Time is of the essence: direct and indirect effects of plant ontogenetic trajectories on higher trophic levels

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Abstract. Physiological and morphological constraints during plant ontogeny affect the expression of numerous plant traits relevant to higher trophic levels, such as nutritional content and physical and chemical defenses. Yet we know little about how temporal variation in these traits can directly and/or indirectly mediate tri-trophic interactions, such as those between plants, their herbivores, and herbivore natural enemies. Using four distinct ontogenetic stages of *Plantago lanceolata* (Plantaginaceae) and the specialist herbivore *Junonia coenia* (Lepidoptera, Nymphalidae), we evaluated how ontogenetic changes in plant quality can: (1) directly alter plant–herbivore interactions through butterfly oviposition choice and caterpillar performance assays, and (2) indirectly alter herbivores' susceptibility to higher trophic levels through caterpillars' iridoid glycoside sequestration and immune defenses. Results showed that plant defensive traits increased over *P. lanceolata* development, with leaf tissues becoming tougher and plant allelochemicals (iridoid glycosides) occurring in higher amounts. Conversely, plant nutritional quality (water and nitrogen content) decreased as plants aged. These ontogenetic trajectories strongly altered both direct and indirect interactions between plants and higher trophic levels. Buckeye butterflies showed a stronger oviposition preference for younger developmental stages of *P. lanceolata*, laying on average 60% more eggs on juvenile than on reproductive plants. Feeding experiments with caterpillars showed that larvae feeding on juvenile plants showed faster relative growth rate and increased digestive efficiency compared with those feeding on plants in the reproductive stage. These individuals, however, acquired lower levels of sequestered chemical defenses than did those feeding on older *P. lanceolata* plants, potentially rendering them more susceptible to predation. Finally, host plant age altered the ability of a caterpillar to mount an immune response against simulated parasitoid eggs. Specifically, caterpillars reared on older plant life stages, and thus with higher levels of sequestered iridoid glycosides, showed a compromised immune response compared to those feeding on younger plant age classes. This study exemplifies how ontogenetic trajectories in plant traits can scale up to directly or indirectly alter tri-trophic interactions, which may have key implications for understanding temporal shifts in herbivore population and community structure.

Key words: aucubin; butterfly oviposition choice; catalpol; caterpillar immune response; iridoid glycosides; Junonia coenia; *nutritional indices; ontogeny;* Plantago lanceolata; *sequestration; tri-trophic interactions.*

Introduction

Recent decades have seen a growing understanding of how the relative strength of bottom-up and top-down forces vary spatially and temporally in terrestrial ecosystems (Hunter and Price 1992, Denno et al. 2005, Gripenberg and Roslin 2007), as well as how both forces are indirectly linked to each other through plant traits (Kos et al. 2011*a*, Marczak et al. 2013). In particular, plant traits including nutritional quality (e.g., water and nitrogen content), physical defenses (e.g., leaf toughness

and density of trichomes or spines), and chemical defenses (e.g., alkaloids, terpenoids, phenolics) are known to govern tri-trophic interactions like those between plants, their herbivores and herbivore natural enemies. Yet, whereas much research has been focused on how phenotypic variation in such plant traits within and between populations mediates higher trophic level interactions (Travis 1996, Bailey et al. 2009), relatively little is known regarding how the strength and direction of plant–herbivore–natural enemy interactions may vary over time, especially across host plant development.

Reflecting the underlying variation in resource acquisition, allocation, and functional priorities (e.g., growth vs. reproduction) as plants develop from seedling to mature stages, termed ontogeny, all major traits that

influence higher trophic levels often vary. In particular, nutritional and defense traits that influence plant quality as food for herbivores often show nonlinear trajectories (Mattson 1980, Boege and Marquis 2005, Hanley et al. 2007, Barton and Koricheva 2010), as do those traits that alter plant attractiveness to herbivores' natural enemies (i.e., volatile organic compounds and shelter and food rewards [reviewed in Quintero et al. 2013]). While it is expected that temporal variation in these plant traits will impact multitrophic interactions, the direction and magnitude of these changes can be hard to predict. One reason underlying this complexity is that often, ontogenetic trajectories encompass shifts in several plant defensive traits at a time, which can show parallel or opposing trajectories (Barton and Koricheva 2010). A second reason lies in the interplay between the direct and indirect effects that these ontogenetic trajectories might have on the behavior and performance of higher trophic levels. Hence, the goal of this study was to evaluate how ontogenetic variation in plant defenses and nutritional quality might affect herbivores (1) directly, through changes in oviposition choice and herbivore performance, and (2) indirectly, through changes in herbivore vulnerability to natural enemies.

In general, invertebrate herbivores have been shown to cause greater damage (Price 1991, Albrectsen et al. 2004, Fonseca et al. 2006) or reach higher density and diversity (Cuevas-Reyes et al. 2004, Boege 2005, Thomas et al. 2010) on younger compared to older plant stages. However, it is unclear whether this trend is the result of changes in herbivore behavior (host selection and preference) or performance (development time, relative growth rate, fitness), or both, as host plants age. Empirical studies examining herbivore host plant selection have revealed highly species-specific responses, with cases in which ovipositing females or free-living larvae prefer either younger (e.g., Pires and Price 2000, Del Val and Dirzo 2003, Cuevas-Reyes et al. 2004) or mature (e.g., Lawrence et al. 2003, Johnson and Zalucki 2005, Heckel et al. 2010) host plant stages. In addition, changes in herbivore performance, leading to differences in the rate of feeding or the absolute amount of tissue lost to herbivores as plants age, can be difficult to predict. In several cases, when more than one trait was assessed within a plant species, contrasting ontogenetic trajectories were described within and among both plant allelochemicals and nutritional quality (e.g., Rehill et al. 2006, McArthur et al. 2010, Quintero and Bowers 2012). As a result, these complex ontogenetic changes can render plants more or less susceptible to a diverse suite of generalist and specialist herbivores (e.g., Kearsley and Whitham 1989).

Herbivore predation risk is also likely to change throughout plant ontogeny, if ontogenetic trajectories in plant traits alter plant cues used by natural enemies to find their prey or indirectly modify prey quality, and thus predator choice and performance. Evidence for the former comes from studies demonstrating that the top-

down control of herbivores can be mediated by ontogenetic trajectories in plant size and architectural complexity (e.g., Van Bael et al. 2003, Boege 2005), release of volatile organic compounds in response to herbivore damage, and changes in the availability of food rewards to predators (reviewed in Quintero et al. 2013). In contrast, studies assessing the indirect effect of ontogenetic trajectories of plant traits on herbivore quality as prey or host for natural enemies are limited. Because natural enemies such as predators and parasitoids may vary in their response to prey quality, many possible scenarios may emerge. For example, in the case of specialist sequestering herbivores, highly defended plant stages can increase prey toxicity or unpalatability, reducing herbivore predation risk by both predators (e.g., Chaplin-Kramer et al. 2011, Kos et al. 2011b) and parasitoids ("the nasty host hypothesis" [Gauld et al. 1992]). Alternatively, since the ability of insect herbivores to successfully encapsulate and kill their endoparasitoids and pathogens depends on their energetic and physiological resources (e.g., Haviola et al. 2007, Bukovinszky et al. 2009, Smilanich et al. 2009a), herbivores exposed to high levels of allelochemicals or poor nutritional quality may experience decreased immunocompetence ("the vulnerable host hypothesis" [Smilanich et al. 2009a]). Hence, as host plants age, the changes in tissue quality and defenses that translate into variation in herbivores' quality as prey or host can indirectly mediate the selective pressure imposed by predators and parasitoids on herbivore population dynamics.

Here, we present a series of experiments that assess the extent to which ontogenetic variation in plant defenses and nutritional quality affect butterfly oviposition, caterpillar performance, and caterpillar vulnerability to natural enemies, as mediated by sequestration of plant allelochemicals and immune defenses against simulated parasitoid eggs. Greenhouse and laboratory experiments were conducted across four distinct ontogenetic stages of the model system, ribwort or narrow-leaf plantain, Plantago lanceolata L. (Plantaginaceae), and one of its specialist herbivores, the buckeye butterfly, Junonia coenia Hübner (Lepidoptera: Nymphalidae). Strong ontogenetic trajectories in nutritional quality and constitutive concentrations of plant allelochemicals have been previously reported in P. lanceolata, changing as much as an order of magnitude over relatively short periods of time (Bowers and Stamp 1993, Fuchs and Bowers 2004, Barton 2007, Quintero and Bowers 2012). Furthermore, given that J. coenia has several generations a year (Brock and Kaufman 2003) and that P. lanceolata forms natural populations with diverse age structures (Shefferson and Roach 2010), herbivores are likely to be exposed to a wide diversity of host age classes in wild populations. We predict strong direct as well as indirect effects of plant ontogenetic trajectories on herbivore behavior and performance,

potentially altering interactions with higher trophic levels such as predators and parasitoids.

MATERIALS AND METHODS

Study system

Plantago lanceolata (Plantaginaceae) is a common short-lived weed (annual or facultative perennial) introduced to North America from Eurasia ~200 years ago (Cavers et al. 1980). It produces iridoid glycosides (hereafter IGs) (Ronsted et al. 2000) as its primary allelochemicals influencing generalist and specialist herbivores (Bowers 1991). In general, high levels of IGs deter or decrease damage inflicted by generalist herbivores, although several specialist herbivores have evolved to overcome, and in some cases sequester, those defenses in both their native and introduced range (reviewed in Dobler et al. 2011). The two major IGs produced by *P. lanceolata* are aucubin and catalpol, which vary from <1% up to 10–12% dry mass, depending on plant ontogeny, genotype, and nutrient availability, among other factors (Bowers and Stamp 1993, Barton 2007, Quintero and Bowers 2012). Besides IGs, *P. lanceolata* also invests in physical defenses such as leaf toughness (Schippers and Olff 2000) and glandular and non-glandular trichomes (de la Fuente 2002).

Junonia coenia (Nymphalidae), the common buckeye butterfly, is a New World butterfly that can have 1–3 broods per year in temperate regions (Brock and Kaufman 2003). *Junonia coenia* is a specialist on plants containing IGs (Bowers 1984), being commonly associated with *P. lanceolata* in the USA (Graves and Shapiro 2003). Adult female butterflies use IGs as oviposition stimulants, choosing host plants or tissues with higher IG content, in particular catalpol (Klockars et al. 1993, Prudic et al. 2005). In addition, *J. coenia* butterflies lay a single egg at a time, and thus, in oviposition tests, each egg represents an individual choice. Furthermore, buckeye caterpillars not only use IGs as feeding stimulants, but are also able to sequester aucubin and catalpol in their hemolymph. Levels of IGs in buckeye caterpillars, which are positively correlated with levels of IGs in their diet, vary normally from <5% to >20% dry mass (Camara 1997). Caterpillars storing higher levels of IGs in their tissues benefit from decreased mortality from generalist invertebrate predators, including wasps (Stamp 2001, Stamp and Meyerhoefer 2004), ants (de la Fuente et al. 1995, Dyer and Bowers 1996), stink bugs (Strohmeyer et al. 1998), and spiders (Theodoratus and Bowers 1999). Nevertheless, some physiological and ecological costs associated with consuming and sequestering high levels of IGs have also been reported, such as decreased performance and sequestration efficiency (Adler et al. 1995, Camara 1997), or increased larval susceptibility to parasitoids by weakening cellular immune responses (Smilanich et al. 2009a, Richards et al. 2012). Buckeye larvae used in this study were from a laboratory colony reared at the University of Colorado at Boulder, originally obtained from a colony maintained at Duke University.

Experimental design

Plants used in these experiments were grown at the University of Colorado greenhouse during the summers of 2009 and 2010. Seeds were collected from >20 maternal plants from a population in Boulder County, Colorado, and mixed before sowing them in seed flats at intervals over the season. Seeds were germinated in Fafard mix and transplanted, after 15 days, to a growth medium of Metro Mix 350 and turface (Sun-Gro Horticulture, Agawam, Massachusetts, USA) in 4.5-L pots. In both years, butterflies (2009) and caterpillars (2010) were exposed simultaneously to all host plant developmental stages, synchronizing the plant stages by germinating seeds at intervals of 30 days from March to June. In this way, although average environmental conditions from sowing to harvest varied among age classes, we ensured that all individual plants were exposed to the same environmental conditions prior to exposure to herbivores or harvest. As explained in Quintero and Bowers (2012), this design reduced potential problems associated with serial sowing by reducing the effects of other confounding environmental factors such as photoperiod and temperature on plant quality and defenses.

To assess the effect of changes during host plant ontogeny on higher trophic levels, we used four distinct developmental stages: J1, which represents young juvenile plants soon after they ended their seedling stage (i.e., containing 5–15 new leaves, and averaging 0.40 g dry mass); J2, juvenile plants that have reached a complete rosette with new, intermediate, and old leaves but have not yet developed any reproductive structures (~1.60–4.30 g dry mass); FL, flowering mature rosette plants that had few to many scapes with buds or open flowers (~6–11.5 g dry mass); and FR, fructifying mature rosette plants with many scapes ranging from fruit development to seed release (~14.20 g dry mass). In both years, we simultaneously grew 20–180 plants in each of these four plant age classes, and replicates were randomly placed on four to six 2.5 × 4 m greenhouse benches, exposed to natural daylight, and watered daily. Scotts Peter's Excel fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, Ohio, USA) mixed in a ratio of 15–5–15 N–P–K with trace micronutrients was supplied to all plants every 3–4 days throughout the duration of the experiment.

Leaf traits across plant ontogeny

In addition to individual plants being of a specific developmental stage, there are also leaf tissues of different age categories within a stage: new, intermediate, and old leaves. The categories are named based on the position that leaves occupy in the rosette and their physiological status (Bowers and Stamp 1993). For all the experiments described below we separated plant

tissues into these three leaf categories plus inflorescences; however, we report combined leaf trait data from new and intermediate leaves for two reasons. First, new and intermediate leaves are the only tissue category present across all four plant developmental stages, allowing comparison across treatments. Second, as previously observed by Klockars et al. (1993), buckeye butterflies preferentially oviposit on new and intermediate leaves (i.e., 94% of their eggs), regardless of their availability in the plant, and caterpillars prefer to consume only tender leaves in the center of the rosette (C. Quintero, *personal observation*). Combined new and intermediate leaf trait data are reported throughout all experiments because, although butterflies were able to discriminate among tissues, caterpillars were fed a mixture of both leaf age classes. This methodology should highlight overall whole-plant ontogenetic trajectories over other confounding factors (i.e., leaf tissue age).

Following harvest, combined new and intermediate leaves from each plant were weighed fresh, oven-dried at 50°C for 48 hours, and weighed again to the nearest 0.01 g. Plant nutritional quality was assessed as variation in leaf water and nitrogen content. Leaf water content was calculated as [(wet mass − dry mass)/wet mass] × 100, while nitrogen content was quantified by Micro-Dumas combustion on a NA1500 C/H/N analyzer (Carlo Erba, Milan, Italy), using ~3 mg of finely ground leaf tissue per sample. To assess variation in concentrations of IGs, all tissues were ground into a fine powder, and 10–30 mg subsamples were processed for IG extraction and analyzed by gas chromatography following previously described methods (Bowers and Stamp 1993, Quintero and Bowers 2012). Finally, to determine leaf toughness we followed two methodologies. For the plants used in the butterfly choice tests (2009), before leaf tissues were oven dried, a Wagner Fruit Tester series penetrometer (Wagner Instruments, Greenwich, Connecticut, USA; Model No. U0801, with a 0.1-mm tip) was used to measure the force required (GF; in grams) to fracture the leaf lamina (Sanson et al. 2001). Leaf toughness was assessed on 3–6 leaves per tissue category and developmental class, taking four measures per leaf in between major leaf veins. In all other experiments involving caterpillars (2010), leaf toughness was measured as specific leaf area (SLA). SLA was calculated as A/M, where A is the area of a disk ~2 cm in diameter (cut with a cork borer) and M is the leaf disk dry mass (Milla et al. 2008). Twenty leaf disks collected from at least 10 leaves per age class were used. SLA has been shown to inversely correlate with fiber concentration, such that lower SLA indicates higher concentrations of fiber, and thus, higher leaf toughness (e.g., Choong 1996, Gras et al. 2005). These methodologies were comparable and highly correlated, with a higher leaf specific area being negatively correlated with leaf toughness ($R = -0.84$, $P = 0.005$, $N = 30$); but methods varied between years due to equipment availability.

Developmental variation in combined new and intermediate leaf biomass, toughness, and water and nitrogen content was analyzed using one-way analyses of variance (ANOVA), followed by Bonferroni post hoc tests to distinguish mean differences among age classes. Multivariate analysis of variance (MANOVA) was used to examine concurrent variation in aucubin and catalpol concentrations as a function of plant age, due to significant correlations between these two variables within tissues. When significant effects were detected, we followed with univariate ANOVAs for each IG, and mean group differences among plant age classes were assessed using Bonferroni post hoc tests. Biomass data were square-root transformed, and percentage of water, nitrogen, and IG concentrations were arcsine square-root transformed to improve normality and homogeneity of variance.

Female oviposition choice

To evaluate buckeye butterfly oviposition preference among developmental stages of *P. lanceolata*, two sets of three-way choice tests were performed from 16 to 30 June 2009: (1) stages J1–J2–FL, and (2) stages J2–FL–FR, with 20 replicates per test. This homogeneous incomplete block design was selected over paired-choice tests in order to better reflect natural conditions, where butterflies are exposed to choices among many plants at once, and to avoid multiple pair comparisons (i.e., six possible combinations) that would decrease replication. In addition, we avoided complete block designs, involving all four plant stages, due to known limitations in the number of treatments among which an animal can actually discriminate, as well as to avoid the undesired effect of the spatial configuration of treatments (Raffa et al. 2002). For each replicate, one plant of each of the three developmental stages was placed inside a circular wired cage (1.2 m diameter × 1 m height), covered with a fine mesh, and secured at the top with clothespins. Plants were equally spaced in a triangular pattern, 30 cm apart from each other and 10 cm from the edge of the cage, and a source of sugar water was placed in the center of the cage. Naïve, virgin, 1–3 day old butterflies, four males and one female, were placed inside each cage for 72 h, allowing sufficient time for the female butterfly to mate and lay eggs. All experiments were performed in the field under natural light and temperature conditions (see Plate 1A, B). Plants were acclimated to the field conditions for a week prior to the experiment. Cages were checked every 12–24 hours to refill the food supply and to water plants as needed. After 72 hours, all plants were removed from the cages, harvested, and aboveground tissues were separated into four categories: new, intermediate, and old leaves, and inflorescences (see Bowers and Stamp 1993, Klockars et al. 1993). The number of leaves and inflorescences per tissue per plant were counted, as well as the total number of eggs laid on each tissue category per plant. Once eggs were removed

from all tissues, leaf traits were assessed as described in *Leaf traits across plant ontogeny*.

Given the lack of independence among choices in preference tests, the non-parametric Friedman test by ranks was used to assess female choice across the two sets of three-way choice tests, followed by Wilcoxon signed-rank tests, with the appropriate Bonferroni adjustment, to test for mean group differences among the three age classes. Total number of eggs laid differed widely among individual females (i.e., 60–370 eggs) and plants of different developmental stages show significant variation in leaf biomass available (see *Results*); therefore, we used the proportion of eggs laid by each female on each plant age class over the available fresh biomass of new and intermediate leaves (i.e., grams of wet tissue, as a proxy for leaf area) as our dependent variable. Moreover, results were similar between analyses that corrected for or did not correct for differences in available biomass between plant developmental stages (data not shown); however, because data trends were clearer when correcting for leaf tissue availability, we report only these data here (Fig. 1).

Caterpillar performance and feeding efficiency

All larval performance experiments were conducted during spring–summer 2010, under controlled growth chamber conditions with a photoperiod of 14D:10N, and day–night temperatures of 27°C–22°C. Individual plants reared at the greenhouse were harvested every 2–3 days in order to supply caterpillars with a constant source of fresh, previously undamaged leaves. Sets of mixed new and intermediate-aged leaves were also saved to measure leaf IGs, nitrogen and water content, and leaf toughness as described previously ($N = 20$–35 sets per age class).

Caterpillar performance.—Neonate larvae in groups of 10 individuals per petri dish were assigned randomly to feed on a mix of new and intermediate leaves from each of the four plant developmental stages (J1, J2, FL, and FR), until pupation. Twelve replicates per age class were performed for each of these non-choice tests for a total of 480 larvae. Larvae were monitored every day and a constant supply of fresh leaves was provided. At the beginning of the experiments, as well as every three days, all live larvae per petri dish were counted and weighed as a group to the nearest 0.01 mg. Four measures of caterpillar performance were calculated: mortality rate, relative growth rate, time to pupation, and pupal mass. Relative growth rate (RGR), dry mass increase per unit dry mass per day, was calculated as $[(W_f - W_i)/W_i]/t$, where W_f is final biomass, W_i is initial biomass, and t is the total number of days from neonate to newly molted fifth instar. Mean individual mass per petri dish (i.e., unit of replication) was used in order to account for changes in the number of surviving caterpillars among treatments over time. Once caterpillars pupated, pupae were weighed fresh to the nearest 0.01 mg. Variation in larval mortality rate, RGR, time
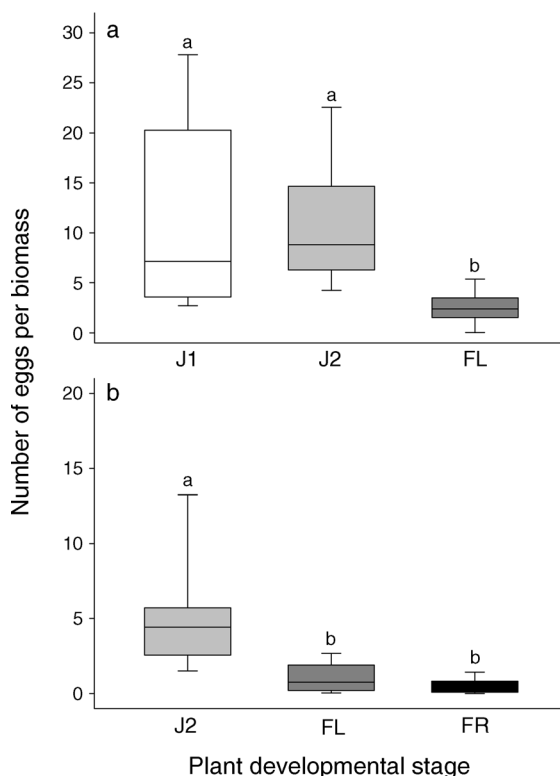

Fig. 1. *Junonia coenia* host selection across two sets of three-way oviposition choice tests, represented as number of eggs per available fresh mass of new and intermediate leaves per plant for (a) J1–J2–FL ($N = 16$) and (b) J2–FL–FR ($N = 18$) *Plantago lanceolata* stages. See *Materials and methods: Experimental design* for description of plant developmental stages. Boxes represent the median and the 25th and 75th percentiles, and bars extend to the 5% and 95% values. Different letters indicate significant differences (Wilcoxon signed-ranks test; $P < 0.05$).

to pupation, and pupal mass were tested by one-way ANOVA followed by Bonferroni post hoc tests. For pupal mass, the group (i.e., each petri dish as unit of replication) was used as a random effect in the model. Time to pupation and pupal mass data were square-root transformed, and proportion data (mortality rate and RGR) were arcsine square-root transformed to improve normality and homogeneity of variance.

Caterpillar feeding efficiency.—In a separate experiment, newly molted fifth-instar caterpillars, reared on J1, J2, FL, and FR plants from neonates as described previously, were used to calculate four nutritional indices according to the standard gravimetric method (Waldbauer 1968): consumption index (CI), approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD). All measurements were gathered over a 24-h interval starting with 20 newly molted fifth-instar larvae per treatment diet, placed individually in small, sealed, plastic containers (160 mm^2), and provided with sufficient leaf material (see Plate 1C). Prior to

this 24-h period as well as after it, caterpillars were starved for 4–8 hours to ensure an empty gut. A separate subset of larvae and leaves from each treatment diet was dried and weighed at the beginning and the end of the experiment to obtain dry mass conversion factors. Nutritional indices were analyzed using one-way analyses of covariance (ANCOVAs) (Raubenheimer and Simpson 1992). The numerator of the formula used to calculate each nutritional index was the dependent variable, while the denominator was used as a covariate; all were square-root transformed. In the case of CI, initial larval mass was used as the covariate.

Caterpillar IG sequestration and immune defenses

Caterpillar IG sequestration.—Following the nutritional indices experiment, all fifth-instar larvae ($N = 20$ per treatment), starved for 4–8 hours to ensure an empty gut, were freeze-killed and kept frozen until they were processed for extraction of sequestered IGs. To measure sequestration of IGs, whole caterpillars were ground with sand in 5 mL MEOH, prepared for IG extraction and quantification using gas chromatography, as described in Richards et al. (2012). Variation in the concentration of IGs sequestered, arcsine square-root transformed, as a function of host plant age, was assessed using a one-way MANOVA with the proportion of dry mass aucubin and catalpol as dependent variables. When a significant effect was detected, we followed with univariate ANOVAs for each compound separately.

Caterpillar immune defenses.—To measure the immune response of fifth-instar *J. coenia* larvae, a separate set of caterpillars previously reared on J1, J2, FL, and FR plants from neonates as described in *Caterpillar performance*, were injected with silica beads (Rantala and Roff 2007, Smilanich et al. 2009a; see Plate 1D). Previous studies have shown that exposing caterpillars with these positively charged glass beads produces a strong encapsulation response, which correlates with both immune response against real parasites and pathogens (Rantala and Roff 2007), and field parasitism rates (Smilanich et al. 2009b). Thus, larval immune response, measured as percent melanization, was assessed following previously described methods (Smilanich et al. 2009a, Richards et al. 2012). Although parasitism and immune responses to parasitoids may vary across larval development (Hawkins et al. 1997, Rantala and Roff 2005, Bukovinszky et al. 2009), in this experiment fifth-instar caterpillars were used for ease of manipulation. A total of 20 larvae per treatment were injected, but final sample size varied between 10 and 16 replicates per treatment due to mortality or lack of feeding after injection. Analyses of covariance (ANCOVA), with number of beads retrieved per caterpillar as a covariate, were used to compare percent melanization as a function of host plant developmental stage. Pearson's correlation coefficients were used to examine associations between percent melanization and

sequestered IGs (aucubin, catalpol, and total IGs). All statistical analyses were performed using SPSS 16.0 (SPSS 2007).

RESULTS

Female oviposition choice

Individual *J. coenia* females laid, on average, 202.5 ± 15.5 eggs (mean ± SE), across all host plant stages. Of the 40 individual females tested, six laid fewer than 20 eggs, and thus were excluded from the analyses. Friedman tests, assessing variation in proportion of eggs laid by females on new and intermediate leaves (with and without being corrected by differences in available biomass between plant developmental stages), showed that butterflies significantly preferred younger over older stages (Fig. 1). In particular, in the case of the choice test J1–J2–FL, butterflies laid approximately six times as many eggs per available gram of fresh biomass on juvenile stages than on reproductive stages ($\chi^2_{2,16} = 15.87$, $P < 0.0001$; Fig. 1a), with no significant differences between the J1 and J2 stages (despite a small reduction in egg number per available fresh biomass) but an important reduction in butterfly preference between J1 or J2 compared to FL plants (Fig. 1a). In the case of the choice test including older plant stages, J2–FL–FR, the differences were even more pronounced (Fig. 1b), with butterflies laying 4–9 times more eggs per gram of available fresh biomass on juvenile than on reproductive stages ($\chi^2_{2,18} = 21.44$, $P < 0.0001$), preferring J2 over either FL or FR stages (Fig. 1b). Butterfly oviposition choice may respond to a combination of traits changing in leaf tissues as plants age, since combined new and intermediate leaves significantly differed in all plant traits measured across plant ontogeny. In both cases (J1–J2–FL and J2–FL–FR), there was a significant increase in biomass, physical, and chemical defenses, while water and nitrogen content decreased as plants aged (Appendix: Table A1).

Caterpillar performance and feeding efficiency

Plant quality and defenses.—Similar to the patterns described in 2009, combined new and intermediate leaves used to feed *J. coenia* caterpillars in 2010 also varied in all measured traits as a function of host plant age (Fig. 2). Specifically, while overall nutritional quality decreased (water content $F_{3,141} = 79.97$, $P < 0.0001$; N content $F_{3,139} = 141.19$, $P < 0.0001$; Fig. 2a, b), physical (SLA $F_{3,76} = 417.44$, $P < 0.0001$; Fig. 2c) and chemical defenses (Wilks' $\lambda = 0.54$, $F_{6,280} = 16.72$, $P < 0.0001$; aucubin $F_{3,141} = 29.47$, $P < 0.0001$; catalpol $F_{3,141} = 28.71$, $P < 0.0001$; Total IGs $F_{3,141} = 32.15$, $P < 0.0001$; Fig. 2d) increased as plants developed.

Caterpillar performance.—Changes in plant quality and defenses across ontogeny did not alter larval mortality, although they strongly impacted caterpillar growth rate, development time, and pupal mass. Mortality rate of immature larvae tended to increase as plant age increased (Fig. 3a), but did not significantly
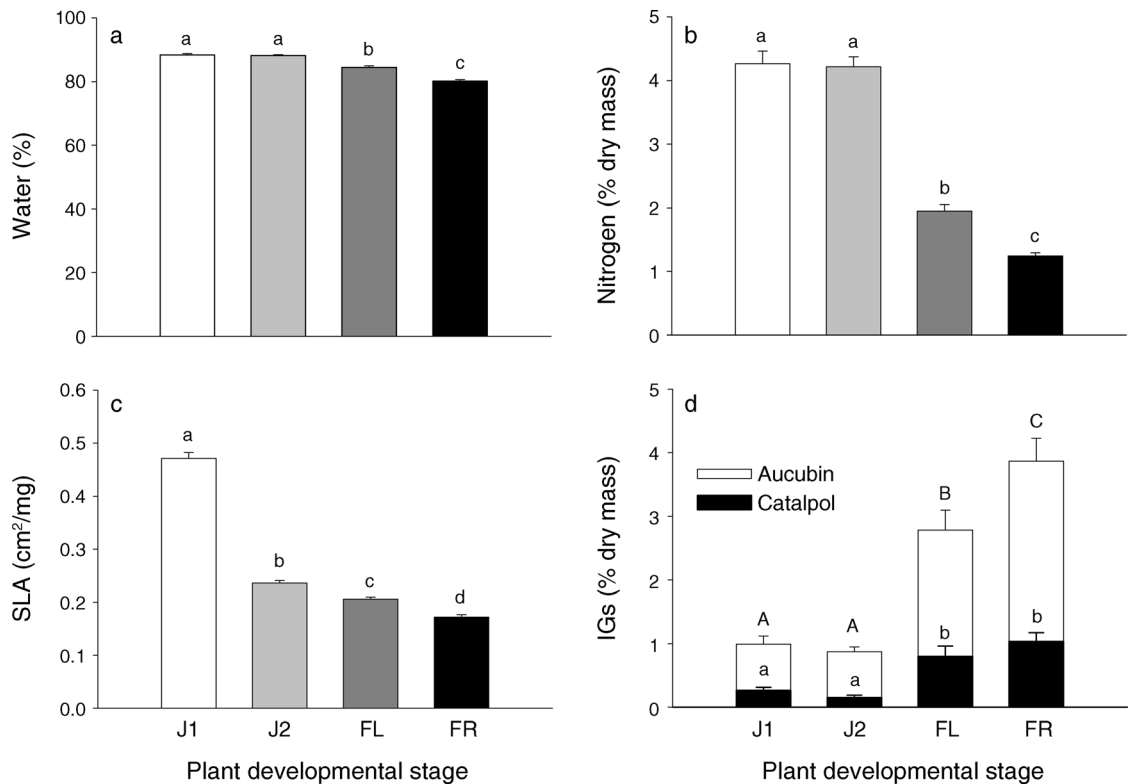
Fig. 2. Ontogenetic variation in new and intermediate leaves of *Plantago lanceolata* used in all larval experiments (mean + SE). Four leaf traits were measured: (a) water content, (b) nitrogen content, (c) physical defenses measured as specific leaf area (SLA, with high values representing low leaf toughness), and (d) chemical defenses measured as percent dry mass of iridoid glycosides (IG; aucubin and catalpol). See *Materials and methods: Experimental design* for description of plant developmental stages. Original data were square-root or arcsine square-root transformed for statistical analyses; actual values are shown for illustrative purposes only. Different letters indicate significant mean group differences as tested by a Bonferroni post hoc test ($P < 0.05$). (d) Capital letters were used to represent group mean differences for aucubin and lowercase letters for catalpol concentrations.

vary as a function of host plant age ($F_{3,44} = 2.21$, $P = 0.1$). In contrast, RGR significantly decreased two to three times for those larvae fed new and intermediate leaves of reproductive stages, as compared with leaves of the same age but from juvenile stages ($F_{3,44} = 13.35$, $P < 0.0001$; Fig. 3b). Time to pupation significantly varied as a function of diet ($F_{3,44} = 69.4$, $P < 0.0001$), with larvae reared on FR plants taking ~30% longer (i.e., 7–10 days) to reach the pupal stage as compared with larvae reared on J1, J2, and FL plants (Fig. 3c). However, only marginally significant differences were detected for pupal mass across host plant treatments ($F_{3,126} = 2.6$, $P = 0.055$; Fig. 3d), suggesting that the extended development time compensated for low-quality diets.

Caterpillar feeding efficiency.—Some measurements of caterpillar feeding efficiency also varied as a function of host developmental stage (Table 1). The consumption index (CI) showed an abrupt increase of two times more leaf material consumed as host plants changed from juvenile to mature stages (Fig. 4). Approximate digestibility (AD), and the pre-digestive measure of efficiency of conversion of ingested food (ECI) did not significantly vary among larvae fed different diet treatments

(Table 1, Fig. 4). In contrast, the post-digestive measure of efficiency of conversion of digested food (ECD) was higher only for larvae feeding on young juvenile plants as compared to those feeding on older juvenile and mature plant stages (Table 1, Fig. 4).

Caterpillar IG sequestration and immune defenses

Caterpillar IG sequestration.—Host plant developmental stage had a significant effect on caterpillar sequestration (Wilks' $\lambda = 0.43$, $F_{9,151} = 6.96$, $P < 0.0001$), with an overall increase in caterpillar IG concentration as host plants aged (Fig. 5a). Univariate ANOVAs also demonstrated a significant effect of plant age on larval aucubin and catalpol concentrations ($F_{3,64} = 17.53$, $P < 0.0001$ and $F_{3,64} = 9.83$, $P < 0.0001$, respectively) as well as on total IG concentrations ($F_{3,64} = 15.98$, $P < 0.0001$) and the proportion of catalpol/total IG ($F_{3,64} = 6.01$, $P < 0.001$). In general, caterpillars feeding on immature plant stages (i.e., J1 and J2) did not differ in their levels of sequestered IGs, but caterpillars feeding on mature reproductive stages acquired 30–50% more defenses in their tissues than those feeding on J1 and J2 plants (Fig. 5a), mirroring the changes seen
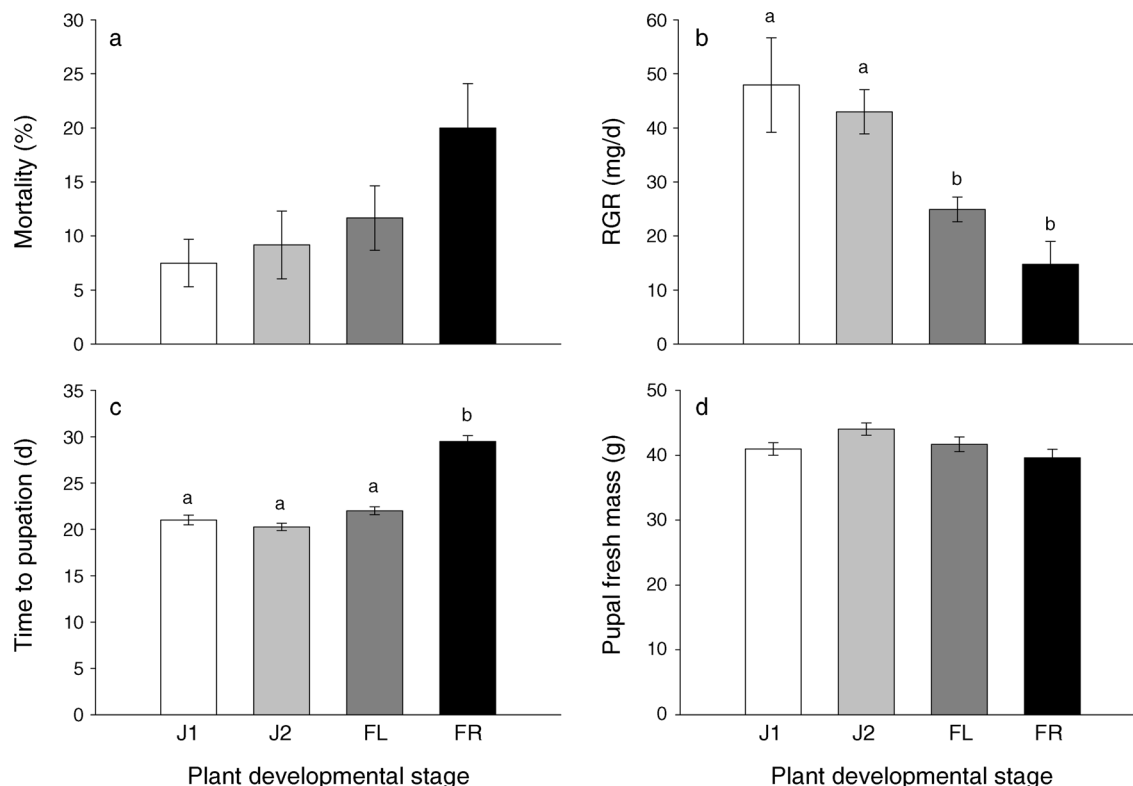
FIG. 3. Performance of *Junonia coenia* caterpillars, reared from neonate to pupation, across four host plant developmental stages (J1, J2, FL, and FR), measured as (a) mortality rate, (b) relative growth rate, (c) time to pupation, and (d) pupal mass. See *Materials and methods: Experimental design* for description of plant developmental stages. Original data were square-root or arcsine square-root transformed for statistical analyses, actual values (mean ± SE) are shown for illustrative purposes only. Different letters indicate significant mean group differences as tested by a Bonferroni post hoc test ($P < 0.05$), when overall ANOVA was significant.

among their diets (Fig. 2). In fact, mean plant IG concentration was highly correlated with mean caterpillar sequestered IGs ($R^2 = 0.97$, $P = 0.01$, data not shown).

Caterpillar immune defenses.—The ability of fifth-instar caterpillars to encapsulate and melanize the glass beads significantly varied across host plant age classes ($F_{3,49} = 2.97$, $P = 0.04$). Specifically, larvae feeding on FR plants showed a significant decrease in percent melanization (up to 30% difference) compared to younger host plant stages (Fig. 5b). The number of

beads recovered per individual caterpillar, used as a covariate, did not affect the melanization response ($F_{1,49} = 1.6$, $P = 0.21$), indicating that the ability to mount an immune defense can be independent of level of attack. Finally, Pearson correlation coefficients demonstrated a negative correlation between caterpillar percent sequestration and percent melanization (total IGs, $R = -0.32$, $P = 0.021$, $N = 52$; aucubin, $R = -0.29$, $P = 0.035$, $N = 52$; catalpol, $R = -0.31$, $P = 0.023$, $N = 52$), indicating that diets rich in IGs, leading to higher IG larval sequestration, diminish caterpillar immune response.

TABLE 1. Summary of one-way ANCOVAs comparing larval nutritional indices as a function of host plant developmental stage (diet treatment: J1, J2, FL, and FR).

	CI			ECI			AD			ECD		
Source of variation	df	F	P	df	F	P	df	F	P	df	F	P
Covariate	1	13.13	**0.001**	1	35.97	**<0.001**	1	85.67	**<0.001**	1	0.29	0.60
Plant age	3	10.34	**<0.001**	3	2.39	0.077	3	0.93	0.43	3	7.73	**<0.001**
Error	63			63			63			63		

Notes: CI is consumption index, ECI is efficiency of ingested food, AD is approximate digestibility, and ECD is efficiency of conversion of digested food. See *Materials and methods* for description of covariate in each case. Significant effects are indicated in boldface type.
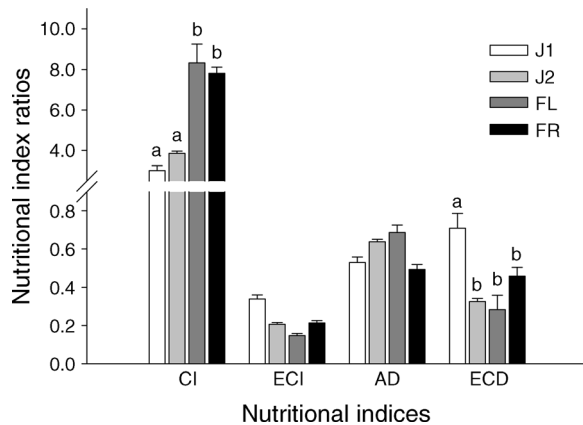
FIG. 4. *Junonia coenia* larval nutritional indices (mean + SE) for newly molted fifth-instar larvae feeding for 24 h on new and intermediate leaves of four host plant developmental stages (J1, J2, FL, and FR; see *Materials and methods: Experimental design* for description of plant developmental stages): consumption index (CI), efficiency of conversion of ingested food (ECI), approximate digestibility (AD), and efficiency of conversion of digested food (ECD). Note that the *y*-axis is broken to allow CI values to fit on the same graph as the other parameters. Different letters indicate significant mean group differences as tested by a Bonferroni post hoc test ($P < 0.05$), when overall ANOVA was significant.

DISCUSSION

Ontogenetic changes in traits that alter host plant quality to herbivores have been repeatedly demonstrated across all plant life forms (reviewed in Barton and Koricheva 2010). In this study, we found that, whereas *P. lanceolata* leaf toughness doubled and levels of IGs in young leaves increased up to 16 times between juvenile and mature stages (i.e., 0.36– 6% dry mass), water content decreased by 17% and nitrogen content decreased by 72% during the same period (Fig. 2; Appendix: Table A1). Similar degrees of change have been shown in other studies as well (Bowers and Stamp 1993, Schippers and Olff 2000, Quintero and Bowers 2012). This shift towards less nutritious and better-defended tissues as *P. lanceolata* develops might render older plant stages less susceptible to *J. coenia* herbivory. Yet here we showed that herbivore performance, and thus expected overall plant damage, throughout *P. lanceolata* development, might depend on the identity, abundance, and behavior of the third trophic level, as the indirect effects of plant ontogenetic trajectories on *J. coenia* defense and immune response (i.e., herbivores' susceptibility to higher trophic levels) might be as strong as their direct effects (i.e., herbivores' host selection and performance).

Plant ontogeny and its direct effects on plant–herbivore interactions

Variation in insect herbivore abundance and damage on certain host plant developmental stages over others may depend on a combination of both host selection and

herbivore performance. In this study, both factors tend to favor younger life stages of the host plant, as adult *J. coenia* butterflies selectively preferred younger developmental stages of *P. lanceolata*, laying 4–9 times more eggs per gram of available fresh biomass on juvenile than on reproductive stages. In accordance with the preference–performance hypothesis (Jaenike 1978), immature larvae also performed better on juvenile stages of their host. In particular, as host plants aged, larvae feeding on older plants decreased by threefold their relative growth rate, took almost twice as long to develop to the pupal stage, and increased by two times the sequestration of IGs in their hemolymph. Nevertheless, despite this considerable variation in larval performance, neither larval mortality nor pupal mass
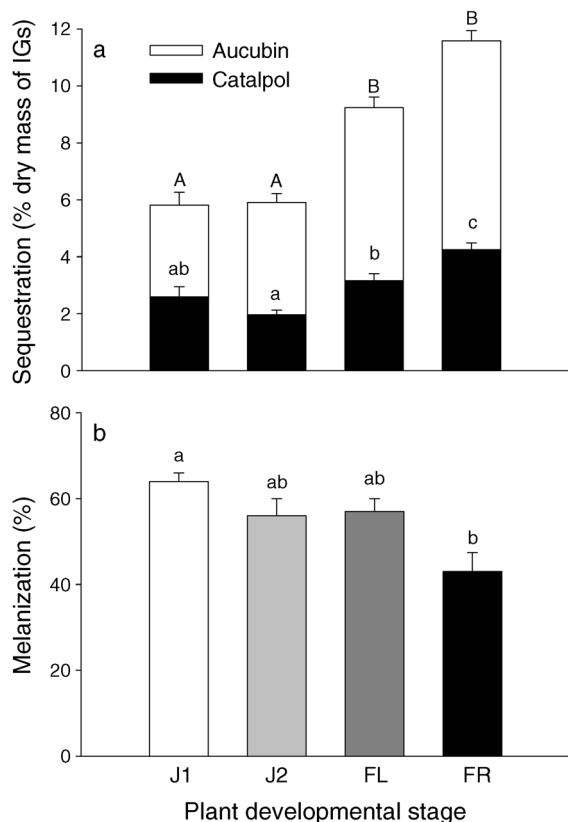

FIG. 5. The percentage of sequestration and melanization of *Junonia coenia* newly molted fifth-instar larvae (mean + SE), feeding from neonate on new and intermediate leaves of four host plant developmental stages (J1, J2, FL, and FR; see *Materials and methods: Experimental design* for description of plant developmental stages). (a) Percent dry mass aucubin (white) and catalpol (black) present in larval hemolymph. (b) Larval immune response to simulated parasitoid eggs measured as percent melanization of injected silica beads. Original data were arcsine square-root transformed for statistical analyses; actual values are shown for illustrative purposes only. Different letters above bars indicate significant mean group differences as tested by Bonferroni post hoc tests ($P < 0.05$). Capital letters were used to represent mean group differences for aucubin, and lowercase letters for catalpol concentrations.

varied significantly across plant ontogeny, suggesting that overall herbivore fitness may not be strongly impacted beyond the potential effects that delayed development time may already confer (e.g., Benrey and Denno 1997).

What plant traits drive female oviposition choice and larval performance across plant ontogeny? In the case of butterfly choice tests, as reported for other lepidopteran species (Honda 1995), a combination of stimulant and deterrent traits is expected. Previous studies have demonstrated that buckeye butterflies use IGs as oviposition cues, and that often higher IG content in leaves, in particular catalpol, increases likelihood of oviposition (Klockars et al. 1993). In addition, female oviposition behavior is also affected by host plant traits other than IG concentration, as females laid more eggs on high-nitrogen, low-IG plants than on low-nitrogen, high-IG plants (Prudic et al. 2005). Lastly, because buckeyes often select younger leaves over older leaves (Klockars et al. 1993), we believe that overall female preference may have been driven by the combination of: (1) larger proportion of new leaves, (2) higher proportion of catalpol despite lower total IG content, and (3) higher nutritional quality (nitrogen and water) and lower leaf toughness in younger juvenile stages.

Larval performance differences may have also resulted from a combination of traits. First, the nutritional quality of forbs, distinguished by their high water (75–95% fresh mass) and nitrogen content (1.5–9.7% dry mass) (Slansky and Scriber 1985), often promotes rapid growth of chewing herbivores (Tabashnik and Slansky 1987). Because insects respond to lower nitrogen content by increasing food consumption or the efficiency of nitrogen use (Tabashnik and Slansky 1987), the observed 10% decrease in foliar water content and threefold decrease in nitrogen content as *P. lanceolata* ages, may well explain the higher consumption rate (CI) and lower larval relative growth rate (RGR) observed while feeding on those tissues. Second, leaf toughness can further decrease larval performance by limiting the size of meals being eaten, slowing gut passage rates, and reducing nutrient supply and efficiency of assimilation of nutrients (Hochuli 1996, Clissold et al. 2009). Therefore, the low efficiency of conversion of ingested and digested food (ECI, ECD) and the extended development time of *J. coenia* reared on older host plant stages may be due, at least in part, to ontogenetic trajectories in leaf physical defenses. Finally, the toxic effect of IGs can also decrease performance, even for this specialist sequestering insect (e.g., Adler et al. 1995, Camara 1997). The toxic effects of IGs are induced when the compounds are activated by enzymes such as the hydrolytic β-glucosidases (Dobler et al. 2011). The resulting iridoid aglycones can denature amino acids, proteins, and nucleic acids, act as enzyme inhibitors, and inhibit the formation of prostaglandins and leucotrienes, all of which may either affect herbivores directly or may reduce the quality of ingested food by rendering proteins undigestible (reviewed in Dobler et al. 2011). Because larvae confined to low-quality diets commonly compensate by consuming more tissues (Tabashnik and Slansky 1987), such a strategy would increase *J. coenia* exposure to defensive compounds, which must subsequently be detoxified or sequestered, diverting energy that otherwise could be allocated to growth (Smilanich et al. 2009a). Hence, we believe that a combination of higher levels of physical and chemical defenses and lower nutritional quality in leaves of older host plant stages slows the growth of this specialist insect by making digestive processes less efficient.

Combined, the direct effects of ontogenetic changes in *P. lanceolata* defensive traits on *J. coenia*'s performance should lead to the expectation of higher damage on younger life stages of *P. lanceolata*. Such bias towards vulnerable young stages might severely impact the population growth of this plant species, although the high tolerance capability of *P. lanceolata*, even at very young life stages such as seedlings (Barton 2008, 2013), might be sufficient to offset these direct effects. Yet overall damage under natural conditions might depend on the ability of female butterflies to locate less conspicuous young stages, the capability of young larvae to disperse to nearby hosts when the original source is depleted, or it may depend on the identity, abundance, and behavior of their natural enemies (see indirect effects in the following section). Finally, these results also suggest that potential mismatches between female oviposition choice and offspring performance (Gripenberg et al. 2010) may depend on the developmental stages available for both adults and larvae in natural populations or experimental assays.

Plant ontogeny and its indirect effects on herbivore–natural enemy interactions

While ontogenetic changes in host plant traits can indirectly alter the strength of the relationship between herbivores and their natural enemies via changes in herbivore performance and palatability, such indirect effects have received less consideration than direct effects. Here, we illustrated how temporal variation in plant traits may lead to opposing effects on predators vs. parasitoids, as mediated by caterpillar ability to sequester host plant chemical defenses.

In terms of predator–prey interactions, because larval sequestration correlates with IG content in the diet (e.g., Camara 1997), and *P. lanceolata* IG content increased from <1% to >4% dry mass between juvenile and mature stages (Fig. 2; Appendix: Table A1), our results suggest a considerable decrease in larval predation risk as host plant age increases. Sequestration of IGs by *J. coenia* larvae not only serves as an effective defense against invertebrate predators such as ants, stink bugs, spiders, and predatory wasps (e.g., de la Fuente et al. 1995, Strohmeyer et al. 1998, Theodoratus and Bowers
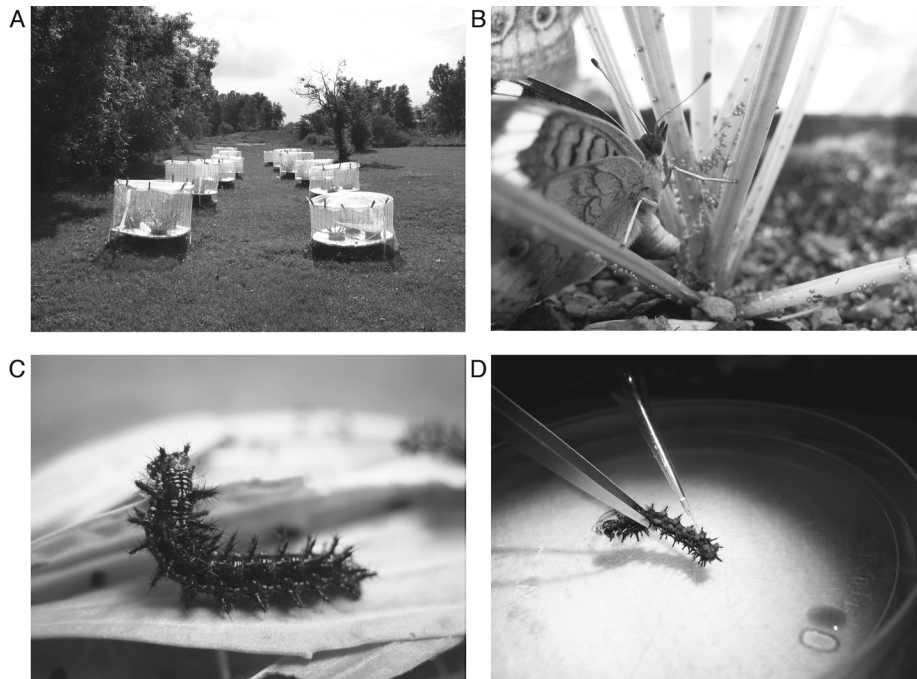
PLATE 1. (A) Butterfly oviposition choice cages in the field, under natural light and temperature conditions, (B) female buckeye butterfly (*Junonia coenia*, Nymphalidae) laying eggs at the base of a *Platago lanceolata* (Plantaginaceae) rosette, (C) fifth-instar buckeye caterpillar eating fresh *P. lanceolata* leaves for the caterpillar feeding efficiency experiments, and (D) fifth-instar buckeye caterpillar being injected with silica beads, under a dissection microscope and using a handmade fine glass needle, for the caterpillar immune defenses experiment. Photo credits: C. Quintero.

1999), but they may also show dose-dependent responses (Dyer and Bowers 1996), with concomitant decreases in predators' performance and fitness if forced to consume highly unpalatable larvae (Strohmeyer et al. 1998, Stamp 2001, Stamp and Meyerhoefer 2004). As a result, although we did not experimentally assess the effect of plant age on subsequent larval mortality, the doubled IG content seen in caterpillars when feeding on fructifying mature plants (~12% dry mass IGs) compared to young juvenile stages (~6% dry mass IGs) (Fig. 5a) should scale up to not only decrease larval predation risk, but also to confer physiological costs to predators that consume them, potentially weakening the top-down control of predators on herbivores as *P. lanceolata* ages.

In contrast, in terms of parasitoid–prey interactions, this increase in larval sequestration ability while feeding in older host plant stages resulted in diminished larval immunocompetence, suggesting higher susceptibility of *J. coenia* to parasitoids and pathogens as their host plant develops. In particular, less chemically defended *J. coenia* larvae reared on juvenile plants achieved 30% higher levels of melanization compared to larvae reared on older mature stages (Fig. 5b). This result agrees with previous evidence that higher levels of IGs in *J. coenia* diets decreased larval ability to mount an encapsulation and melanization response (Smilanich et al. 2009a, Richards et al. 2012). Although our study cannot discriminate whether this response is a consequence of

increased larval ingestion and sequestration of plant allelochemicals (Haviola et al. 2007, Smilanich et al. 2009a) or poor diet quality (Ojala et al. 2005, Klemola et al. 2007), it is likely that the increase in IGs as host plants aged is the primary driver of the changes seen in melanization. Previous studies with *J. coenia* reared on artificial diets or using IG supplementation (i.e., same nutritional quality but different IG levels) demonstrated that high IG content, principally catalpol, significantly reduced larval immune response (Smilanich et al. 2009a, Richards et al. 2012). Because parasitoids are considered one of the most important sources of mortality for many caterpillars (Hawkins et al. 1997), and immune response is clearly one of the most effective defenses that caterpillars have against parasitism (Smilanich et al. 2009b), a 30% decrease in mortality due to enhanced immunocompetence can be ecologically relevant. Furthermore, given that susceptibility to parasitism can be stronger at earlier life stages of larval development (Remmel et al. 2011), probably due to diminished immunocompetence (Rantala and Roff 2005, Buko-vinszky et al. 2009), our results may therefore be underestimating the actual successful immune response to parasitoids over host plant ontogeny. Yet, overall, our results support the "vulnerable host hypothesis," adding to the increasing evidence that defenses that are effective against predators may render larvae more

susceptible to parasitoids (Gentry and Dyer 2002, Smilanich et al. 2009*b*).

Conclusions

Our research revealed that ontogenetic trajectories in plant defenses and nutritional quality can scale up, potentially altering tri-trophic interactions over time. What is more remarkable is that the differences reported here for *J. coenia* host selection, performance, sequestration, and estimated predation risk on different developmental stages of *P. lanceolata* can surpass the variation seen in previous studies due to environmentally or genetically mediated phenotypic variation in *P. lanceolata* quality and defenses (Adler et al. 1995, Bowers and Stamp 1997, Prudic et al. 2005). Therefore, ontogenetic shifts due to climate change, for example, which can alter the relative timing of species interactions, can have critical effects on the population dynamic of herbivores as well as on the community composition of higher trophic levels (Yang and Rudolf 2010).

Finally, because spatiotemporal variation in direct and indirect species interactions plays key roles in structuring communities (Poelman et al. 2008), studies assessing ontogenetic trajectories in plant defensive traits should be cautious when scaling up their predictions across trophic chains. Host plant ontogenetic trajectories can have not only strong direct, but also indirect, effects on higher trophic levels, illustrating why bottom-up and top-down controls of herbivore populations are context dependent or even hard to demonstrate. This was particularly true in this study system, where the effect size (i.e., the maximum raw difference between group means) of direct effects of *P. lanceolata* ontogeny on *J. coenia* host selection (0.74) and performance (i.e., RGR 0.92) were similar to its indirect effects (i.e., IG sequestration −0.75 and immunocompetence against simulated parasitoid eggs 0.65). However, it is unknown how direct and indirect effects may enhance, inhibit, or counterbalance each other. For instance, while lower levels of sequestered defenses should enhance larval susceptibility to predators, faster development times achieved while feeding on *P. lanceolata* juvenile stages (i.e., ~10 days faster) may decrease larval chances of being found by enemies (Benrey and Denno 1997), resulting in potentially similar levels of predation risk across plant ontogeny. Furthermore, because ontogenetic trajectories in host plant quality may vary not only in magnitude but also in direction (i.e., allelochemicals vs. nutritional quality), we expect that the responses of associated herbivores will vary substantially among species, with diet breadth, and with degree of dietary specialization. This species-specific variation may lead to nonoverlapping herbivore distributions across host plant stages, otherwise interpreted as competitive exclusion. Consequently, studies incorporating field surveys are necessary to shed light on how the direct and indirect effects of plant ontogenetic trajectories on plant–herbivore–natural enemy interactions might combine to shape temporal variation in overall levels of tissue damage, plant fitness, and the resulting arthropod community. Nonetheless, above all, as has been stated for spatial structure (Gripenberg and Roslin 2007), we argue that the strength of almost every bottom-up and top-down force is likely to vary over time (i.e., seasonally and throughout plant ontogeny).

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SUPPLEMENTAL MATERIAL

Appendix

Nutritional quality and defensive traits of combined new and intermediate *Plantago lanceolata* leaves harvested following butterfly oviposition choice experiments (*Ecological Archives* E095-226-A1).