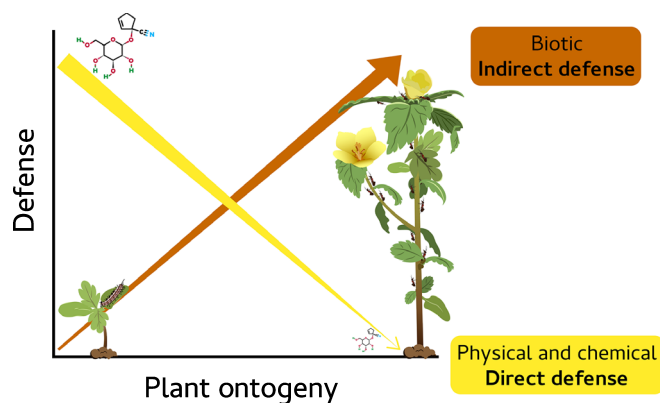


Fig. 1 Prediction for the ontogenetic trajectories in plant indirect and direct defenses in myrmecophytic species.

adaptive value across the ontogeny of a myrmecophytic plant species. Because early ontogenetic stages do not produce rewards for patrolling ants, we predicted that physical and/or chemical defense should be favored by natural selection at these stages. By contrast, later during plant development, when plants are able to produce rewards for ants, the adaptive value of these indirect defenses should increase as a result of the protection by patrolling ants (Rutter & Rausher, 2004), and because they can be less costly than direct resistance traits (Kessler & Heil, 2011). Accordingly, ontogenetic differences in the amount of plant defenses were expected to influence herbivore efficiency to assimilate plant tissues and hence their biomass accumulation.

In a previous study, we reported that risk of attack and leaf damage by herbivores on *Turnera velutina* plants used in this study were both greater for seedlings than for older stages (Ochoa-López *et al.*, 2018), which suggests differences in the intensity of natural selection by herbivores across plant ontogeny. In this study, we assessed how the simultaneous phenotypic expression of three main defensive traits (hydrocyanic acid, trichome density (TD) and sugar in extrafloral nectar (SEFN) associated with ant patrolling) at three ontogenetic stages (seedling, juvenile and reproductive) influence the number of seeds produced (i.e. fitness) when plants reached their reproductive stage. We report ontogenetic changes in the direction and strength of natural selection on these defenses and the adaptive value of their joint expression across plant development. Lastly, to further assess the effectiveness of those defenses against the main herbivore, the specialist *Euptoieta hegesia*, we quantified herbivore assimilation efficiency, sequestration of hydrocyanic acid and performance (biomass gain) when larvae fed on tissues from different ontogenetic stages of *T. velutina*.

Materials and Methods

Study system

Turnera velutina Presl. (Passifloraceae) is a myrmecophytic shrub endemic to Mexico growing in coastal sand dunes and tropical dry forests (Arbo, 2005; Villamil *et al.*, 2013). *Turnera velutina* produces axillary flowers, and their fruits are dehiscent capsules

with an average of 36 seeds (Sosenski *et al.*, 2017). Flowering occurs mostly from May to June. In this species, significant ontogenetic trajectories have been described for the production of hydrocyanic acid, TD and SEFN (Ochoa-López *et al.*, 2015). Heritable variation has been reported only for the expression of TD (Ochoa-López *et al.*, 2018). In addition, risk of attack by herbivores has been found to be greater in young seedlings than in reproductive plants, as females of the specialist butterfly *Euptoieta hegesia* Cramer (Lepidoptera: Nymphalidae) prefer to oviposit on leaves of the younger stage (Ochoa-López *et al.*, 2018). Accordingly, the percentage of leaf damage by herbivores has been reported to be 1.5- to two-fold greater for seedlings than for older stages (Ochoa-López *et al.*, 2015).

Experimental population

An experimental population of *T. velutina* was established in the coast of Veracruz, Mexico (19°36'N, 96°22'W), within the grounds of the Centro de Investigaciones Costeras La Mancha (CICOLMA). The climate in this area is warm sub-humid, with an average annual temperature ranging between 21.1°C in January and 27.3°C in June, and an annual precipitation ranging from 899 to 1829 mm (Travieso-Bello & Campos, 2006), mostly occurring between June and September. In June 2013, we produced an F₁ generation ($N=300$ plants) of *T. velutina*, using seeds from 20 maternal plants naturally growing in the shaded parts of the sand dunes. Flowers were self-crossed and excluded from pollinators and fruits were collected when ripe. After removing the elaiosomes, seeds from 20 maternal families were placed in germination trays in a mixture of local soil and vermiculite (1:1). Trays were bottom-watered for 3 wk until germination. F₁ plants were then transplanted into 21 pots and watered until their reproductive stage. In June 2014, between nine and 16 F₁ plants per family were self-pollinated (using several flowers from each plant) to obtain a total of 2000 (F₂) full-sib seeds. We germinated 75 seeds/family as previously described to produce 1200 seedlings. On August 2014, we established 20 plots (1 × 1 m) in the sand dune under the partial shade of vegetation canopy. When plants produced their first true leaf, between three and 10 individuals per family were transplanted to experimental plots using a 10 × 10 cm grid, where they were exposed to all biotic interactions and natural environmental variation.

Ontogenetic trajectories in plant defense

Although we generated plants from 20 genetic families, we only used 16 of them, with at least six surviving plants per ontogenetic stage. Subsets of plants were used to describe the ontogenetic changes in plant defense at three different ontogenetic stages. We quantified hydrocyanic acid (HCN), as a measure of the cyanogenic potential of leaves (Ballhorn *et al.*, 2005), TD, and the amount of sugar produced in extrafloral nectar (SEFN) as a reward for patrolling ants. HCN and TD were quantified once plants reached one of three ontogenetic stages: seedlings (plants

with the first two true leaves fully expanded, $N=213$), juvenile (plants with the 10th leaf fully expanded, $N=220$), and reproductive (plants bearing the first flowers, $N=225$). SEFN was quantified only in the latter two stages, as seedlings do not produce extrafloral nectaries. Although we are aware that development is a continuous process (Ellner & Rees, 2007; Rees & Ellner, 2009; Merow *et al.*, 2014), we chose these ontogenetic stages because they represent three key transitions in the functional priorities of plants (Boege & Marquis, 2005). Specific methods for assessing each defense are described in Ochoa-López *et al.* (2018). Briefly, using a colorimetric test with sodium picrate, we assessed cyanogenic potential (HCN content) on the most apical fully expanded leaf of each ontogenetic stage (i.e. plants were measured only once) (Schappert & Shore, 1995). To estimate HCN content ($\mu\text{g HCN g}^{-1}$ DW), we used the formula $\mu\text{g HCN} = (\text{optical density of sample at } 590 \text{ nm} - \text{optical density of blank}) / 0.0001236$, obtained from a standard curve ($r^2 = 0.93$, $P < 0.0001$) using sodium cyanide (Code 7660-1; Caledon Laboratories Ltd, Halton Hills, Canada) as a source of HCN. TD (no. of trichomes mm^{-2}) was measured in the penultimate fully expanded leaf of plants at each ontogenetic stage, using a stereoscopic microscope. Indirect defense was estimated by quantifying SEFN in the three most apical fully expanded leaves (only for juvenile and reproductive plants).

Plant fitness

In plants that reached maturity in the experimental population, we quantified seed number from ripe fruits collected monthly between October 2014 and February 2016.

Herbivore performance

A colony of *E. hegesia* was established in cages inside the glasshouse at the biological field station of CICOLMA. Caterpillars were collected ($N=84$) shortly after hatching and reared in individual plastic containers, where they were randomly assigned to a diet consisting of either fully expanded leaves of reproductive plants or whole seedlings obtained from the same F₂ genotypes (but different from the ones used for the natural selection experiment). Dehydration was avoided by covering petioles and seedling roots with a cotton ball. Plant material was offered to larvae *ad libitum* for 6 d. We estimated larval performance by registering their weight, length and head diameter on a daily basis. During this period, weight of fresh plant tissue was registered when added or subtracted from each container. Assimilation efficiency was estimated as the weight gained by the caterpillar divided by the weight of fresh food eaten during the 6 d. Because *E. hegesia* has been reported to sequester cyanogenic glycosides in *Turnera ulmifolia* (Schappert & Shore, 1999), we assessed whether this was the case for the larvae feeding on *T. velutina*. We assessed HCN contents in the bodies of each larva after feeding on either seedlings ($N=25$) or reproductive plants ($N=25$). Caterpillars were previously, frozen, lyophilized and dissected. Guts were removed to make sure that the released cyanide was indeed in the caterpillar's tissues. Released cyanide of

the larvae tissue was measured using the same protocols as previously described for leaf tissue (Schappert & Shore, 1995; Ochoa-López *et al.*, 2018).

Statistical analyses

Influence of plant defenses across ontogeny on seed production We first evaluated if the three defensive traits changed along plant development, using a MANOVA and *post hoc* ANOVAs per defensive trait. Models included ontogenetic stage as a fixed factor, and family as a random explanatory variable. Then, to assess if the phenotypic effects of each plant defense on plant fitness varied as a function of the ontogenetic stage in which they are expressed, we adjusted two generalized mixed models with a Poisson distribution (LME4 package, R; Bates *et al.*, 2015). The first was used to assess linear effects of each defense on seed production, considering family as a random explanatory variable, and ontogenetic stage, cyanogenic potential, TD, SNEF, and their interactions as explanatory fixed variables. The interaction between ontogeny and SEFN was not included in the model, as seedlings and most juvenile plants did not produce nectar. The second model was used to assess nonlinear effects of the expression of plant defense across ontogeny on seed production. In addition to the previously described fixed and random variables, the quadratic terms of each defense and their interaction with ontogeny were included in the model. Values of each defensive trait were standardized as $\frac{(x_i - \bar{x})}{\sigma}$, where \bar{x} and σ represent the mean value and standard deviation, respectively, for each defense. Relative fitness values (number of seeds produced by each individual/population average of seed production) were considered as the response variable. A significant interaction between a given defense and ontogeny was considered as evidence that their adaptive value varied as a function of the ontogenetic stage in which they were expressed.

Ontogenetic changes in the intensity of phenotypic natural selection To assess ontogenetic changes in the direction and/or intensity of natural selection on the three defenses, we performed phenotypic selection analyses, one for each ontogenetic stage, using a multiple linear regression model (Lande & Arnold, 1983), and standardized values of each defensive trait to predict relative plant fitness. After finding a lack of multivariate normal distribution of the three defenses (package MVN; Korkmaz *et al.*, 2014) and exploring their collinearity through pairwise correlations and variance inflation factors, (package CAR, R software; Fox & Weisberg, 2011), we followed the approximation of Palacio *et al.* (2019) for multiple phenotypic traits with skewed distributions. We performed two independent models at each ontogenetic stage to obtain unbiased linear and nonlinear selection gradient estimates. Whereas linear models included only the defensive traits as explanatory variables, nonlinear models included the defensive traits, their interactions and quadratic terms. Because the residuals of our models departed from a normal distribution, to assess significance of the selection gradients we used nonparametric bootstrapping to estimate SEs with 95% bias-corrected bootstrap confidence intervals (Efron &

Tibshirani, 1994) with the package BOOT (Canty & Ripley, 2017). Significant selection gradients were defined as those not including zero values within the confidence intervals.

Phenotypic selection analyses are useful to understand the ecological effects of the expression of multiple defenses across development on plant fitness, but do not allow the inference of the potential evolutionary responses of such ontogenetic trajectories. For those traits with significant genetic variation, considering genotypic means rather than phenotypic values ensures that the correlation between fitness and a given trait is genetically determined and not influenced by the environment (Rausher, 1992). Hence, genotypic selection gradients were calculated for TD, for which significant genotypic variation and heritability have been previously reported in the studied population (Ochoa-López *et al.*, 2018). With this purpose, a standard selection analysis was performed for each ontogenetic stage using a linear regression model (Lande & Arnold, 1983) with the standardized values of the family means of TD per stage as explanatory variables, and the average relative fitness for each maternal family.

Defensive ontogenetic strategies To explore the idea that different genotypes may express particular defensive ontogenetic strategies, which in turn should influence plant fitness, we assessed if particular combinations of the three defenses at each ontogenetic stage had an adaptive value. With this aim, we used the genotypic means of the three defense values per ontogenetic stage and performed a principal component analysis (PCA). Given the lack of genetic correlations among the three defenses across plant ontogeny (Ochoa-López *et al.*, 2018), the mean genotypic value of each defense at a given ontogenetic stage was considered as an independent variable of the same defense in the other ontogenetic two stages. To visualize the ontogenetic strategies of defense for each genotype, we plotted the scores of the first and second principal components (PC1, PC2). This plot allowed the visualization of the functional space of multi-trait defensive strategies across plant ontogeny and revealed how plants expressed singular combinations of the three defenses at each ontogenetic stage. Then, we used the scores of the first principal component (which explained *c.* 30% of the total variation) to perform a multiple linear regression analysis, considering the relative fitness as the response variable, and the score of each ontogenetic stage as explanatory variables. Significant effects of the PC scores at a given stage would mean that the particular combination of defenses at that stage influenced plant fitness. Interactions among the scores of the three ontogenetic stages were also included to assess the adaptive value of multi-trait defensive strategies across plant ontogeny.

To evaluate herbivore performance as a function of plant ontogenetic stage, we used a generalized linear mixed model with repeated measures (Bates *et al.*, 2015), considering day and ontogenetic stage of their host plant as fixed factors and caterpillar individual number as a random factor. An ANOVA was used to estimate the effect of diet on assimilation efficiency, considering the initial caterpillar weight as a covariate. The amount of HCN sequestration when feeding on seedlings or reproductive plants was assessed using an ANOVA with the ontogenetic stage of their

food as fixed factor and considering the total amount of fresh leaf biomass consumed by larvae as a covariate. All statistical analyses were performed in R v.3.6.0 (R Development Core Team, 2019).

Results

Ontogenetic trajectories in plant defense

After 2 years in the field, *T. velutina* plants from 16 genotypes showed significant ontogenetic trajectories in the expression of three defensive traits (Wilks' $\lambda = 0.2642$, $F_{2,639} = 200.73$, $P < 0.0001$; Fig. 2), as previously reported (Ochoa-López *et al.*, 2018). The main pattern was that cyanogenic potential decreased almost twice from the seedling to the reproductive stage ($F_{2,51} = 7.52$, $P = 0.002$; Fig. 2a), TD increased four-fold ($F_{2,51} = 386.9$, $P = 0.0001$; Fig. 2b) and SEFN was produced only in reproductive plants (Fig. 2c). This pattern was consistent for all genotypes (family, Wilks' $\lambda = 0.9243$, $F_{19,639} = 0.892$, $P = 0.702$).

Ontogenetic changes in the adaptive value of defensive traits

When considering the simultaneous expression of the three defenses across plant ontogeny, we found that the influence of TD and cyanogenic potential on seed production varied as a function of the ontogenetic stage in which they were expressed (ontogeny \times either defensive trait; Table 1), suggesting that the adaptive value of each direct defense changed across plant development. After performing the phenotypic selection analyses, including both defenses at each ontogenetic stage (and SEFN in the case of reproductive plants), we found that, indeed, the targets, direction and intensity of natural selection changed across plant ontogeny. Specifically, negative directional and disruptive selection was found on the cyanogenic potential at the seedling stage ($\beta = -0.3504$, $\gamma = 0.3385$; Table 2; Fig. 3a). Intermediate values of TD were also favored at this stage ($\gamma = -0.6789$; Table 2; Fig. 3b). For juvenile plants, we found negative directional and disruptive selection on TD ($\beta = -0.4621$, $\gamma = 0.7871$; Table 2; Fig. 3e). Finally, at the reproductive stage, high phenotypic values of TD were favored both at the phenotypic ($\beta = 0.4527$; Table 2; Fig. 3h) and genotypic ($\beta = 0.358$, $P = 0.01$) levels. In addition, a positive correlational selection gradient was detected acting on cyanogenic potential and sugar content in extrafloral nectar ($\gamma = 0.3081$; Table 2; Fig. 4).

After performing a series of bioassays with the specialist herbivore *E. hegesia*, we found greater amounts of HCN in the tissues of larvae that fed on seedlings than on reproductive plants ($F_{1,48} = 25.53$, $P < 0.01$; Fig. 5a). In addition, larvae consumed more leaf biomass ($F_{1,48} = 24.82$, $P < 0.01$) and grew faster when fed on high cyanogenic seedlings than on leaves from reproductive plants (Fig. 5b). This can be explained by their assimilation efficiency, which was 35% greater when feeding on the younger plant stage ($F_{1,48} = 10.67$, $P < 0.01$; Fig. 5c).

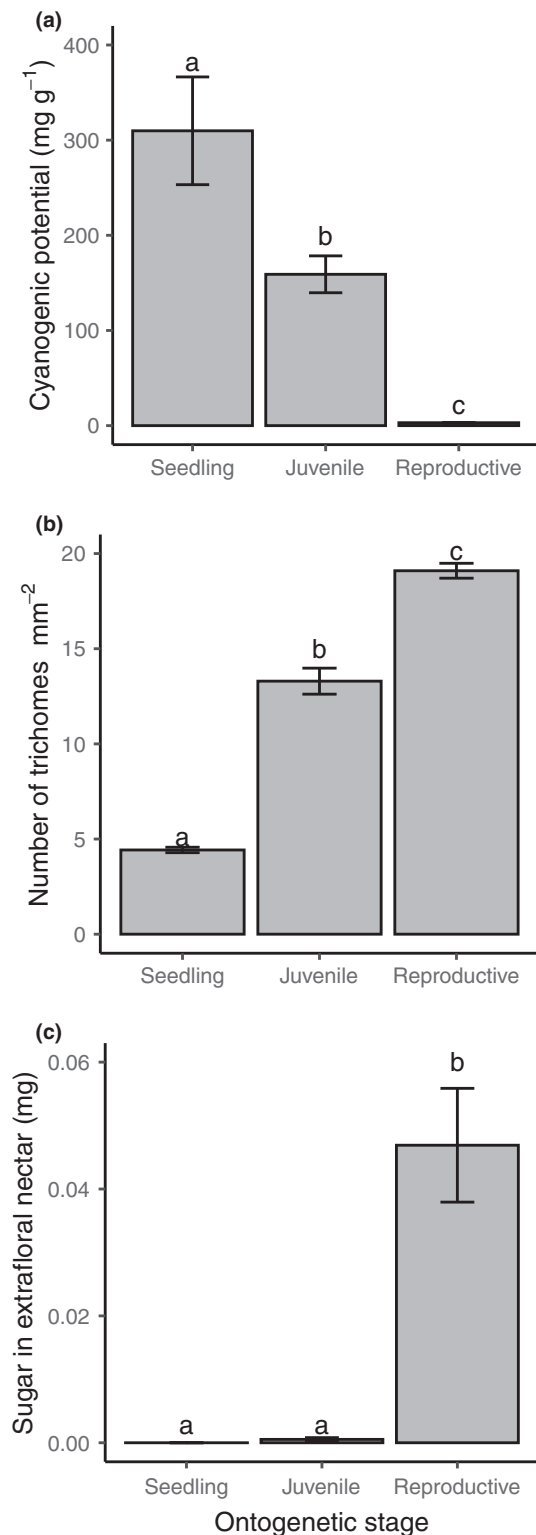


Fig. 2 Ontogenetic trajectories of defensive traits (cyanogenic potential (a), trichome density (b), and sugar content in extrafloral nectar (c)) in *Turnera velutina* plants growing in the experimental plots. Different letters above bars indicate significant differences among ontogenetic stages. Values correspond to means \pm SE.

Table 1 Full factorial mixed-model ANOVA testing for the effects of ontogenetic stage and defensive attributes of plant fitness (number of seeds) in *Turnera velutina*.

Effect	df	χ^2	P-value
Ontogenetic stage	2	1.980	3.714
Cyanogenic potential	1	0.791	0.3737
Extrafloral nectar	1	2.556	0.1098
Trichome density	1	10.744	0.0010
Cyanogenic potential × extrafloral nectar	1	12.340	0.0004
Cyanogenic potential × trichome density	1	0.839	0.3596
Trichome density × extrafloral nectar	1	3.255	0.0711
Ontogenetic stage × cyanogenic potential	2	12.881	0.0015
Ontogenetic stage × trichome density	2	73.355	< 0.0001
Cyanogenic potential²	1	4.424	0.0354
Extrafloral nectar ²	1	0.347	0.555
Trichome density²	1	33.505	< 0.0001
Ontogenetic stage × cyanogenic potential²	2	20.853	< 0.0001
Ontogenetic stage × trichome density²	2	38.242	< 0.0001

Significant values are shown in bold.

Multi-trait ontogenetic strategies in plant defense

By using a PCA, we visualized the functional space of multi-trait defensive strategies of each genotype across plant ontogeny and revealed how plants expressed singular combinations of defensive traits at each ontogenetic stage (identified with different ellipses and colors in Fig. 6). Greater values of PC1 denote high values of TD and SEFN, and low values of HCN. High scores of PC2 represent high values of SEFN and HCN (Table 3; Fig. 6). When analyzing the covariation between the scores of the first PC on the relative fitness of each genotype, we found a significant effect of particular combinations of defensive traits expressed at the reproductive stage on genotype mean fitness. Interestingly, we found that the three-way interaction term PC1 seedling × PC1 juvenile × PC1 reproductive ($F_{1,8} = 13.49$, $P = 0.0063$; Table 4) had a significant effect on the production of seeds.

Table 2 Phenotypic selection gradients describing linear (β) and nonlinear (γ) selection gradients on cyanogenic potential (HCN), trichome density (TD), and sugar content in extrafloral nectar (SEFN) of *Turnera velutina* at each ontogenetic stage.

Ontogenetic stage	Trait	β	CI	γ	CI
Seedling	HCN	-0.3504 ± 0.17	-0.5886, -0.1753	0.3385 ± 0.27	0.0999, 0.6809
	TD	0.1891 ± 0.17	-0.0195, 0.4745	-0.6789 ± 0.26	-1.2226, -0.3652
	HCN × TD			-0.2268 ± 0.22	-0.5319, 0.0240
Juvenile	HCN	0.012 ± 0.18	-0.2478, 0.3040	-0.6189 ± 0.39	-2.6893, 0.2113
	TD	-0.4621 ± 0.18	-0.9557, -0.1043	0.7871 ± 0.25	0.2688, 1.7611
	HCN × TD			0.1305 ± 0.29	-0.3925, 0.7711
Reproductive	HCN	-0.0614 ± 0.09	-0.3137, 0.0986	0.1880 ± 0.21	-0.1282, 0.6623
	TD	0.4527 ± 0.09	0.2737, 0.7015	0.1437 ± 0.18	-0.2539, 0.6362
	SEFN	0.0131 ± 0.09	-0.1463, 0.1989	0.0726 ± 0.10	-0.1773, 0.3359
	HCN × TD			0.0753 ± 0.09	-0.1351, 0.2459
	HCN × SEFN			0.3081 ± 0.15	0.0458, 0.6739
	SEFN × TD			-0.1582 ± 0.13	-0.4335, 0.0843
	HCN × TD × SEFN			-0.0379 ± 0.12	-0.2563, 0.1726

Significant selection gradients were determined with 95% bias-corrected bootstrap confidence intervals. Significant values are shown in bold.

Discussion

Ontogenetic changes in the adaptive value of defensive traits

We present experimental evidence showing that the targets, direction and intensity of natural selection on three defense traits changed across the ontogeny of *T. velutina* plants. Specifically, low cyanogenic potential was favored at the seedling stage, while high TD was unfavored at the juvenile stage but favored at the reproductive stage. Although previous studies have reported variation in the strength and direction of selection as a result of environmental and temporal variation (Grant & Grant, 2002; Siepielski *et al.*, 2009), this is the first study reporting changes in the targets of natural selection on different defenses across plant ontogeny. In addition, we present evidence suggesting that specific combinations of the three defenses at each ontogenetic stage can influence seed production.

Both the risk of attack and foliar damage by the specialist herbivore *E. hegesia* in the field are greater early during the ontogeny of *T. velutina*, when plants are not large enough to attract patrolling ants (Ochoa-López *et al.*, 2018). These ontogenetic changes in herbivore damage were similar among genotypes ($F_{19,329} = 0.68$, $P = 0.84$). Given this selective pressure and the lack of ant defense in seedlings, we expected natural selection to favor greater amounts of either HCN or TD as alternative defenses at the youngest stage. Surprisingly, we found directional selection against cyanogenic potential and stabilizing selection for TD at this stage. Although seedlings had the highest concentration of HCN compared with other stages (Fig. 2a; Ochoa-López *et al.*, 2018), individuals with high cyanogenic potential produced fewer seeds when they reached maturity (Fig. 3a). Although the few individuals with the highest cyanogenic potential had a small increment in fitness, this was not comparable with individuals with low cyanogenic potential. The lack of effectiveness of HCN as a defense could be a result either of the costs associated with its synthesis (Goodger *et al.*, 2006; Kessler &

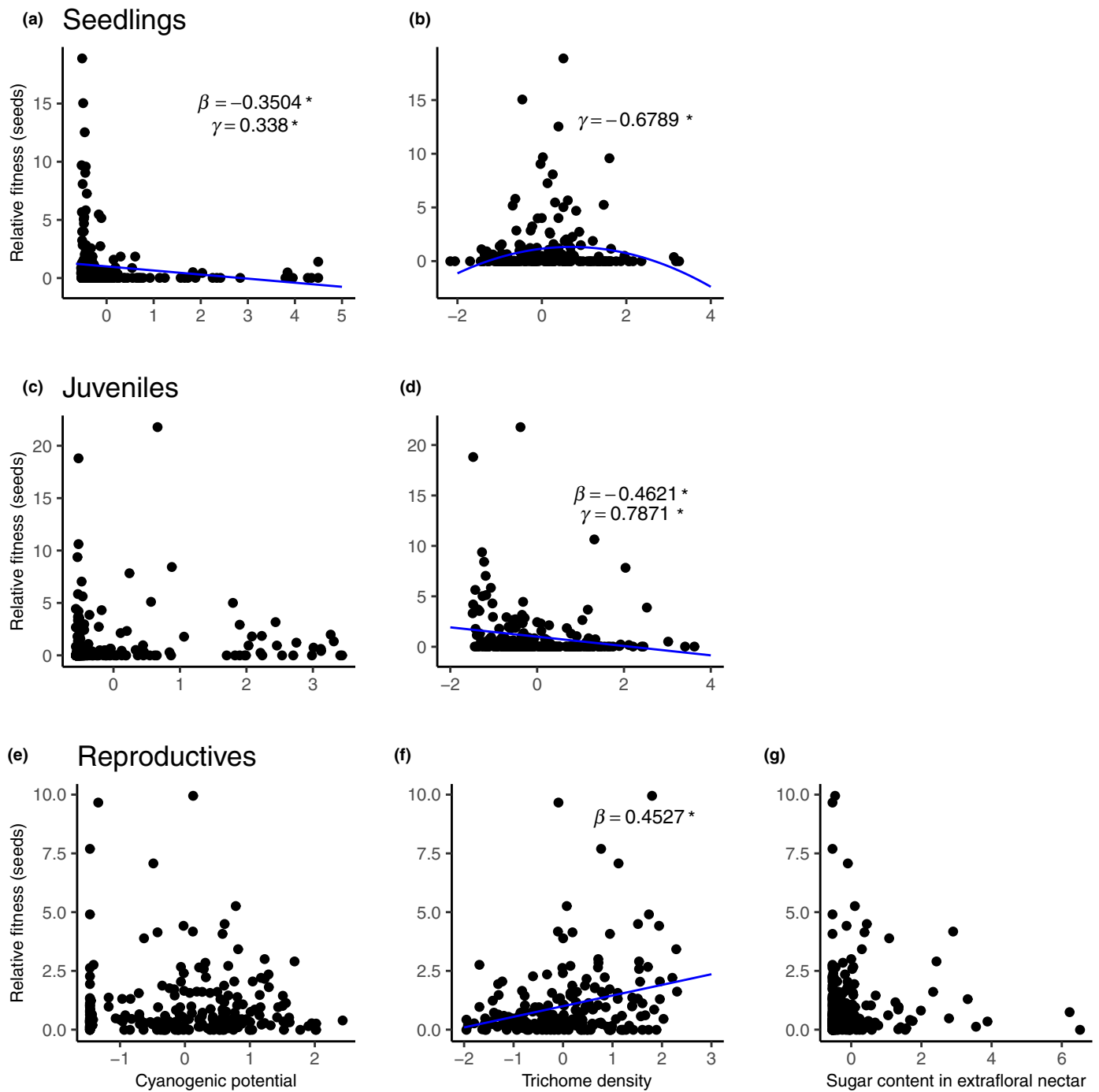


Fig. 3 Phenotypic selection gradients for cyanogenic potential (a, c, e), trichome density (b, d, f) and sugar content in extrafloral nectar production (SEFN) (g) of *Turnera velutina* at different ontogenetic stages growing in natural conditions. See text for details on the significance of selection coefficients.

Heil, 2011; Neilson *et al.*, 2013) or due to the ability of the specialist herbivore *E. hegesia* to sequester cyanogenic glycosides (Schappert & Shore, 1999; this study). Although cyanogenic glycosides have been related to an effective reduction of leaf damage by generalist herbivores in some plant species (Ballhorn *et al.*, 2005; Thompson & Johnson, 2016), specialist herbivores commonly show an array of strategies to detoxify or sequester these compounds (Engler *et al.*, 2000; Urbanska *et al.*, 2002; Pentzold *et al.*, 2014), rendering them ineffective at reducing herbivore

damage (Schappert & Shore, 1999; Gleadow & Woodrow, 2002; Ballhorn *et al.*, 2005, 2007; Shlichta *et al.*, 2014; Hernández-Cumplido *et al.*, 2016). Sequestration of cyanogenic glycosides is a well-known mechanism in specialist caterpillars feeding on cyanogenic plants to reduce risk of predation (Nahrstedt & Davis, 1983; Nahrstedt, 1985; Schappert & Shore, 1999; Nishida, 2002), which seems to be the case for *E. hegesia* as well.

Indeed, *E. hegesia* performed better when feeding on younger stages of *T. velutina*, which could be associated with a greater

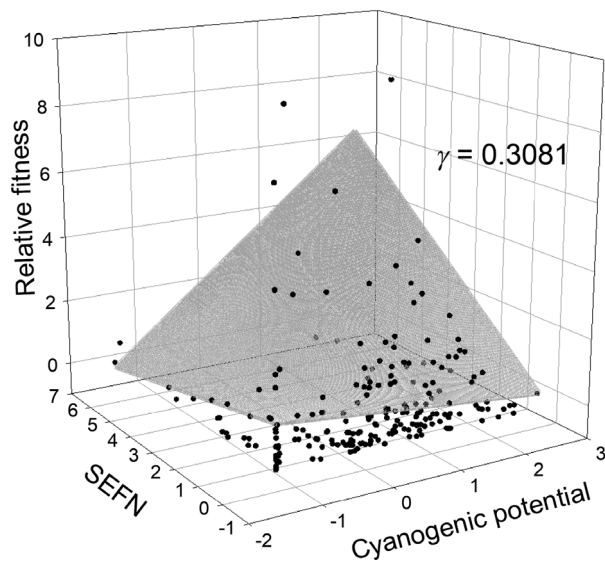


Fig. 4 Adaptive surface of the correlative selection gradient for cyanogenic potential and sugar content in extrafloral nectar (SEFN) in reproductive *Turnera velutina* plants.

nutritional value of leaves at this stage. In fact, a previous study reported greater concentration of nitrogen in leaves of juveniles than in leaves from reproductive plants of *T. velutina* (Damián *et al.*, 2018). Hence, even if cyanogenic glycosides could have a negative effect on caterpillars, it seems to be offset by the benefits of feeding on more nutritious leaf tissue. Although alternative mechanisms of plant defense, such as compensatory responses, have been reported in *T. velutina* seedlings (Ochoa-López *et al.*, 2015), the replacement of lost tissue at the seedling stage does not seem to be enough to avoid a reduction in seed production when these plants start reproducing. Hence, the observed high contents of HCN in seedlings might be a result of current selection by generalist herbivores and/or of past selection events, before the specialist herbivore was able to sequester cyanogenic compounds. If this is the case, we suggest that *E. hegesia* has currently overcome the potential arms race with *T. velutina* on the coevolution of chemical defenses and counter-defenses (Mello & Silva-Filho, 2002).

Trichome density has been previously associated with reduced herbivore damage (Kärkkäinen *et al.*, 2004; Kaplan *et al.*, 2009) and increased plant fitness (Valverde *et al.*, 2001; Bingham & Agrawal, 2010). In fact, previous studies have found positive selection on TD in *Asclepias incarnata* (Agrawal *et al.*, 2008a) and *Arabidopsis* spp. (Mauricio & Rausher, 1997; Kärkkäinen *et al.*, 2004; Loe *et al.*, 2007). In the case of *T. velutina*, we found that the direction and intensity of the selection gradient changed along plant ontogeny (Fig. 3; Table 2). In seedlings, where resources are limited and risk of herbivory is high (Boege & Marquis, 2005), we found that intermediate TDs were favored (Fig. 3b), which could be an alternative to low values of HCN. Whereas in juvenile plants TD was mostly unfavored by selection (Fig. 3e; again, except for a few individuals with high TD and relative fitness), it was significantly favored in reproductive plants (Fig. 3h). Because trichomes play an important role in

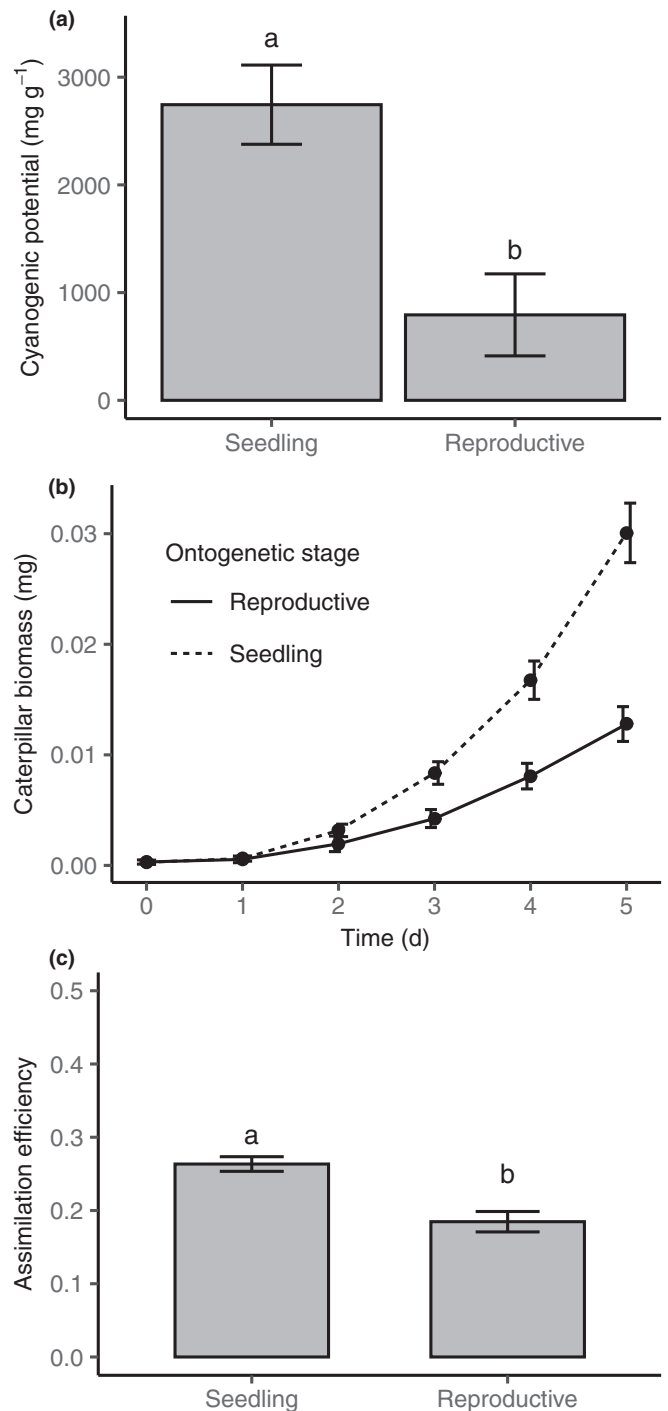


Fig. 5 Herbivore performance of *Euptoieta hegesia* after feeding for 6 d on either seedlings or reproductive *Turnera velutina* plants. (a–c) Content of hydrocyanic acid (HCN) in the larvae bodies (a), relative growth gain of caterpillars (b) and their assimilation efficiency (c). Different letters above bars indicate significant differences among ontogenetic stages. Values correspond to means \pm SE of each variable.

controlling evapotranspiration, their adaptive value could be related to both a reduction in herbivore damage (Agren & Schemske, 1993; Mauricio & Rausher, 1997; Kärkkäinen *et al.*, 2004; Loe *et al.*, 2007) and water stress avoidance (Ehleringer *et al.*, 1976; Skaltsa *et al.*, 1994; Espigares & Peco, 1995;

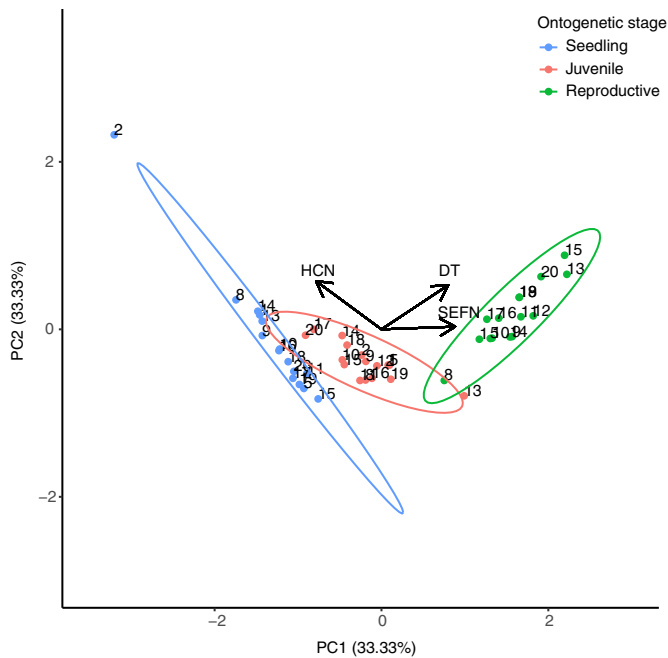


Fig. 6 Functional space of ontogenetic strategies in plant defense. Scores of the first and second principal components (PC1 and PC2, respectively) of genotypic means of the three defensive traits (hydrocyanic acid (HCN), trichome density (TD) and sugar content in extrafloral nectar (SEFN)) at three ontogenetic stages (seedling, juvenile and reproductive) of *Turnera velutina*. Ellipses are drawn with 95% of interval of confidence.

Table 3 Results of principal component analysis (PCA) performed with the genotypic means of the defensive traits in 16 *Turnera velutina* genotypes at each ontogenetic stage.

Defensive trait	PC1	PC2
HCN	-0.549	0.736
SEFN	0.561	0.675
TD	0.619	0.041
Variance explained	0.333	0.333
Cumulative variance	0.333	0.667

Defensive traits used were hydrocyanic acid (HCN), sugar content in extrafloral nectar (SEFN) and trichome density (TD). PC1/2, first/second principal components.

Sandquist & Ehleringer, 1998, 2003a,b; Agrawal *et al.*, 2008a). Hence, these three ontogenetic stages might have different selective pressures by herbivores, resource availability and water stress. Disentangling the role of these factors, however, warrants further investigation. Because TD had significant genetic variation and heritability in the studied population (Ochoa-López *et al.*, 2018), it is the only trait for which the observed ontogenetic trajectories could potentially evolve through natural selection. By contrast, despite their influence on plant fitness, the evolutionary responses of HCN and SEFN seem to be constrained by environmental heterogeneity. Hence, whereas the ontogenetic trajectories of some defenses could be the result of evolutionary responses to natural selection, others are likely instead to be shaped by plastic

Table 4 Full factorial ANOVA testing for the effects of combinations of defense traits (first principal component scores, PC1) on plant fitness (number of seeds) in *Turnera velutina*.

Effect	df	F	P-value
PC1 seedling	1, 8	5.264	0.0509
PC1 juvenile	1, 8	2.452	0.1560
PC1 reproductive	1, 8	13.821	0.0059
PC1 seedling × PC1 juvenile	1, 8	0.955	0.3571
PC1 seedling × PC1 reproductive	1, 8	0.150	0.7086
PC1 juvenile × PC1 reproductive	1, 8	0.053	0.8231
PC1 seedling × PC1 juvenile × PC1 reproductive	1, 8	13.486	0.0063

Significant values are shown in bold.

responses under variable environments (Whitman & Agrawal, 2009).

For myrmecophytic plants, a reduction in SEFN can decrease ant recruitment, increase herbivore damage and reduce fitness (Kessler & Heil, 2011). Resource-mediated attraction of natural enemies of herbivores generally results in a clear top-down control of herbivore populations, with positive fitness effects for the plant (Miller, 2007). However, direct tests of selection on these indirect defenses are still rare. Previous studies have reported a positive relationship between fitness and the number and size of extrafloral nectaries and the volume of nectar produced (Rudgers, 2004; Rutter & Rausher, 2004). Here, we report a positive correlative selection gradient for sugar content in extrafloral nectar and cyanogenic potential at the reproductive stage of *T. velutina*, when the interaction between plants and ants is more intense. Although reproductive plants had the lowest cyanogenic potential compared with younger stages, the positive correlative gradient could be the result of more vigorous plants producing more defenses of different kinds. Even when we hypothesized that older plants with active ant colonies should not rely on direct defenses, if there are exploiter ant species that do not provide an effective defense against herbivores, the expression of some amounts of chemical defense should be beneficial. For example, in the myrmecophytic species *Volchisia hindsi*, the ontogenetic decrease in HCN has been found to vary as a function of the identity of ants colonizing the plants, which can be either true mutualists or opportunistic ant species (Fonseca-Romero *et al.*, 2019). In the case of *T. velutina*, further investigation is needed to assess whether variation in ant identity and efficiency corresponds to the differential expression of HCN and ant rewards.

A previous study showed that greater amounts of extrafloral nectar are produced in leaves bearing flowers (Villamil, 2017). Therefore, SEFN seems to be related to the attraction of patrolling ants that protect not only leaves, but also flowers, which offer highly nutritious tissues and are highly vulnerable to other consumers (Villamil, 2017). Recent studies also suggest that greater production of SEFN in leaves bearing flowers represent a mutualist–management strategy, by distracting ants from flower nectar to avoid ant–pollinator conflicts (Villamil *et al.*, 2018, 2019).

Ontogenetic strategies in plant defense

Ontogenetic changes in the intensity and targets of natural selection can have important consequences for the evolution of lifetime plant strategies, because relative fitness does not depend on the expression of a single defense at one ontogenetic stage, but rather on what plants do earlier or later during their development. Successful genotypes would be those adjusting their phenotypes to match the best defensive strategy at each ontogenetic stage (Fig. 6). Although we found a significant effect of the expression of different combinations of defenses at each ontogenetic stage (as indicated by the significant three-way interaction among the PCs for each stage), further investigation is needed to interpret the adaptive value of particular ontogenetic strategies in plant defense.

Although other studies have already reported ontogenetic switches in defensive strategies from resistance to tolerance (Boege *et al.*, 2007), from chemical to biotic defense (Ochoa-López *et al.*, 2015; Ochoa-López *et al.*, 2018), or in the expression of different secondary metabolites (Goodger *et al.*, 2013), to our knowledge, this is the first report on how changes of defensive strategies along ontogeny are related to differential reproductive outputs. These ontogenetic strategies can represent adjustments in the expression of each defensive trait when they are more effective or less costly. The optimal defense (Bryant *et al.*, 1992) and the growth-differentiation (Herms & Mattson, 1992) theories predict that selection should favor the production of defensive traits when herbivory risk is higher, in this case the seedling stage. On the other hand, as plants grow, the pool of resources is divided on the different physiological needs of the plant. Later ontogenetic stages have greater availability to acquire resources (and, in the case of myrmecophytic plants, attract mutualistic ants), but also have more physiological needs (e.g. reproduction). We have shown, however, that the adaptive value of defensive traits at different ontogenetic stages also depends on the expression of other defensive traits earlier or later during plant development. Incorporating a demographic approach could further enlighten our understanding of these ontogenetic strategies, as it can reveal how the expression of particular defensive traits can affect plant survival, the transitions among ontogenetic stages and overall plant fitness.

Concluding remarks

Studies on the evolution of plant defense have provided evidence of natural selection on heritable defensive traits (Berenbaum *et al.*, 1986; Simms & Rausher, 1987; Mauricio & Rausher, 1997; Shonle & Bergelson, 2000). Here, we further demonstrated the influence of ontogeny as source of variation determining the adaptive value of three defenses in plants growing in their natural environment over 2 yr. There are three main take-home messages arising from this work: the targets of selection and the adaptive value of different defensive traits can change across plant ontogeny; some ontogenetic trajectories in plant defense are more likely to show evolutionary response than others; and ontogenetic strategies involving the expression of multiple defensive traits influence the

overall effects of natural selection. These findings suggest that we need to reconsider how and when selection analyses are performed, to fully understand the evolution of phenotypes.


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Author contributions

SO-L, JF, CAD and KB conceived and designed the experimental population and the statistical analyses. SO-L and XD collected data from the experimental population and SO-L performed the statistical analyses. KB and RR designed the experiments of herbivore performance. RR performed the experiments of herbivore performance and analyzed the data. SO-L and KB led the writing of the manuscript, and XD, JF and CAD contributed with further editorial enhancements.

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Capítulo 3.

Effect of ontogenetic trajectories of defense on the vital rates of plant population in *Turnera velutina*.

Abstract

Although ontogeny has been identified as a great source of phenotypic variation in plant defense against herbivores, it has not been fully incorporated into plant demographic studies estimate survival, which usually estimate growth and fecundity vital rates using as predictors the size or age of individuals, and the species or population average values of functional traits. By including the ontogenetic changes of these functional traits, we could fully understand the partial effects of the average values of plant defense on population dynamics, to a more holistic view considering the dynamism in resource allocation and ecological interactions imposed by plant development. Using an experimental population established in the coastal dunes located in La Mancha, Veracruz, Mexico, we assessed how the ontogenetic trajectories in three plant defenses influenced the growth, survival and fecundity rates in *T. velutina* population. We found that ontogenetic trajectories of defensive traits had differential effects on vital rates. High values of trichome density along ontogeny decreased plant growth and fecundity, but increased survival. Greater values of cyanogenic potential decreased the three vital rates and finally, higher values of sugar content in extrafloral nectar increased fecundity vital rates. From these results, we suggest that the impact of ontogenetic changes in defense on growth, survival, and fecundity vital rates. Ontogenetic changes in any trait should be included in the assessment of population process to fully comprehend the evolution of life story traits.

Introduction

The performance of any individual depends on the limited amount of resources available to fulfill different functions (Cody 1966), which can translate into population vital rates (survival, growth, and fecundity), determining population dynamics and shape the evolution of life histories (Lande 1982a). In the case of plants, herbivory can strongly influence individual growth, survival, and reproductive success (reviewed in Crawley 1988) and these effects are usually reflected in low survival of individual plants and reduced population growth (Halpern & Underwood 2006). As a response, plants have evolved several traits to reduce herbivore damage or its negative impacts. However, plant defenses commonly have associated resource allocation costs, related to trade-offs between the expression of defenses and growth (Coley *et al.* 1985; Simms & Rausher 1987; Herms & Mattson 1992; Cipollini & Sipe 2001; Cipollini *et al.* 2014; Huot *et al.* 2014; Züst & Agrawal 2017). In addition, ecological costs related with other biotic interactions such as competition, pollination, herbivory and/or biotic defense (Mauricio 1998; Cipollini & Sipe 2001; Koricheva 2002; Osier & Lindroth 2006) can also contribute with the impacts of the expression of particular traits on population vital rates. For example, the expression of defensive traits can diminish plant fecundity by interfering with the mutualist behavior of pollinators (Adler & Irwin 2005, 2012; Kessler *et al.* 2008, 2011; Cipollini & Heil 2010; Hernandez-Cumplido *et al.* 2010), the plant's ability to compete for resources (Van Dam & Baldwin 1998) or the plant's ability to respond to the abiotic environment (Cipollini 2004, 2005, 2010). However, to fully understand the magnitude of these ecological costs, more information is required on how these biotic interactions act synergistically along the development of plants.

Traditionally, plant demographic studies estimate survival, growth and fecundity vital rates using as predictors the size or age of individuals and the species or population average values of functional traits (McGill *et al.*

2006; Shipley *et al.* 2006). This approach, however, does not consider trait variation within species and along plant development (Bolnick *et al.* 2011; Laughlin *et al.* 2012; Carmona *et al.* 2016). The later source of variation in plant phenotypes is of great relevance, as ontogenetic variation in defensive traits has been proven to be ubiquitous in most plant species (Boege & Marquis 2005; Barton & Koricheva 2010), and can influence the adaptive value of such traits (Cope *et al.* 2019; Ochoa-López *et al.* 2020).

If natural selection modifies resource allocation to functions and traits that increase plant fitness (Cody 1966; León 1976), plants expressing certain combination of defensive traits along ontogeny could have greater probability of survival, and increase growth and/or higher fecundity in the population. However, the influence that individuals with changing phenotypes across ontogeny can have on population dynamics have not been fully explored. For example, Struckman *et al.* (2019) evaluated how functional traits affect vital rates and population growth of *Asclepias syriaca*. Although they found that changes in the mean values and variation in some of the traits, particularly in cardenolides concentration, affected population growth through the individual effects on vital rates, they considered static values of the studied traits along plant development, which does not consider that ontogeny as an important source of phenotypic of most traits. In this context, the aim of this study was to determine how changes in the expression of three defensive traits along plant development can influence population vital rates of the myrmecophytic perennial shrub *Turnera velutina*. Changes in physical, chemical, and biotic defensive traits have been previously reported in this species, with significant impacts on plant fitness (Ochoa-López *et al.*, 2020), which provides a suitable system to explore how ontogenetic trajectories of defensive traits modify the vital rates of the population. We expected that ontogenetic changes in plant defense would influence growth rate, survival and/or reproduction probability. Given our previous results (Ochoa-López *et al.* 2018; 2020), we expected that the defensive strategies that include high values of cyanogenic potential should decrease survival and fecundity, while the ones with high values of trichome density should increase growth rate and fecundity. This approach allows a transition from the understanding of the partial effects of the average values of plant defense on population dynamics, to a more holistic view considering the dynamism in resource allocation and ecological interactions imposed by plant development.

Methods

Turnera velutina and interacting species.

Turnera velutina Presl. (Passifloraceae) is a long-lived myrmecophytic shrub endemic to Mexico, that grows in coastal sand dunes and tropical dry forests (Arbo 2005; Villamil *et al.* 2013). The studied population is located along the established coastal sand dune at the field station Centro de Investigaciones Costeras La Mancha (CICOLMA), Veracruz, Mexico (19°35'N, 96°22'W, < 100 m above sea level). This area has a warm subhumid climate, with temperatures ranging between 21.1°C in January and 27.3°C in June, and an annual precipitation between 899 and 1829 mm (Travieso-Bello & Campos 2006); which mainly occurs between June and September. *T. velutina* produces axillary flowers, fruits that are dehiscent capsules with an average of 36 seeds (Sosenski *et al.* 2017). Flowering occurs mostly between May to June. The seeds have elaiosomes as rewards for foraging ants. At the study site, *T. velutina* interacts with 25 ant species (Cuautle *et al.* 2005), but not all of them imply a positive interaction for the plant. For example, whilst *Forelius analis* removes 64% of seeds and enhances germination rate; *Monomorium cyaneum* only eats the elaiosomes without removing the seed from the plant (Salazar-Rojas *et al.* 2012). *Turnera velutina* has one specialist herbivore, *Euptoieta hegesia* Cramer

(Lepidoptera: Nymphalidae) (Cuautle *et al.* 2005) that prefers and performs better when feeding on young seedlings than in reproductive *T. velutina* plants (Ochoa-López *et al.* 2018). Accordingly, leaf damage by herbivores is between 1.5 to 2 times greater for seedlings than for older stages (Ochoa-López *et al.* 2015). *Crociosema plebejana* (Noctuidae) has been reported as a pre-dispersal seed predator (Torres-Hernández *et al.* 2000).

Turnera velutina defensive strategies change through plant developmental (Villamil *et al.* 2013; Ochoa-López *et al.* 2015, 2018, 2020). As plant age, cyanogenic potential decreases whereas trichome density and extrafloral nectar sugar content increase. Previous studies have shown that specific combinations of the three defenses at each ontogenetic stage, considered as an ontogenetic defensive strategy, influence plant fitness (Ochoa-López *et al.* 2020). Furthermore, some of these ontogenetic defensive trajectories have genetic heritable variation and have the potential to evolve through natural selection (Ochoa-López *et al.* 2018).

Experimental population.

We established an experimental population of *T. velutina* within the native population of the study site. We first produced 1200 seedlings F2 generation seedlings in a shadehouse, using 20 maternal F1 plants that were self-pollinated the previous year (for further details see Ochoa-López *et al.* 2020). To germinate the seeds, we manually removed elaiosomes and placed them in germination trays in a mixture of local soil and vermiculite (1:1), which we watered from the bottom for 3 weeks until germination. In August 2014, we established twenty 1 × 1 m plots in four sites of the sand dune under the partial shade of vegetation canopy, into which F2 seedlings were transplanted when they flushed their first leaf. Seedlings were randomly arranged in the experimental plots using a 10 × 10 cm grid, where they were exposed to all biotic interactions and natural environmental variation during the rest of their development.

Ontogenetic trajectories in plant defense.

Although development is a continuous process (Ellner & Rees 2007; Rees & Ellner 2009; Merow *et al.* 2014), to assess ontogenetic changes in plant defense we defined three ontogenetic stages based on three key transitions in the functional priorities of plants (Boege & Marquis 2005): seedlings (plants with up to the first two true leaves fully expanded, n= 213), juvenile (plants with up to the 10th leaf fully expanded, n=220), and reproductive (plants bearing the first flowers, n=225). When plants from the experimental plots reached each of these ontogenetic stages, we measured hydrogen cyanide (HCN), as a proxy of the cyanogenic potential (Ballhorn *et al.* 2005), trichome density (TD), and sugar content in extrafloral nectar (SEFN) as a reward for patrolling ants. Specific methods for assessing each of these defenses are described in Ochoa-López *et al.* 2018. We also recorded the basal diameter of each plant each month, as a measure of their size, the time at which the reproductive stage was reached, and the number of seed from fruits collected between October 2014 and February 2016 within the experimental population.

Model selection analysis.

To evaluate the effect of ontogenetic trajectories of defense on population vital rates, we constructed models for each vital rate (growth, survival, fecundity) as a function of plant size at the beginning of each month, defined as basal diameter, and the values of HCN, TD and SEFN. Generalized linear models (GLMs) were used for the survival and fecundity models, and linear mixed models were used for the growth model. In both cases, the random variable was the maternal genotype and the month. We used the size at the end of each month as the response variable for the growth model. For the survival model, we considered if a plant was dead or alive

in the next month. For the fecundity models, we considered if the onset of plant reproduction had occurred (reproduction model), and if so, the number of seeds produced at the following month (seed production model). We were only interested in double interaction effects between defensive strategies and plant size because they represent direct trade-offs that could arise between these traits, so the remaining interaction terms were not considered. We used backwards model selection using AIC to define the minimum adequate model for each vital rate (following Zuur et al. 2009). Models were adjusted in R version 3.6.3 (R Core Team, 2020). using *glmmTMB* (Brooks *et al.*, 2017) and *lme4* (Bates *et al.*, 2015). Model selection were performed using *MuMIn* (Barton, 2009).

To visualize the effect of different ontogenetic defense strategies, once the models of the population vital rates were selected, we used them to visualize the growth rate, the probability of survival, the probability of reproduction and average seed production (Fig. 2). To do so, we considered three ontogenetic strategies of defense: 1) low defense, using the minimum genotypic values of each defensive trait per month as predictor values; 2) average defense, using the mean values of each defensive trait in the plant genotypes per month as predictor values; and 3) high defense considering the maximum genotypic values of each defensive trait per month as predictor values. Then, we plotted model predictions of each vital rate to compare the performance of each ontogenetic defense strategy (Fig. 2).

Results

We found that the ontogenetic trajectories of the defensive traits had differential effects on vital rates of *T. velutina* population. Trichome density was the only defensive trait improving the fit for regressions of all vital rates, although the magnitude of its effects varied between growth, survival and fecundity. Higher values of trichome density along plant ontogeny decreased plant growth (Fig. 1a) and fecundity (Fig. 1c and d), but increased survival (Fig. 1b). Greater values of cyanogenic potential decreased the three vital rates in which it was selected as part of the minimal adequate model (Fig. 1b, c and d), and finally, higher values of sugar content in extrafloral nectar increased fecundity vital rates (Fig. 1c and d).

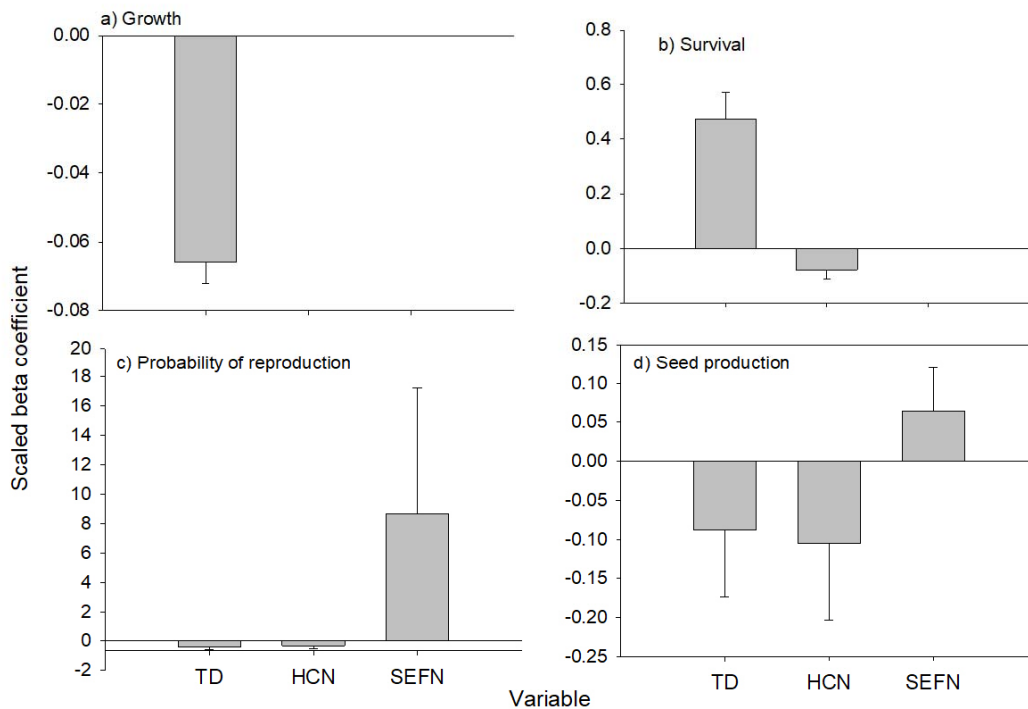


Fig. 1. Coefficient values \pm s. e. for the demographic vital rate regressions of a) growth, b) survival, c) probability of reproduction and d) seed production by defense variables: trichome density (TD), cyanogenic potential (HCN) y sugar content in extrafloral nectar (SEFN).

Estimates for the different ontogenetic defense strategies allowed us to test how the population vital rates changed as a function of the investment in each defense (with low, average, or high values) along plant development. For the growth model, the interaction term being selected as part of the minimal adequate model suggests that the effect of the trichome density in plant growth depends on the size of the plant (Fig. 2a). In particular, when plants reached their juvenile stage, those with high investment in trichomes increased their size more rapidly than the plants with other strategies. In contrast, the probability of survival of plants was affected by two defensive traits: trichome density and cyanogenic potential; plants with low investment in both traits had greater probability of survival (Fig. 2b). Lastly, the probability of plant reproduction was influenced by plant size (Fig. 2c) and particular ontogenetic strategies in plant defense: plants with low investment in all three defensive traits when they were young (smaller than 3 cm in diameter), but a high investment when they grew, had greater probability of reproduction than plants with other ontogenetic strategies in plant defense. In the case of seed production, plants with low investment in all three defensive traits had greater number of seeds, compared with average and high investment in plant defense (Fig. 2d).

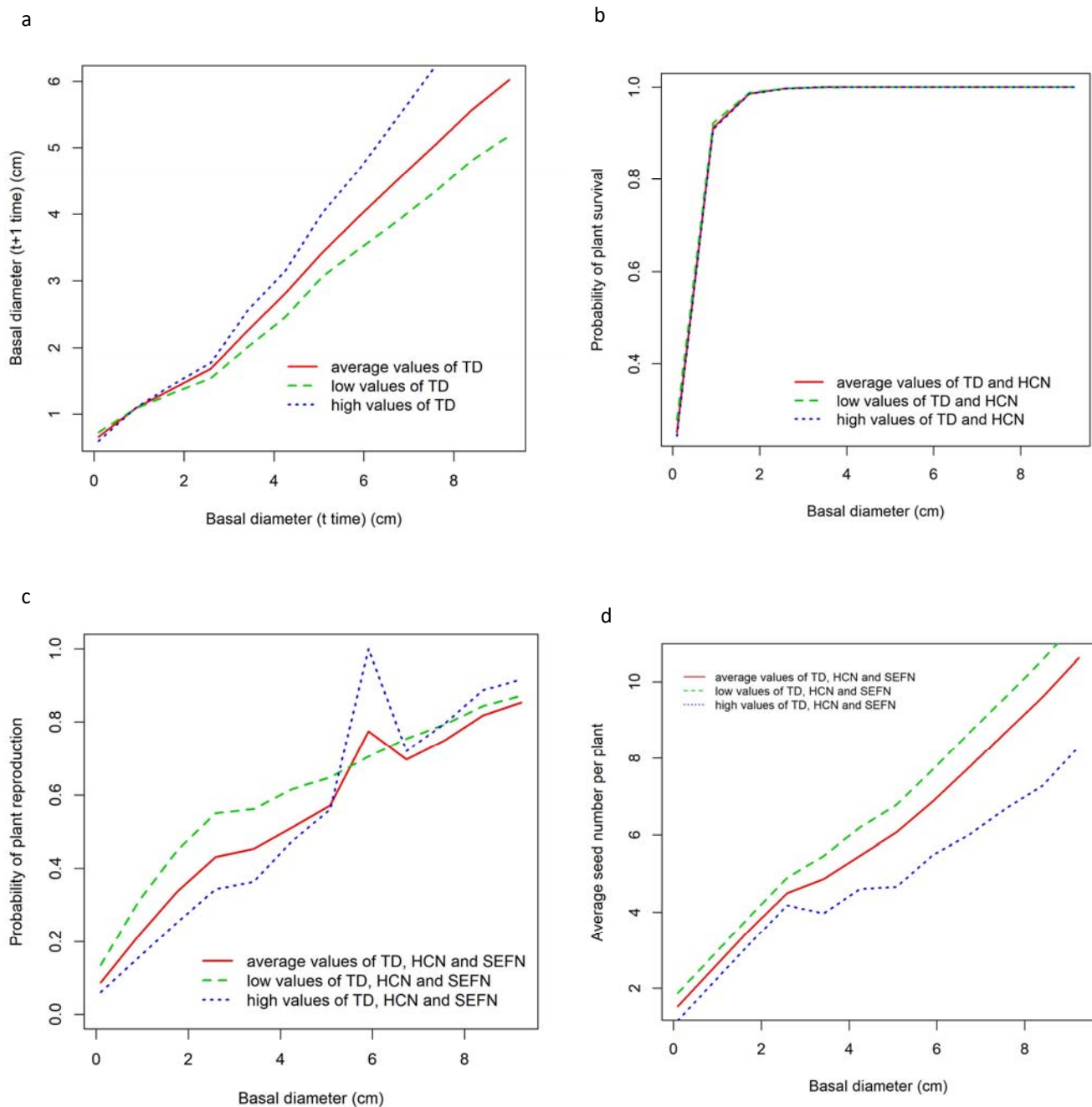


Fig. 2. Vital rate regression predicted for plants with different ontogenetic defense strategies: a) Growth model predictions at low (dashed), average (solid), and high (dotted) values of trichome density (TD). b) Probability of survival predictions at low (dashed), average (solid), and high (dotted) values of trichome density (TD) and cyanogenic potential (HCN). c) Probability of reproduction and d) seed production at low (dashed), average (solid), and high (dotted) values of trichome density (TD), cyanogenic potential (HCN) and sugar content in extrafloral nectar production (SEFN).

Discussion

We found that population vital rates of *T. velutina* are influenced by particular ontogenetic strategies in plant defense. Whereas a high investment strategy in defensive traits increased growth, it decreased seed production and the probability of survival (Fig. 2), suggesting resource allocation related costs. However, when analyzing each defensive trait individually, we found that each defense had differential effects on population vital rates (Fig. 1). Cyanogenic potential across plant development had a negative effect on all vital rates, trichome density decreased plant growth, and fecundity, but increased the probability of survival, and sugar content in extrafloral nectar increased both the probability of reproduction and seed production. This adds to previous evidence on how the costs of defensive traits can translate into negative impacts on population vital rates (Struckman *et al.* 2019). However, our study highlights that if we only consider the effects of the individual's average expression of defenses on population vital rates, we cannot fully comprehend their impact on plant population dynamics, for which the consideration of ontogenetic changes on multiple defensive traits adds valuable information.

Assessing how the expression of defenses along plant ontogeny has particular impacts on plant performance is required to have a better understanding of the effects of defensive traits on plant demography. In this study, we describe how particular defensive strategies expressed along development can modify population vital rates. Plants with high defense expressed along the whole development had higher growth rate, but less seed production; meanwhile, plants with low defense values had lower growth rate, but higher fecundity rate. Both growth and reproduction processes in plants depend on the development of meristems, and are mutually exclusive (Geber 1990), so tradeoffs between both functions are expected. However, the genotypic variation within the relationship between these vital rates can also influence this relationship. For example, fecundity can increase with plant size, but this relationship can change as a function of the environment (Sultan & Bazzaz 1993; Clauss & Aarssen 1994; Bazzaz *et al.* 2005). These dynamics warrant further investigation.

The findings in this study add to previous evidence reporting the effect that defense traits can have on the expression of vital rates, commonly described as the cost of defense production (Coley *et al.* 1985; Simms & Rausher 1987; Herms & Mattson 1992; Cipollini & Sipe 2001; Cipollini *et al.* 2014; Huot *et al.* 2014; Züst & Agrawal 2017). In a demographic context, however, there is scarce information on how defensive traits can shape population vital rates. For *T. velutina*, we found that the highest probability of reproduction occurred through a particular defensive strategy along ontogeny, involving low defense at young ontogenetic stages but high defense in the older ones. Hence, our approach allows expanding our view of the demographic consequences of plant defense at a single moment of a plants life, to a more comprehensive understanding after including the continuous ontogenetic changes in plant defense that can influence population vital rates. With this approach we highlight that the connection between demography and evolution follows from the recognition that the life cycle is the unit of description of the organism and is a part of its phenotype. As such, life cycle is as much subject to be adaptative as is the anatomy, physiology or behavior of the organism (Lande 1982b).

A previous study reports ontogenetic changes in the direction and intensity of natural selection of physical defenses in *T. velutina*, showing that intermediate levels of trichome density were favored at the seedling stage, low values were promoted at the juvenile stage, and a positive directional gradient was found for reproductive plants (Ochoa-López *et al.* 2020). This suggests that ontogenetic defense strategies involve

changes in the expression of a particular defensive trait along ontogeny to optimize plant fitness. The results found in this study further support this, as we report that the maintenance of high densities of trichomes across the entire ontogeny can result in lower seed production. Previously, we have found that adult plants selection favored high values of trichome density, whereas in juvenile plants it favored low values (Ochoa-López *et al.* 2020). Since in juvenile low values of trichomes has been associated to an increased gas exchange for carbon fixation (Bickford 2016), typical of the a rapid growth strategy in this ontogenetic stage. Hence, the adjustment in the expression of this trait across ontogeny seems to be adaptive. Both microevolutionary and demographic approaches highlight the value of assessing the relationship of multiple traits over plant fitness, not only at one ontogenetic stage, but across the entire development. In particular, the use of simulations allowed to modify the values of three defensive traits at once and explore how they can influence population vital rates. Incorporating more than one defensive trait into the analysis of how plant defenses influence plant fitness allows the consideration of the complexity of life story traits. Further efforts could incorporate individual effects of multiple traits along ontogeny on vital rates in a demographic model.

We need to further consider that the relationship between any trait and a vital rate is influenced by the environment and the overall multivariate phenotype of an individual (Yang *et al.* 2018), in addition to the interactions that plants have with other species (pollinators, herbivores, etc.). For example, we currently know that herbivores can limit plant population growth through plant survival and fecundity along plant development (Bastrenta *et al.* 1995; Ehrlén 1995; Stowe *et al.* 2000; Struckman *et al.* 2019), but what remains unclear is how ontogenetic changes of multiple plant traits can influence the effect of herbivores on plant population dynamics considering other biotic interactions and environmental challenges. Other studies exploring the individual effects of functional traits on vital rates have reported that functional traits such as seed mass, specific leaf area and stomatal density are positively related to growth rate (Liu *et al.* 2016; Visser *et al.* 2016), meanwhile survival rate is negatively related to seed mass (Visser *et al.* 2016) and positive related to wood density and leaf lifespan (Adler *et al.* 2014). In this study, we provide some evidence that adjustments in the expression of plant defense across plant development can indeed change population vital rates. By incorporating molecular, physiological, and ecological approaches, we could further identify the mechanisms that link ontogenetic trajectories of defensive traits population growth, through their impacts on vital rates.

This study reinforces the idea that individual level effects of traits on vital rates are not good quality proxies of their impacts on plant population dynamics, and consequently no robust demographical conclusions can be drawn from studies at particular ontogenetic stages. We need to consider the ontogenetic variation of individuals' traits, in addition with their trade-offs or synergies. To our knowledge, we report for the first time how population vital rates are influenced by particular ontogenetic strategies in plant defense. The inclusion of demographic models when studying the impact of defensive traits and herbivore damage on plant populations could disentangle how demographic trade-offs and plasticity within an individual affect the distributions of phenotypic traits over time and space.

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Discusión general

El estudio de la evolución de las características de los organismos tradicionalmente se ha enfocado en momentos particulares de su desarrollo, dejando de lado el hecho de que la selección natural actúa de manera continua sobre los organismos durante todo su desarrollo, y que el valor adaptativo de los fenotipos puede cambiar durante el mismo. En este estudio se resalta la importancia de la ontogenia como una fuente de variación que debe considerarse al estudiar cualquier atributo de los organismos, en particular de las defensas en plantas. La expresión de los atributos defensivos en cada estadio ontogenético tiene asociados costos y beneficios (Boege & Marquis 2005; Orians *et al.* 2010; Barton & Boege 2017), por lo que las trayectorias ontogenéticas de la defensa pueden surgir como resultado de las presiones de selección a las que se exponen las plantas en sus hábitats. En este sentido, la expresión de los atributos de defensa durante el desarrollo de las plantas puede ser considerada en si como un fenotipo complejo, con las características de los rasgos adaptativos bajo selección natural: tener variación genética heredable, y que su expresión tenga un efecto significativo sobre la adecuación de las plantas.

Para explorar este enfoque utilicé un sistema no modelo como *T. velutina* para estudiar los patrones de defensa en un ambiente en el cual las plantas se encuentran expuestas a todas las condiciones ambientales de una población natural. Encontré que las trayectorias ontogenéticas de las defensas en *Turnera velutina* tienen un valor adaptativo que cambia durante el desarrollo de las plantas. Sin embargo, de las tres defensas estudiadas, sólo la densidad de tricomas presentó variación genética heredable en los tres estadios ontogenéticos analizados, mientras que el potencial cianogénico solo tuvo variación genética heredable durante el estadio plántula. Encontré también que los blancos de la selección natural cambian durante el desarrollo de la planta. En las plántulas se favorecieron valores intermedios de densidad de tricomas y valores bajos de potencial cianogénico, mientras que en las plantas juveniles se favorecieron valores bajos de densidad de tricomas y en estadio reproductivo se favorecieron valores altos de densidad de tricomas, y una combinación de altos valores del potencial cianogénico y de producción de azúcar en el néctar extrafloral.

Los estudios de la evolución de las defensas de las plantas han provisto evidencia de selección natural sobre atributos heredables (Berenbaum *et al.* 1986; Simms & Rausher 1987; Mauricio & Rausher 1997; Shonle & Bergelson 2000; Agrawal 2005). Sin embargo, sólo existe un estudio que analiza si las trayectorias ontogenéticas de la defensa pueden ser sujetas a selección natural y pueden ser adaptativas. En dicho estudio, la relación entre las trayectorias ontogenéticas de defensa y la adecuación en árboles de *Populus tremuloides* fue diferente entre los sexos de las plantas y el tipo de atributo químico defensivo (Cope *et al.* 2019). En el presente trabajo se reporta también la influencia de la ontogenia como una fuente de variación que determina

el valor adaptativo de tres atributos defensivos expresados en su ambiente durante un experimento de dos años.

Cambios en las presiones de selección durante la ontogenia de Turnera velutina.

Plántulas

En *T. velutina*, las plántulas constituyen un estadio ontogenético que se caracteriza porque que existe una mayor presión por el herbívoro especialista *Euptoieta hegesia*, así como una menor disponibilidad de recursos energéticos, por lo que esperaríamos la defensa expresada sea suficiente para poder sobrevivir. De las defensas estudiadas, las plántulas tuvieron los menores valores de densidad de tricomas, los valores más altos de potencial cianogénico y no presentaron de nectarios extraflorales. Además, en estudios previos se reporta que las plántulas tienen la capacidad de expresar respuestas compensatorias, es decir, reemplazan el tejido perdido (Ochoa-López *et al.* 2015).

Dadas estas características, el potencial cianogénico parece ser la defensa principal para este estadio, que además presentó variación genética heredable (Ochoa-López *et al.* 2020). Contrario a las predicciones, sin embargo, encontré una selección direccional negativa contra el potencial cianogénico en este estadio. La aparente falta de efectividad del potencial cianogénico como defensa puede ser resultado de su costo de producción (Goodger *et al.* 2006; Kessler & Heil 2011; Neilson *et al.* 2013) o debido a la habilidad del herbívoro especialista *E. hegesia* para secuestrar los glucósidos cianogénicos (Schappert & Shore 1998; Ochoa-López *et al.* 2020). Si bien el potencial cianogénico se ha relacionado con una reducción del daño foliar por herbívoros generalistas (Ballhorn *et al.* 2005; Thompson & Johnson 2016), los herbívoros especialistas tienen varias estrategias de detoxificación de estos compuestos (Engler *et al.* 2000; Urbanska *et al.* 2002; Pentzold *et al.* 2014), haciéndolos inefectivos para reducir el daño foliar (Schappert & Shore 1999; Gleadow & Woodrow 2002; Ballhorn *et al.* 2005, 2007; Shlichta *et al.* 2014; Hernández-Cumplido *et al.* 2016).

Este secuestro de glucósido cianogénicos es un mecanismo que utilizan los herbívoros para reducir el riesgo de depredación (Nahrstedt & Davis 1983; Nahrstedt 1985; Schappert & Shore 1999; Nishida 2002), el cual parece ser el caso de *E. hegesia*, ya que las larvas que se alimentan de plántulas tienen un mejor desempeño en comparación de las que se alimentan de estadios reproductivos (Ochoa-López *et al.* 2020). En este sentido, los patrones actuales de selección podrían ser resultado ante la presión actual o pasada de herbívoros generalistas, previo a que el especialista fuera capaz de secuestrar los compuestos cianogénicos, por lo que *E. hegesia* puede estar actualmente ganando la carrera armamentista con *T. velutina* en la coevolución de defensas químicas y contra defensas (Mello & Silva-Filho 2002).

Plantas juveniles

Las plantas juveniles tienen la disyuntiva de crecer y sobrevivir, para lo cual la asignación de recursos puede incrementar hacia funciones de diferenciación de tejido para crecer y desarrollar defensas contra herbívoros (Herms & Mattson 1992). En *T. velutina*, las plantas juveniles tuvieron un incremento en la densidad de tricomas en comparación con las plántulas, atributo defensivo con variación genética heredable (Ochoa-López *et al.* 2018). En este estadio, los nectarios extraflorales apenas comienzan a producir néctar extrafloral y se observó una disminución en el potencial cianogénico.

Si bien los tricomas foliares están relacionados con una disminución del daño foliar por herbívoros (Kärkkäinen *et al.* 2004; Kaplan *et al.* 2009), y un incremento en la adecuación de las plantas (Valverde *et al.* 2001; Bingham & Agrawal 2010), encontré que en las plantas juveniles de *T. velutina* se favorecen una densidad baja de tricomas (Ochoa-López *et al.* 2020). Aunque trabajos previos han reportado una selección direccional positiva sobre la densidad de los tricomas (Mauricio & Rausher 1997; Kärkkäinen *et al.* 2004; Loe *et al.* 2007; Agrawal *et al.* 2008), estos estudios fueron realizados con plantas adultas, cuando las presiones de selección pueden ser diferentes. Los tricomas foliares, además de tener un papel como defensa contra herbívoros, también se han reportado como un mecanismo para reducir la pérdida de agua en las hojas (Woodman & Fernandes 1991; Bickford 2016), por lo que su expresión puede ser consecuencia de varias presiones de selección actuando al mismo tiempo sobre este atributo. Bajas densidades de tricomas se han asociado con un incremento en el intercambio gaseoso para la fijación de carbono (Bickford 2016), lo cual se puede reflejar en una estrategia de rápido crecimiento, característico de estadios juveniles.

Plantas reproductivas

Durante el estadio reproductivo, las plantas de *T. velutina* tienen el menor riesgo de ataque por herbívoros (Ochoa-López *et al.* 2018) y un menor porcentaje de daño foliar (Ochoa-López *et al.* 2015). Además, tienen una mayor capacidad de asimilar recursos, aunque también tienen más funciones fisiológicas a las que deben asignarlos (crecimiento, defensa, reproducción). En este estadio, las plantas de *T. velutina* tuvieron los valores más altos de densidad de tricomas y de producción de néctar extrafloral, y los más bajos de potencial cianogénico (Ochoa-López *et al.* 2020). Asimismo, en este estadio es donde se ha establecido por completo la relación con las hormigas mutualistas, las cuales aprovechan tanto el néctar extrafloral como los elaiosomas de las semillas como recompensas (Torres-Hernández *et al.* 2000; Cuautle *et al.* 2005; Salazar-Rojas *et al.* 2012).

En este estadio, sólo la densidad de tricomas tuvo variación genética heredable (Ochoa-López *et al.* 2018) y también tuvo un gradiente de selección positivo (Ochoa-López *et al.* 2020) sugiriendo que tienen el potencial

de tener cambios evolutivos por selección natural. Los tricomas foliares están relacionados con la reducción del daño foliar por herbívoros (Kärkkäinen *et al.* 2004; Kaplan *et al.* 2009), e incrementan la adecuación de las plantas (Valverde *et al.* 2001; Bingham & Agrawal 2010). Sin embargo, también tienen un papel importante en controlar la evapotranspiración, por lo que su valor adaptativo podría estar relacionado tanto a la disminución del daño por herbivoría (Agren & Schemske 1993; Mauricio & Rausher 1997; Kärkkäinen *et al.* 2004; Loe *et al.* 2007) como a evitar el estrés por agua (Ehleringer *et al.* 1976; Skaltsa *et al.* 1994; Espigares & Peco 1995; Sandquist & Ehleringer 1998, 2003b, a; Agrawal *et al.* 2008). En trabajos previos, se ha reportado una selección direccional positiva sobre la densidad de los tricomas (Mauricio & Rausher 1997; Kärkkäinen *et al.* 2004; Loe *et al.* 2007; Agrawal *et al.* 2008), pero para discernir cuales son las presiones que promueven este atributo se requiere más estudios.

Las plantas reproductivas tuvieron los valores más bajos de potencial cianogénico comparado con estadios más tempranos. Sin embargo, encontramos un gradiente de selección correlativo positivo entre el potencial cianogénico y la producción de néctar extrafloral (Ochoa-López *et al.* 2020). Este gradiente puede ser indicador de que plantas vigorosas pueden producir más defensas de varios tipos. La producción de defensa química puede ser benéfica en las plantas reproductivas, sobre todo si entre las colonias de hormigas con las que se relaciona se incluyen algunas especies explotadoras que no ofrecen el servicio de patrullaje contra herbívoros. Esto se ha reportado previamente en otras especies mimercófilas, por ejemplo, en *Volchisia hindsii*, la producción de HCN responde a la identidad de las hormigas que colonizan la planta, las cuales pueden ser mutualistas o especies oportunistas que explotan el recurso sin brindar el servicio de patrullaje (Fonseca-Romero *et al.* 2019).

Las asociaciones simbióticas establecidas por recompensas suelen atraer enemigos naturales de los herbívoros y resultar en un control descendiente de las poblaciones de éstos, lo que se refleja positivamente sobre la adecuación de la planta (Miller 2007). En plantas mimercófilas, una reducción en la producción de recompensa puede disminuir el reclutamiento de hormigas, incrementar el daño por herbívoros y disminuir la adecuación (Kessler & Heil 2011). En la población de estudio, se ha reportado que *T. velutina* interactúa con 25 especies de hormigas (Cuautle *et al.* 2005), aunque no todas tienen un papel de simbiote al disminuir el impacto de los herbívoros sobre la planta (Torres-Hernández *et al.* 2000; Cuautle *et al.* 2005; Salazar-Rojas *et al.* 2012). En esta población, la presencia de hormigas grandes disminuye el daño por herbivoría y en aquellas plantas con especies de hormiga grandes (e.g., *Camponotus abdominalis*) ocurre una mayor producción de frutos (Torres-Hernández *et al.* 2000), lo que sugiere este tipo de relación mutualista entre hormigas y *T. velutina*. Esto puede explicar parcialmente la falta de variación genética y heredabilidad en la expresión del SEFN ya que se ha reportado que la calidad del NEF está relacionada con la presencia e identidad de las hormigas simbiotes (Heil

et al. 2009; Bixenmann *et al.* 2011). Además, para las defensas bióticas solo se han reportado un componente genético significativo, pero solo sobre el número (Wooley *et al.* 2007) y la morfología (Rudgers 2004) de los nectarios extraflorales, no sobre la calidad y cantidad de las recompensas.

Por otro lado, la producción del néctar extrafloral en las plantas reproductivas puede variar en distintas etapas reproductivas de *T. velutina*. En particular, se ha reportado que mayor producción de néctar en hojas con flores (Villamil 2017), que tienen altos valores nutricionales y son vulnerables para otro tipo de consumidores. La producción de néctar extrafloral también ha sido sugerida como una estrategia de manejo de mutualistas, distraendo a las hormigas y evitando que consuman néctar floral y que interfieran negativamente con las visitas de polinizadores (Villamil *et al.* 2018, 2019). Estos factores podrían contribuir también a la variación observada en este atributo en la población experimental.

Estrategias ontogenéticas de defensa como ajuste en la expresión de atributos.

Las estrategias ontogenéticas de defensa pueden representar ajustes en la expresión de cada atributo defensivo cuando es más efectivo y/o menos costoso. Las teorías de defensa óptima (McKey 1974; Rhoades & Cates 1976) predice que los estadios tempranos al tener mayor valor para la adecuación deberían tener mayor producción de defensa, mientras que la hipótesis de balance de crecimiento y diferenciación (Herms & Mattson 1992) predice una mayor producción de defensas en plantas reproductivas al tener más recursos por asignar. Estas dos hipótesis nos permiten explorar cuáles son los factores que pueden moldear la expresión de las defensas en las plantas incluyendo la presencia de restricciones inherentes al proceso de desarrollo y los cambios en las presiones de selección de factores bióticos y abióticos. Si consideramos las presiones de selección consideradas por estas hipótesis, la teoría de defensa óptima se basa en que los patrones de defensa son resultado de la presión que ejercen los herbívoros, mientras que la hipótesis de balance de crecimiento-diferenciación se centra más en las restricciones fisiológicas que le permiten a una planta expresar o no ciertas defensas. Sin embargo, en este trabajo reportó que, de entrada, ninguna teoría sirve para generalizar los patrones esperados y que hay que analizar cada una en función de sus costos y beneficios y plasticidad.

Encontré que, cambios en las presiones de selección ejercida por los herbívoros, promovieron distintos gradientes de selección, como se discute en el segundo capítulo. Las plántulas tienen una mayor depredación por parte de los herbívoros, y a pesar de que este estadio tiene los valores promedio más altos de potencial cianogénico (defensa química). Sin embargo, al explorar la propuesta de la hipótesis de balance de crecimiento y diferenciación, se predice una mayor producción de defensas durante el estadio adulto ya que cuenta con más recursos. Si sólo se considera el patrón de defensa química de *T. velutina*, esta predicción no se cumple,

sin embargo, con el patrón de las defensas físicas si se observa y coincidentemente también se encontró un gradiente positivo para este atributo, donde los genotipos con una mayor densidad de tricomas también tienen una mayor adecuación.

Esto nos lleva reconsiderar la propuesta de la hipótesis de la defensa óptima, dado que a pesar de que un estadio sea el más vulnerable, se deben considerar otros factores internos y externos, incluyendo el impacto que la herbivoría puede tener sobre la capacidad de las plantas de asimilar y asignar recursos. La herbivoría puede tener un efecto diferente sobre las plantas en función del estadio ontogenético, por ejemplo en plantas jóvenes puede tener un efecto negativo sobre el crecimiento, mientras que en plantas adultas afecta la reproducción (Massad 2013). Considerando estos procesos, la hipótesis de balance crecimiento-diferenciación (Herms & Mattson 1992) propone que las plantas no pueden invertir simultáneamente en crecimiento y producción de defensas, por lo que las defensas son sintetizadas, después de cubrir las necesidades energéticas del crecimiento. En *Turnera velutina* encontramos que los patrones de defensa física y biótica siguen esta predicción donde las plantas expresaron los valores altos de estas defensas al alcanzar el estadio adulto. Para profundizar en esta discusión, debemos analizar como los factores internos y externos interactúan durante cada ontogenético, donde las presiones de selección y la cantidad de recursos disponibles interactúan con otros factores internos y externos.

A manera de conclusión podemos afirmar que ninguna de las teorías puede explicar por completo los patrones de defensa observados, lo cual puede interpretarse como que las predicciones propuestas por estas hipótesis no son generalizables para todas las defensas. Esto puede reflejar que tanto las presiones de selección como las restricciones fisiológicas determinan en conjunto la producción de defensas a lo largo de la ontogenia de las plantas.

Plasticidad fenotípica de las trayectorias ontogenéticas de la defensa.

Encontré que existe plasticidad fenotípica en la expresión de las trayectorias ontogenéticas de las defensas, es decir, los genotipos tienen la capacidad de cambiar las trayectorias ontogenéticas en función de las condiciones ambientales. Se encontró que el potencial cianogénico tuvo trayectorias contrastantes entre la población experimental y la del invernadero, en este último el potencial disminuyó con la ontogenia, mientras que en el invernadero sólo hubo un ligero incremento con el desarrollo. En contraste, las otras defensas mantuvieron la misma trayectoria ontogenética en dirección, pero la magnitud fue mayor en la población experimental para la densidad de tricomas y para las plantas de invernadero en el contenido de azúcar en el NEF. Con estos resultados, se muestra que las plantas de *T. velutina* tienen la capacidad de modificar la expresión de las

trayectorias de defensa en presencia de factores ambientales cambiantes. En la población de origen, *T. velutina* está expuesta a una gran heterogeneidad ambiental que se reflejan en microambientes con diferentes niveles de intensidad lumínica y de disponibilidad de agua. Además, *T. velutina* es principalmente dispersada por hormigas de la especie *Forelius analis* y las semillas que son manipuladas por estas hormigas, tienen mayor tasa de germinación (Salazar-Rojas *et al.* 2012), por lo que los sitios donde estas plántulas pueden germinar y establecerse están asociados con la ubicación de los nidos de las hormigas. En este escenario ambiental heterogéneo, si las plantas tienen la capacidad de modificar la expresión de los atributos, tendrán una mayor probabilidad de sobrevivir al interactuar con los factores bióticos y abióticos del micrositio donde hayan sido descartadas por las hormigas. La plasticidad de las trayectorias ontogenéticas representará entonces el resultado del efecto del ambiente sobre el desarrollo de los diferentes genotipos y pueden generar una diversidad de fenotipos en los organismos adultos.

Efectos de las trayectorias ontogenéticas de la defensa sobre las tasas vitales

Encontré que las estrategias ontogenéticas de la defensa de *T. velutina* tuvieron un efecto sobre las tasas vitales de la población. Particularmente, las plantas que expresan una estrategia defensiva con valores altos de defensa incrementaron su tasa de crecimiento, mientras que disminuyeron su supervivencia y producción de semillas. También encontré efectos negativos de los atributos individuales sobre las tasas vitales, específicamente, el potencial cianogénico disminuye todas las tasas vitales mientras que la densidad de tricomas disminuye el la tasa de crecimiento y de fecundidad, pero aumenta la tasa de supervivencia.

En estudios previos se han reportado los efectos individuales que los atributos defensivos pueden tener sobre las tasas vitales (Struckman *et al.* 2019). Por ejemplo, se ha reportado que la tasa de crecimiento está relacionada positivamente con la masa de las semillas (Visser *et al.* 2016), la masa foliar específica y la densidad estomatal (Liu *et al.* 2016), pero negativamente con la densidad de la madera (Visser *et al.* 2016). También se ha reportado que la tasa de supervivencia puede ser influenciada negativamente con la masa de las semillas (Visser *et al.* 2016), pero positivamente por la densidad de la madera y el tiempo de vida de las hojas (Adler *et al.* 2014). Finalmente, la tasa de fecundidad se pudo incrementar con atributos como la masa foliar específica y el contenido de nitrógeno (Adler *et al.* 2014). Sin embargo, al enfocarse solamente en los efectos individuales, sin considerar la variación ontogenética de estos atributos, no podremos comprender su impacto sobre las dinámicas poblacionales de las plantas.

Previamente había sugerido que la estrategia de defensa involucra cambios en la expresión de atributos particulares de la defensa a lo largo de la ontogenia para optimizar la adecuación de las plantas (Capítulo 2). Los resultados encontrados en este capítulo apoyan esta visión, ya que reportamos que la expresión de valores

altos de defensa durante el desarrollo puede resultar en una disminución de la producción de las semillas. Por lo tanto, el ajuste de la expresión de este atributo durante la ontogenia parece ser necesario. Al incorporar los efectos de atributos individuales de múltiples defensas a lo largo del desarrollo sobre las tasas vitales mediante un modelo demográfico, pudimos profundizar el enfoque del análisis de selección clásico, y resaltar el valor de evaluar la relación de múltiples atributos sobre la adecuación a lo largo del desarrollo de los individuos.

Mediante este tipo de aproximaciones, podremos identificar que estrategias ontogenéticas de las defensas contribuyen más al crecimiento poblacional, mediante el efecto sobre la supervivencia, crecimiento y fecundidad de las plantas dentro de las poblaciones. Se ha demostrado que los herbívoros pueden impactar a las poblaciones de plantas mediante un efecto sobre su crecimiento y retrocesos (Knight 2004), y sobre efectos sobre la supervivencia y fecundidad de las plantas entre estadios ontogenéticos (Bastrenta *et al.* 1995; Ehrlén 1995; Stowe *et al.* 2000), no se conoce el efecto entre trayectorias ontogenéticas de atributos defensivos sobre estos procesos demográficos.

Estrategias ontogenéticas de la defensa

Finalmente, propongo que las plantas expresan estrategias ontogenéticas de defensa que se reflejan en la adecuación de la planta, es decir, es la expresión combinada de varias defensas durante toda la ontogenia de la planta es la que puede reflejar un beneficio en términos de adecuación para los individuos. Encontré que existe un efecto de la expresión de diferentes combinaciones de defensas en cada estadio ontogenético, al obtener una triple interacción entre los componentes principales significativa (Ochoa-López *et al.* 2020). Esto sugiere que los genotipos exitosos son aquellos que puedan ajustar su fenotipo a la mejor estrategia defensiva en cada momento del desarrollo. Previamente se habían reportado cambios en las estrategias de defensa de la resistencia a la tolerancia (Boege *et al.* 2007), de defensa química a biótica (Ochoa-López *et al.* 2015), o entre diferentes defensas químicas (Goodger *et al.* 2013). Sin embargo, este trabajo constituye una primera evidencia cómo los cambios de estrategias defensivas a lo largo del desarrollo están relacionados con valores de adecuación diferente.

Conclusión

Las conclusiones puntuales de este trabajo son: 1) las trayectorias ontogenéticas de las defensas son plásticas; 2) los blancos y la intensidad de la selección ejercida por los herbívoros y el valor adaptativo de las defensas pueden cambiar durante la ontogenia de las plantas; 3) algunas trayectorias ontogenéticas de defensa son más propensas a expresar una respuesta evolutiva que otras; 4) las estrategias ontogenéticas involucran la expresión de múltiples atributos defensivos y estas influyen en el efecto promedio de la selección natural. Además, con los resultados de este trabajo sugiero que las trayectorias ontogenéticas de la defensa son

fenotipos complejos que pueden evolucionar por selección natural, y que tienen un efecto sobre los procesos demográficos de las poblaciones.

El aporte original de mi tesis radica en ser una primera aproximación experimental que aborda de manera integral el efecto de las trayectorias ontogenéticas de defensa con un enfoque tanto evolutivo como demográfico. El trabajo presenta evidencia de que las trayectorias ontogenéticas de la defensa pueden cambiar en función del ambiente biótico y abiótico en el cual se expresan. Asimismo, incorporando los resultados de los distintos capítulos se sugiere que la estrategia de defensa involucra cambios en la expresión de atributos particulares de la defensa a lo largo de la ontogenia para optimizar la adecuación de las plantas. Sin embargo, el estudio tiene algunas limitaciones impuestas por el diseño experimental. En particular, al exponer a las plantas desde estadios muy tempranos en las parcelas experimentales, no es posible controlar efectos de variación en la fenología de las interacciones ni el historial de herbivoría previo en función de la ontogenia. Estas limitantes, sin embargo, abren posibilidades para futuros estudios en los que podría considerarse una medida que estime la trayectoria (e. g. medida cualitativa o cuantitativa de cambio en la expresión de las defensas entre etapas) y relacionarla con la adecuación. Finalmente, cuando comparamos lo que nos indican los resultados del enfoque clásico de evolución y el enfoque demográfico podría parecer contradictorio. Ya que por un lado se reportan gradientes positivos para tricomas en plantas adultas y negativos para compuestos cianogénicos en plántulas, y por otro al simular estrategias defensivas con valores bajos de las tres defensas se observa una mayor fecundidad. Sin embargo, debemos tener presente que los patrones de selección reflejan predicciones a largo plazo y los análisis de rasgos vitales o demográficos patrones a una escala de más corto plazo.

En este estudio queda pendiente analizar la selección sobre la magnitud de los cambios ontogenéticos que observamos, posiblemente mediante el uso de medidas de cambio entre estadios ontogenéticos. Así mismo podría evaluar el efecto de las trayectorias ontogenéticas de defensa sobre la herbivoría, como un paso intermedio que informe los resultados de selección sobre rasgos. Además, dentro del área es necesario generar más estudios que evalúen una mayor claridad en cuales son y como funcionan los mecanismos detrás los patrones ontogenético que observamos en muchas especies, y no sólo en términos de atributos defensivos, sino en la expresión de cualquier atributo. Finalmente, un área fructífera en años venideros es la investigación que vincule los estudios moleculares de la expresión de los atributos con sus consecuencias en términos de desempeño y adecuación con estudios de campo.

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Anexo. Artículo requisito "*Plant defence as a complex and changing phenotype throughout ontogeny.*"

Plant defence as a complex and changing phenotype throughout ontogeny

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- **Background and Aims** Ontogenetic changes in anti-herbivore defences are common and result from variation in resource availability and herbivore damage throughout plant development. However, little is known about the simultaneous changes of multiple defences across the entire development of plants, and how such changes affect plant damage in the field. The aim of this study was to assess if changes in the major types of plant resistance and tolerance can explain natural herbivore damage throughout plant ontogeny.
- **Methods** An assessment was made of how six defensive traits, including physical, chemical and biotic resistance, simultaneously change across the major transitions of plant development, from seedlings to reproductive stages of *Turnera velutina* growing in the greenhouse. In addition, an experiment was performed to assess how plant tolerance to artificial damage to leaves changed throughout ontogeny. Finally, leaf damage by herbivores was evaluated in a natural population.
- **Key Results** The observed ontogenetic trajectories of all defences were significantly different, sometimes showing opposite directions of change. Whereas trichome density, leaf toughness, extrafloral nectary abundance and nectar production increased, hydrogen cyanide and compensatory responses decreased throughout plant development, from seedlings to reproductive plants. Only water content was higher at the intermediate juvenile ontogenetic stages. Surveys in a natural population over 3 years showed that herbivores consumed more tissue from juvenile plants than from younger seedlings or older reproductive plants. This is consistent with the fact that juvenile plants were the least defended stage.
- **Conclusions** The results suggest that defensive trajectories are a mixed result of predictions by the Optimal Defence Theory and the Growth–Differentiation Balance Hypothesis. The study emphasizes the importance of incorporating multiple defences and plant ontogeny into further studies for a more comprehensive understanding of plant defence evolution.

Key words: Compensatory growth, extrafloral nectaries, herbivory, ontogenetic trajectories, plant defence, plant–animal interaction, tolerance, *Turnera velutina*, Passifloraceae.

INTRODUCTION

Plant development involves changes in the expression of different plant functions, such as resource assimilation, plant growth, defence, maintenance, storage and reproduction (Herms and Mattson, 1992; Farnsworth, 2004; Weiner, 2004). Most of these changes are driven by resource availability and allocation trade-offs among functions prioritized at each ontogenetic stage. For example, developmental variation in the expression of different defensive strategies against herbivory has been widely reported (Boege and Marquis, 2005). However, a meta-analysis suggests that the patterns in investment of anti-herbivore defences are not predictable because the direction of ontogenetic changes depends on plant life history, the type of defence and the ontogenetic stages compared. For example, in woody plants, physical defences increase as plants age, while secondary metabolites in herbs and grasses decrease with plant development (Barton and Koricheva, 2010). In addition, whereas constitutive defences usually increase, induced

defences decrease throughout plant development. Barton and Koricheva (2010) also revealed that insect herbivores prefer to feed on young plants (Barton and Hanley, 2013), but mammal herbivores prefer the older plant stages (Jachmann, 1989; Swihart and Bryant, 2001).

Although it is clear that plant development is an important source of variation in the expression of anti-herbivore defence, we still know very little regarding the mechanisms promoting such variation. Currently we lack general patterns of the ontogenetic changes in plant defence (hereafter ‘ontogenetic trajectories’). We believe such deficits could be due to the fact that most plant species simultaneously express a combination of multiple defensive and nutritional traits (Fornoni *et al.*, 2004; Carmona and Fornoni, 2013) also defined as defence syndromes (Agrawal and Fishbein, 2006). Moreover, experimental evidence suggests that each defensive trait can follow different ontogenetic trajectories. For instance, opposite ontogenetic trajectories of chemical defences have been reported within the

same individuals of *Eucalyptus froggattii*: terpenoids increase whereas phenolic compounds decrease (Goodger *et al.*, 2013). The concentration of glucosinolates decreases while tolerance to damage increases during the ontogeny of *Raphanus sativus* (Boege *et al.*, 2007). Physical and chemical defences can also have opposite ontogenetic trends, as has been recently shown for *Plantago lanceolata* (Hanley *et al.*, 2007; Quintero *et al.*, 2014). Different ontogenetic trajectories in plants expressing multiple defensive strategies could be a consequence of: (a) changes in fitness costs and/or benefits of each defensive trait at particular ontogenetic stages (Barton, 2014); (b) differences in herbivore preferences (Quintero *et al.*, 2014); and/or (c) genetic correlations and trade-offs among different defensive strategies (Boege *et al.*, 2007). More research on the factors influencing ontogenetic variation in plant defence could shed light on our understanding of plant–herbivore–natural enemy interactions and defence evolution. For example, ontogenetic changes in different defensive and nutritional traits in *P. lanceolata* have been associated with differences in oviposition preferences and greater growth rates of specialist herbivores. In this case, plant ontogeny also influenced the immune responses of herbivores against parasitism (Quintero *et al.*, 2014).

An additional challenge to find general patterns in the ontogenetic trajectories of plant defence relies on the fact that very rarely is the entire ontogeny of plants considered (i.e. from seedling to reproductive stages, but see Goodger *et al.*, 2013; Barton, 2014; Quintero *et al.*, 2014). Because the direction of ontogenetic changes depends on the number of ontogenetic stages compared (Barton and Koricheva, 2010), trends resulting from considering only a portion of these stages can obscure the actual ontogenetic patterns of defensive traits (Barton and Koricheva, 2010). Furthermore, since most assessments have been performed in greenhouses (but see Boege, 2005; Quintero *et al.*, 2014), we also lack evidence on how the expression of multiple defensive traits affects herbivore damage in the field. Within this context, the aim of our study was to (1) describe the simultaneous ontogenetic changes of six defensive traits throughout six major stages of plant development, from seedling to the reproductive stage; and (2) evaluate whether ontogenetic trajectories in plant defence can explain patterns of leaf damage in the field. We were able to identify different patterns in the ontogenetic trajectories of various resistance and tolerance attributes, and associated these patterns with leaf herbivore damage observed in the field.

MATERIALS AND METHODS

Study site

The study was conducted at the Centro de Investigaciones Costeras La Mancha (CICOLMA) located on the coast of Veracruz, Mexico (19°36'N, 96°22'W, elevation <100 m). The climate is warm sub-humid, with a rainy season during the summer (June to September), an annual precipitation of 1100–1500 mm and a mean annual temperature ranging between 24 and 26 °C.

Study system

Turnera velutina (Passifloraceae) is a Mexican endemic perennial shrub that grows in coastal sand dune scrubs and in

tropical dry forests, from sea level to an altitude of 1300 m (Arbo, 2005). *Turnera velutina* has lanceolate and pubescent leaves with paired extrafloral nectaries (EFNs) on the petiole. The extrafloral nectar is made up of sucrose, glucose and fructose in similar percentages (Elias *et al.*, 1975), and is consumed by at least seven ant species (Cuautle *et al.*, 2005) and two wasp species (Torres-Hernández *et al.*, 2000). Some of these ants also disperse *T. velutina* seeds, which have elaiosomes as rewarding structures. The germination rate is approx. 15 %, and most seeds take 2–4 weeks to germinate. After germination, plants can spend up to 3 weeks at the cotyledon stage and they produce the first pair of true leaves on their fourth to sixth week. About 3 weeks later, they produce another pair of leaves. Flowering occurs when plants are approx. 4 months old, and continues until senescence, which occurs after 2 or 3 years (S. Ochoa and K. Boege, pers. obs). *Euptoteia hegesia* Cramer (Lepidoptera: Nymphalidae) is the main foliar herbivore, and its activity peaks from June to August (Cuautle and Rico-Gray, 2003), although it can be found all year round. Other orthopteran and coleopteran herbivores have occasionally been observed in reproductive plants (S. Ochoa and P. Zedillo, pers. obs.).

To characterize the ontogenetic trajectories of anti-herbivore defence in *T. velutina*, we measured physical, chemical and biotic resistance attributes, as well as tolerance expressed through compensatory growth after artificial leaf damage. We assessed plant defence in six major ontogenetic stages that included seedlings (cotyledon and two-leaf stages), juveniles (six- and ten-leaf stages) and reproductive plants (flowering and fruiting stages).

Ontogenetic patterns of resistance: greenhouse assessment

To describe the ontogenetic trajectories of defensive traits, 286 plants of *T. velutina* were grown in a greenhouse located at the study site. We collected seeds from 20 randomly selected plants growing in the sand dunes between June and September 2010. After manually removing their elaiosomes, 60 randomly selected seeds per maternal family were sown in germination trays with a mix of *in situ* soil and vermiculite substrate (50:50). Trays were bottom watered for approx. 3 weeks, until germination. A total of 12 seedlings per family were transplanted into individual 2 L pots, which were large enough to avoid pot binding at the oldest ontogenetic stages. To assess multiple defences, plants were divided into groups of 40 (2–4 plants per family) and each group was measured at one of the following six ontogenetic stages: cotyledon stage (C; 2-week-old plants only bearing cotyledons); infant stage (I; 4- to 6-week-old seedlings with the second true leaf fully expanded); six leaves juvenile stage (J1; 9- to 10-week-old young plants with the sixth leaf fully expanded); ten leaves juvenile stage (J2; 14- to 15- week-old plants with the tenth leaf fully expanded); flowering stage (F1; approx 16-week-old plants with their first flower in anthesis); and fruiting stage (Fr; approx 19-week-old mature plants bearing their first fruit).

The physical attributes of plant resistance considered at each ontogenetic stage were trichome density, leaf toughness and water content, all of which have been considered effective resistance traits (Agrawal, 2004). To control for leaf age, these

attributes were quantified in the penultimate fully expanded leaf of each plant. For stages Fl and Fr, we selected a fully expanded leaf bearing the first flower or fruit, respectively. Trichome density was estimated by counting the number of unicellular trichomes with a stereoscopic microscope (Carl Zeiss Stemi SV6) ($\times 10$) on eight observation fields (18 mm^2), four on the upper side and four on the underside of the leaf blade. The average number of trichomes per unit of area in each leaf side was calculated. Leaf area was measured using a portable scanner (CI-202; CID, Inc., Camas, WA, USA). Leaves were dried at room temperature until no weight changes were recorded, and dry weight was quantified with an analytical balance (VIC303, Acculab Sartorius Group, MA, USA). Leaf mass per area (LMA) was estimated as the dry weight (mg)/area (mm^2) ratio and was used as a proxy of leaf toughness, as it expresses the amount of mass per unit of area (Onoda *et al.*, 2011). Water content (W) was quantified as follows: $W = [(FW - DW)/FW \times 100]$; where FW is the fresh weight and DW is the dry weight.

We qualitatively determined the presence of hydrogen cyanide (HCN) at each ontogenetic stage using a colorimetric test (Schappert and Shore, 1995). To estimate the presence of HCN in cotyledon and infant stages, an additional group of 84 plants were germinated following the protocol previously described. Due to logistical and space limitations, the rest of the ontogenetic stages had to be assessed in field plants. For juvenile plants (6–10 leaves), pre-reproductive (>10 leaves) and reproductive stages (bearing flowers), plants were randomly selected from the field population ($n = 17, 28$ and 56 , respectively). To assess the presence of HCN, a fully expanded cotyledon or leaf was chosen. Using a hole punch, we obtained six leaf discs (approx. 0.6 cm^2). These discs were crushed in an Eppendorf tube with $7 \mu\text{L}$ of chloroform. A piece of picrate paper (filter paper soaked in a 5 % NaCO_2 and 0.5 % picric acid solution) was suspended inside the tube above the plant material, avoiding direct contact with it. Tubes were sealed and left in darkness for 24 h at room temperature ($25\text{--}30 \text{ }^\circ\text{C}$). The picrate paper changed from yellow to orange, rust or dark brown colour due to the presence and amount of HCN released (Schappert and Shore, 1995). We scored the change of colour categorically (i.e. yellow, orange, rust or dark brown), where no changes in colour indicated the absence of HCN.

To estimate plant investment in biotic resistance, we quantified the average number of EFNs and the sugar content in nectar produced in the last four apical, fully expanded leaves. Extrafloral nectar was collected using $1 \mu\text{L}$ microcapillary pipettes, and the volume was measured using a digital calliper. Sugar concentration ($\text{mg } \mu\text{L}^{-1}$) was determined from the values retrieved by a hand-held refractometer (Leica, Buffalo, NY, USA).

Ontogenetic patterns of compensatory growth

We estimated compensatory growth after an event of artificial damage. Due to logistical reasons, for this experiment, we only included three ontogenetic stages: infant (I; two leaves), juvenile (J; ten leaves) and reproductive (R; bearing one flower). Following the procedure described above, we germinated and grew 576 plants from ten genetic maternal families.

Each plant was randomly assigned to one of two groups: (1) control (undamaged plants); or (2) treatment (artificial damage on all leaves) at each ontogenetic stage ($n = 10$ plants per treatment per stage). Experimental plants were damaged by removing 50 % of each leaf blade with scissors (without damaging the midrib), and severely puncturing the remaining half with a needle. This procedure has been reported to have similar effects on the induction of extrafloral nectar secretion to those caused by natural herbivore damage and jasmonic acid application in *T. velutina* (Alonso-Salgado, 2015). Thirty days after being damaged, plants from both groups were harvested, separately collecting roots, stems and leaves. Plant material was dried at room temperature and weighed to estimate compensatory growth and above-/below-ground biomass allocation.

Herbivore damage in the field

In three different years, we assessed plant palatability at each ontogenetic stage by calculating the quantity of leaf tissue eaten by herbivores in field plants. We assessed the potential impacts of herbivore damage on plant performance by estimating the percentage of leaf area consumed in these plants. Two censuses were performed in the field population during June of 2009 and 2010, randomly selecting 30 plants of each of the following three ontogenetic stages: juvenile (J2; plants with 7–10 leaves), pre-reproductive (PR; plants with >10 leaves but without reproductive structures) and reproductive (R; plants bearing reproductive structures or scars left by flowers and fruits $n = 30$). Seedlings and infant plants were not included in these censuses because they were not found in sufficient numbers. A third census was carried out in July 2014, this time including five ontogenetic stages: cotyledon (C), infant (I), juvenile (J2), pre-reproductive (PR) and reproductive plants (R), as previously defined. In all censuses, leaf damage was assessed on all leaves (for C, I and J2 stages) or in 15 leaves for PR and R plants. For the latter cases, leaves were systemically chosen every $x/15$ leaves, x being the total number of leaves per plant. In all censuses, the width (W) and length (L) of each leaf were measured to estimate the original leaf area (LA), using the equation $LA = 0.7 + (0.64 \times L \times W)$, $R^2 = 0.97$, $P < 0.0001$). This relationship was previously established measuring 100 undamaged leaves from all stages. The area consumed by herbivores (CA) was estimated using a 0.5 cm transparent plastic grid. The percentage of leaf area consumed by herbivores was calculated as $CA/LA \times 100$.

Statistical analyses

We used a multivariate analysis of variance (MANOVA) to evaluate if the set of all defensive traits differed between the six ontogenetic stages. The model included ontogenetic stage as the explanatory variable, and trichome density, water content, LMA, number of EFNs, and sugar content in extrafloral nectar as response variables. Hydrogen cyanide was not included in the analysis because it was only found in the two youngest stages. Afterwards, we performed univariate ANOVAs including ontogenetic stage as the explanatory variable, family as a random factor and each defensive trait as the response variable. In all cases, post-hoc contrast analyses were performed to

define which ontogenetic stages were significantly different in the expression of each defence. In the case of trichome density, data were transformed using the logarithmic function $[\log(x + 1)]$ and the side of the leaf blade was included in the model as an explanatory variable, to assess if there were differences between the upper side and the underside of the leaf. To visualize the general ontogenetic trends in all five resistance traits, values of each trait were standardized as $(\bar{x}_i - \bar{x})/(\text{s.d. of } x)$, where \bar{x}_i is the average of the defensive trait x at the i th ontogenetic stage and \bar{x} is the average of that trait across all ontogenetic stages. Hence, deviations from zero can be viewed as the ontogenetic change relative to the population mean and can be used to compare trends of all defences across all ontogenetic stages.

To assess the impact of damage on plant biomass, we performed a two-way ANOVA including family as a random factor, ontogenetic stage, damage treatment and their interactions as explanatory variables, and total biomass as response variable. A significant effect of the interaction between damage and ontogenetic stage was interpreted as ontogenetic differences in the ability of plants to compensate for tissue losses. To verify if plant biomass is a good predictor of plant reproductive success of *T. velutina*, we used 45 additional plants randomly selected from the natural population. We harvested the whole plant and quantified the number of reproductive structures (i.e. flowers and fruits). To assess total plant biomass, we dried leaves, stems and roots at 60 °C for 48 h and weighed them. Using a regression analysis, we found that, indeed, plant biomass was positively associated with the reproductive effort of *T. velutina* ($r^2 = 0.865$, $P < 0.0001$). Considering biomass as a component of plant fitness, we estimated tolerance when plants were damaged at the infant (T_I), juvenile (T_J) and reproductive (T_R) stages for each maternal family. Tolerance was calculated as the difference in total plant fitness (biomass) between the damaged and the control (non-damaged) group at each ontogenetic stage: $T_I = (W_d - W_c)$, $T_J = (W_d - W_c)$, $T_R = (W_d - W_c)$; where W_d is the average biomass of damaged plants and W_c is biomass of undamaged plants at the infant (I), juvenile (J) and reproductive (R) stages, respectively. Hence, tolerance is an attribute of each maternal family, where positive values indicated greater tolerance than smaller and/or negative values (Strauss and Agrawal 1999; Boege et al. 2007). Ontogenetic differences in tolerance among ontogenetic stages were assessed using a generalized lineal model with a normal distribution, including ontogenetic stage as the explanatory variable of the degree of tolerance inherent to each maternal family, with maternal family being the unit of replication. To evaluate if resource allocation to different plant structures changed after damage, we used two-way ANOVAs. In this case, family, ontogenetic stage, damage and their interactions were included as explanatory variables and either root, stem or leaf biomass as response variables. All previously described statistical analyses were performed using R software (R Development Core Team, 2008).

Finally, ontogenetic differences in the mean leaf area consumed and the percentage of foliar area damaged by herbivores were assessed using non-parametric Kruskal–Wallis tests because data could not be normalized. Post-hoc Mann–Whitney tests were used to compare all pairs of ontogenetic stages within each census. These analyses were performed in JMP 10.0 (SAS Institute Inc., 2012).

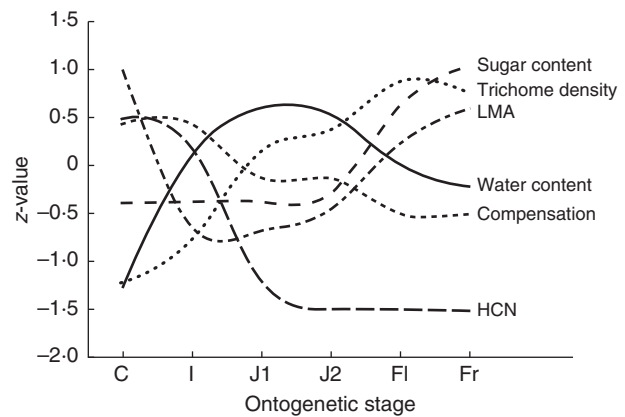


Fig. 1. Standardized values of multiple defensive traits (see text for a detailed explanation) across plant development of *Turnera velutina*. Ontogenetic stages are cotyledon (C), infant (I), juvenile six leaves (J1), juvenile ten leaves (J2), flowering (Fl) and fruiting (Fr).

RESULTS

Ontogenetic patterns of resistance

We found different ontogenetic trajectories for physical, chemical and biotic resistance (Wilks' $\lambda = 0.209$, $P < 0.0001$, Fig. 1). Both trichome density and leaf toughness increased throughout plant development ($F_{5,502} = 1229.23$, $P < 0.001$ and $F_{5,223} = 33.38$, $P < 0.001$; Fig. 2A, B). In particular, ontogenetic changes in trichome density were more pronounced on the underside than on the upper side of the leaf, with an increase up to 5-fold from seedlings to reproductive stages ($F_{1,227} = 434.22$, $P < 0.001$; Fig. 2A). Except for an initial decrease in the transition from cotyledon to the infant stage, leaf toughness increased approx. 60 % from the juvenile to the fruiting stage ($F_{1,227} = 59.818$, $P < 0.0001$; Fig. 2B). Water content also showed a non-linear ontogenetic trajectory, as juvenile plants had 5 and 9 % more water content than seedling and reproductive stages, respectively ($F_{1,195} = 157.61$, $P < 0.001$ and $F_{1,195} = 34.00$, $P < 0.001$; Fig. 2C). Chemical resistance displayed a negative ontogenetic trajectory, as the presence of HCN was detected only in early stages (cotyledon and infant), and was absent in older stages. Finally, we found a positive ontogenetic trajectory in biotic resistance associated with the abundance of EFNs ($F_{5,251} = 23.41$, $P < 0.001$). These glands were absent in cotyledon and infant plants and were differentiated only starting from the third leaf. The number of EFNs was constantly low at both juvenile stages (J1 and J2), but increased up to 33 % in reproductive plants (Fl and Fr) ($F_{1,167} = 5.84$, $P = 0.01$; Fig. 2D). Sugar content in extrafloral nectar had a similar pattern to that of gland abundance. Secretion from reproductive plants contained ten times more sugar than that from juvenile plants ($F_{1,153} = 31.84$, $P < 0.001$; Fig. 2E).

Ontogenetic patterns in tolerance

Artificial damage reduced plant biomass ($F_{2,409} = 1629.31$, $P < 0.001$) as a function of plant age ($F_{2,409} = 5.05$, $P < 0.006$). While seedlings were able to compensate fully, juvenile and reproductive plants were less tolerant to damage

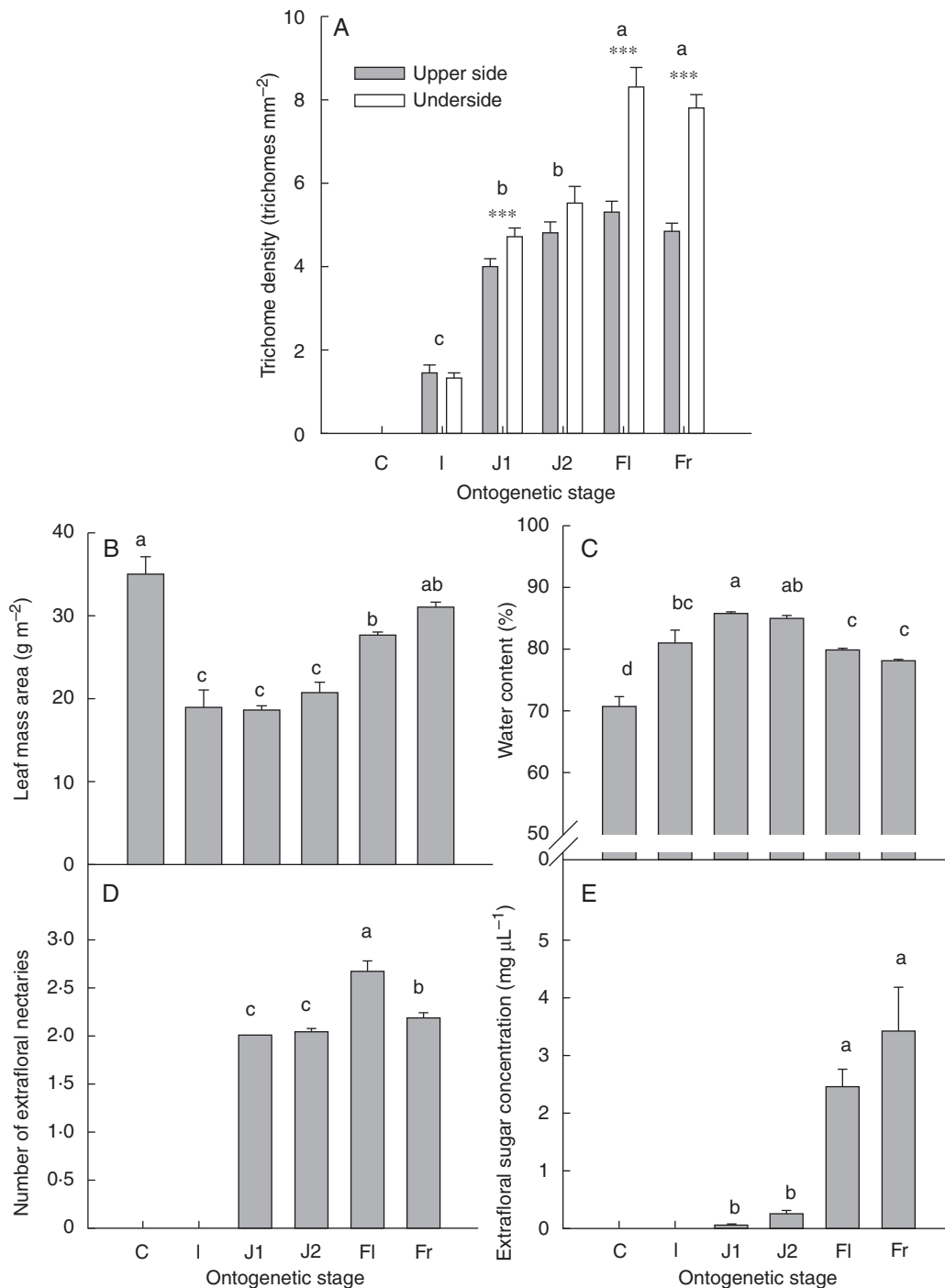


FIG. 2. Ontogenetic trajectories of physical, chemical and biotic defences in *Turnera velutina*. (A) Trichome density, (B) leaf mass area (as a proxy of toughness), (C) water content, (D) number of extrafloral nectaries and (E) extrafloral nectar sugar content. Ontogenetic stages are cotyledon (C), infant (I) juvenile six leaves (J1), juvenile ten leaves (J2), flowering (FI) and fruiting (Fr). Different letters above bars indicate significant differences among ontogenetic stages. Values correspond to the mean \pm s.e. ***Significant differences between leaf blades at $P < 0.0001$.

($F_{2,23} = 6.34$, $P = 0.01$; Fig. 3A). We found differences in resource allocation to different plant parts as a function of ontogeny and leaf damage. Damage promoted an increase in the root/shoot + leaves ratio ($F_{2,409} = 17.47$, $P < 0.001$; Fig. 3B), suggesting a reallocation of resources to produce compensatory responses (Barton, 2008; Barton and Hanley, 2013).

Leaf damage in the field

Leaf area damaged by herbivores in field plants was influenced by plant ontogeny in all censuses (2009, $\chi^2 = 8.80$, d.f. = 2, $P = 0.012$; 2010, $\chi^2 = 11.06$, d.f. = 2, $P = 0.004$; and 2014, $\chi^2 = 28.07$, d.f. = 4, $P < 0.001$; Fig. 4A). In general, juvenile plants had up to three times more leaf area eaten by

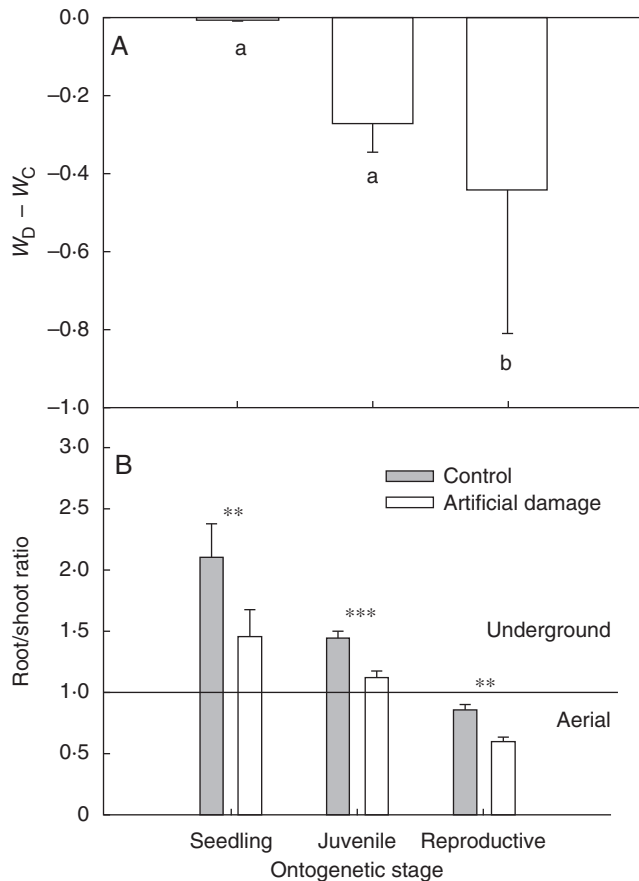


Fig. 3. (A) Differences in tolerance across plant ontogeny measured as the difference between the average biomass of artificially damaged plants (W_D) and the average biomass of control, undamaged plants (W_C) (mean \pm s.e.). Different letters indicate significant differences among ontogenetic stages. (B) Root:shoot ratio throughout ontogeny in control and artificially damaged plants. Values above the horizontal line denote relatively more biomass underground than above-ground, and values below the line represent plants with greater biomass above-ground than underground. Asterisks denote overall differences in biomass between treatments at each ontogenetic stage. ** $P < 0.01$, *** $P < 0.0001$.

herbivores than reproductive plants, which was consistently the least damaged stage. Damage in pre-reproductive plants varied across years: from having equivalent damage to juvenile plants in 2009, to lower values similar to that in reproductive plants in 2010 and 2014. Damage per leaf was significantly lower in juvenile plants than in cotyledon and infant stages, which had equivalent damage to that observed in pre-reproductive plants. However, because leaf area was significantly smaller in these young stages (even when leaves were of the same age), the percentage of leaf area damaged by herbivores was significantly greater early than later during plant ontogeny, at least during the first two censuses (2009, $\chi^2 = 10.91$, d.f. = 2, $P = 0.0043$; 2010, $\chi^2 = 16.38$, d.f. = 2, $P = 0.0003$; and 2014, $\chi^2 = 1.73$, d.f. = 3, $P = 0.78$; Fig. 4B).

DISCUSSION

The expression of different defensive traits had contrasting trajectories across the development of *T. velutina*. Whereas some traits increased throughout ontogeny, others decreased or

showed non-linear trends (Fig. 1). This variation in the ontogenetic trajectories of multitrait defensive strategies could be promoted by resource allocation constraints and changes in the costs and benefits of each defensive trait during plant development (Boege *et al.*, 2007). According to the temporal changes in plant defence, herbivore damage also varied as plants developed in the field. Juvenile plants received greater damage than younger seedlings or older reproductive plants. Our results on the simultaneous changes in tolerance and in the three main types of resistance traits (physical, chemical and biotic defences) add to the scarce evidence regarding the ontogenetic changes in multiple defences (see Goodger *et al.*, 2013; Barton, 2014; Quintero *et al.*, 2014). Studying the expression of different plant defences across plant ontogeny can enhance our understanding of when natural selection should favour the expression of particular combinations of defensive traits and influence herbivore foraging behaviour.

The Growth–Differentiation Balance Hypothesis proposes that the assimilation and allocation of resources limits the production of defences in early stages of ontogeny, and that defences should be greater in mature stages that have acquired enough resources for this function (Herms and Mattson, 1992; Boege and Marquis, 2005). In contrast, one hypothesis of the Optimal Defence Theory (Rhoades and Cates, 1976) predicts that young tissues or ontogenetic stages should be highly defended due to their greater susceptibility to herbivore attack than older stages and their crucial contribution to plant fitness (Bryant *et al.*, 1992). This apparent contradiction between the patterns expected by both hypotheses is reflected in the contrasting ontogenetic trajectories in plant defence that have been described for many plant species (Boege and Marquis, 2005; Barton and Koricheva, 2010). Our results suggest that both predictions can explain the ontogeny of plant defence even in a single plant species, depending on which defensive traits are being assessed. We found that trichome density, leaf toughness and biotic resistance (number of EFNs and production of extrafloral nectar) increased throughout ontogeny (Fig. 1). It is likely that young seedlings, indeed, had resource limitations that compromised the production of structural defences, as predicted by the Growth–Differentiation Balance Hypothesis. Trichome density and leaf toughness have been proven to be costly in the absence of damage at some ontogenetic stages (Hanley *et al.*, 2007), but it would be interesting to assess formally the costs of these defences throughout plant development. Nectar production also requires a considerable amount of resources (Wäckers and Wunderlin, 1999; Heil *et al.*, 2002; Heil, 2011), in particular carbohydrates competing with plant growth. Moreover, a recent study has reported that EFNs of juvenile *T. velutina* plants do not fully produce extrafloral nectar due to anatomical constraints on the secretory epidermis and exit pore of the EFNs (Villamil *et al.*, 2013). Hence, in addition to resource limitation, developmental and anatomical restrictions could be at play in promoting the observed ontogenetic delay in the production of extrafloral nectar.

In contrast to the increasing patterns of physical and biotic defences across plant ontogeny, we found that cyanogenesis and tolerance had decreasing trajectories throughout ontogeny. These trajectories are consistent with the Optimal Defence Theory, and could be explained by the need that young plants have to defend themselves at low costs, and the ability of older

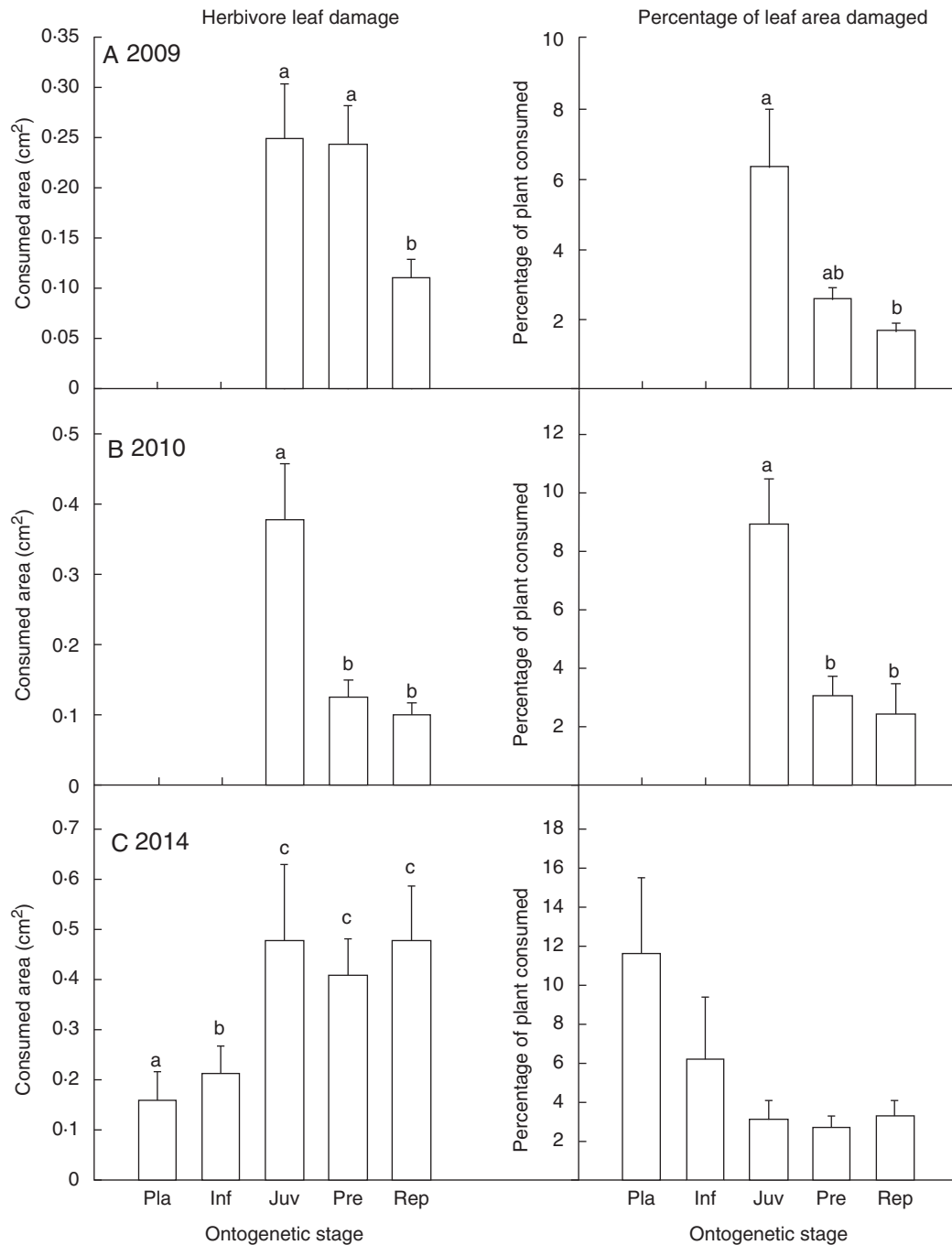


FIG. 4. Damaged leaf area and percentage of leaf area consumed by herbivores (mean \pm s.e.) in *Turnera velutina* plants in the field. Different letters indicate significant differences among ontogenetic stages. Ontogenetic stages are seedling (S), infant (I), juvenile (J), pre-reproductive (P) and reproductive (R).

plant to produce alternative defensive mechanisms. Fast growing plants allocate more resources to chemical defences with small molecules in low concentrations, which have low biosynthetic costs and fast turnover rates (e.g. cyanogenic glycosides and alkaloids; Bryant and Julkunen-Tiitto, 1995; Stamp, 2003). Young plants, which have faster growth rates than older stages, could benefit by producing these types of metabolites. In particular, some studies suggest that cyanide glycosides are an ideal form of defence for young stages since these compounds can be

safely stored in cell compartments (Siegler, 1991) readily available as defences after germination, if required. In the absence of damage, cyanide glycosides serve as a source of nitrogen, usually a limited resource and a key building block for protein synthesis, especially important during germination and seedling establishment (Selmar *et al.*, 1988; Sieglar, 1992). Furthermore, cyanogenic compounds in seeds can be translocated from parental plant tissue (Frehner *et al.*, 1990). Being a maternally inherited trait, this defence could be free of cost for the young

seedling, but costly to afford later during ontogeny. Negative ontogenetic trajectories in cyanogenesis had been previously reported in some populations of the sister species *T. ulmifolia* (Schappert and Shore, 1995). In this species, the production of cyanogenic compounds was associated with a reduced flower production, which suggests a cost for plant reproduction (Shappert and Shore, 2000). Further studies in *T. velutina* should quantitatively assess the concentration of HCN under the same conditions (i.e. not combining greenhouse and field plants), and investigate if the costs of such compounds change throughout plant ontogeny (see Boege et al., 2007).

Additional defences in early stages of plant ontogeny could provide young plants with a mechanism to cope with specialist herbivores adapted to cyanogenic compounds (Barton, 2008). Compensatory growth, which allows plants to replace promptly the tissue lost due to herbivory, would be particularly advantageous in early stages in which a greater proportion of leaf area is lost due to herbivores. Change in resource allocation after herbivore damage is a common response that endows compensatory growth. This response has been previously reported, suggesting that plasticity in biomass allocation is an important mechanism that enables tolerance (Barton, 2008, 2013). Our results are consistent with these findings since we observed that a reduction in root growth maximized shoot growth after herbivore damage.

Seedlings were chemically defended and fully compensated for tissue lost after leaf damage, while reproductive plants were defended by patrolling ants and through the expression of trichomes. In contrast, juvenile plants presented intermediate levels of all these defences. This either could promote an overall lower protection against herbivores or, alternatively, could represent a different anti-herbivore strategy: a combination of intermediate levels of all defensive traits that could provide an effective protection against a greater diversity of herbivores. The first scenario seems to be the case for *T. velutina*, as levels of herbivory found in the field were up to three times greater at the juvenile stage than in younger or older stages. A reduced ability of juvenile plants to produce defences could be due to the depletion of maternal resources available at the seedling stage, caused by resource trade-offs between defence and growth (Herms and Mattson, 1992; Bryant and Julkunen-Tiitto, 1995), and/or due to costs for later reproduction. In addition, juvenile plants do not have the architectural and anatomical features required to interact effectively with defensive ants (Villamil et al., 2013), as has been previously shown for many other myrmecophytic systems (Del Val and Dirzo, 2003; Falcão et al., 2003; Kobayashi et al., 2008; Chamberlain and Holland, 2009; Quintero et al., 2013).

Reduced plant defence at the juvenile stage could have important evolutionary consequences for plant–herbivore interactions. Juvenile plants were the least defended and, accordingly, the most attacked stage in the field. From the herbivore perspective, this can greatly impact their life history evolution: natural selection should favour the synchronization of herbivore development with plant phenology, so that herbivores can find and feed on the less defended host plant stages. In addition, the ontogenetic patterns reported here could promote early damage by specialized herbivores able to deal with cyanogenic compounds, followed by generalist herbivore damage later during plant development. Seasonal patterns in herbivore communities

have been associated with changes in plant quality through the induction of defences (Poelman et al., 2008; Kessler et al., 2012), but there is no information regarding the influence of ontogenetic changes in plant constitutive defences on such seasonal patterns. Hence, the study of ontogenetic changes in plants with multiple defensive strategies offers new contexts for understanding the assemblage and seasonality of herbivore communities. It has been proven in some species that the quantity and composition of plant volatile compounds can change as plants grow (but see Quintero et al., 2013). However, it still remains unknown whether insects can detect volatile compounds that enable them to differentiate among ontogenetic stages of *T. velutina*, as has been previously reported for other species (Hanley et al., 2013).

From the plant perspective, ontogenetic changes in different defensive traits can have important demographic consequences. Because the percentage of leaf area damaged by herbivores was greater for seedlings and juvenile plants than for older stages, the period of transition from seedlings and juveniles to older stages should be quite vulnerable and might have strong impacts on plant fitness and population growth, as has been reported for different systems (Parker and Salzman, 1985; Parker, 2014). In addition, even small amounts of tissue damage in young stages can be more costly in the long term than damage to older plants (Barton and Hanley, 2013). Detailed studies of plant performance and fitness components matched with the production of multiple plant defences spanning across the ontogeny of plants would contribute to our understanding of the evolutionary consequences of the expression of ontogenetic trajectories in multiple defensive traits.

We have described the simultaneous expression of tolerance and the three major types of resistance (biotic, chemical and biotic defensive traits) across plant ontogeny. The multivariate ontogenetic changes in plant defence could be the result of the optimization of resource allocation during plant development. Such optimization could be a function of the costs and benefits of each defensive trait, and of changes in the physiological priorities across ontogeny. If such optimization is adaptive, natural selection should favour those ontogenetic trajectories maximizing plant fitness. However, to demonstrate the adaptive value of switching from one defensive strategy to another during plant development, it is necessary first to assess formally the costs of multiple defences at each ontogenetic stage. Then, genetic variation and heritability of the trajectories must be investigated to determine if such trajectories can be targets of natural selection. Finally, proving their adaptive value requires the detection of significant relationships between particular ontogenetic trajectories and relative fitness. This approach opens up exciting avenues of research that can provide a complex, but more realistic understanding of plant defence evolution. Exporting this knowledge to applied areas could also have some benefits. For example, the discrimination between naturally vulnerable and well-defended stages of cultivars could lead to a more efficient use of pesticides and improved management practices.

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