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SPECIAL TOPIC: FROM PLANTS TO HERBIVORES

Plant and herbivore ontogeny interact to shape the preference, performance and chemical defense of a specialist herbivore

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Abstract

The amount of damage that herbivorous insects impose on plants varies as a function of plant ontogenetic trajectories in tissue quality and defenses, and the herbivores' own developmental trajectories in body size, mandible shape and detoxification enzymes, among others. However, little is known about how host plant and herbivore ontogeny interact. Using four ontogenetic stages of *Plantago lanceolata* (Plantaginaceae) and three to five larval stages of the specialist caterpillar *Junonia coenia* (Nymphalidae), we evaluated how ontogenies in both of these trophic levels shape: (i) caterpillar feeding choice, (ii) performance, and (iii) sequestration of plant allelochemicals. Plant physical (leaf toughness) and chemical (iridoid glycosides) defenses increased, while nutritional quality (water and nitrogen content) decreased, as plants aged. These plant ontogenetic trajectories strongly altered the behavior and physiology of this specialist herbivore, but the magnitude of the response varied with larval stage. In feeding experiments, while first instar larvae showed little preference among plant stages, older larvae significantly preferred juvenile over reproductive stages. In turn, larval consumption increased and digestive efficiency decreased, potentially explaining their decrease in relative growth rate, as larvae and host plant aged, but differences were greater for younger than older caterpillars. Finally, sequestration of plant allelochemicals increased through plant and larval development; however, the major differences due to diet occurred earlier during larval development. Our results highlight that changes in plant ontogeny most strongly influence early herbivore instars, emphasizing the need to consider the developmental stage of both trophic levels to better understand temporal variation in herbivore damage.

Keywords Iridoid glycosides · *Junonia coenia* · Nutritional indices · *Plantago lanceolata* · Sequestration

Introduction

Despite the longstanding interest in understanding temporal variation in herbivore damage, the direct and indirect mechanisms that could explain these patterns have been rarely investigated (Kos et al. 2011; Campos et al. 2016). Recent work has revealed that ontogenetic trajectories in plant nutritional quality, and physical and chemical defenses have strong effects on herbivores' host selection (Johnson and Zalucki 2005; Fonseca et al. 2006; Hanley et al. 2013), larval performance, and larval susceptibility to natural enemies (Boege 2005; Quintero et al. 2014). On the other hand, the substantial change in body size exhibited throughout ontogeny by herbivorous insects is expected to constrain their morphology, physiology and feeding behavior (Clarke and Zalucki 2000; Hochuli 2001; van Dam et al. 2001; Johnson and Zalucki 2007), modifying the type and amount of damage that they are able to impose on vegetative tissue. Relatively little, however, is known about how host plant and herbivore ontogeny interact to affect insect herbivore behavior and physiology and, ultimately, temporal variation in herbivore damage.

Plant and herbivore ontogeny can interact in complex ways. Insect herbivore host plant selection, and the resulting herbivore community, will vary over time due to the

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constantly changing phenotype of multiple plant defensive traits across plant ontogeny (e.g. McArthur et al. 2010; Quintero and Bowers 2012; Goodger et al. 2013; Ochoa-Lopez et al. 2015), and due to the ontogenetic changes in herbivores' traits that enable insects to locate suitable hosts and initiate feeding (Clarke and Zalucki 2000; Zalucki et al. 2002; Schäpers et al. 2015). Subsequently, the amount of damage that this community will exert depends on the interaction between vegetative tissue's nutritional quality and resource allocation to defense traits throughout plants' ontogeny (Mattson 1980; Boege and Marquis 2005; Hanley et al. 2007; Barton and Koricheva 2010) and the developmental stage of the consumers. As they grow, most invertebrate herbivores experience strong shifts in head and body size, mandible shape, symbiotic microbial biota, and detoxification enzymes, among other morphological, behavioral and physiological changes (Gaston et al. 1991; Hochuli 2001; Johnson and Zalucki 2007; Mittapalli et al. 2007; Bowler and Terblanche 2008; Woods 2013; Hammer et al. 2014; Johnston and Rolff 2015). Hence, regardless of plant phenotype changes over time, even the same herbivore community and/or herbivore abundance may exert variable amounts of damage over time, as bite size, consumption index, efficiency in nutrient extraction, assimilation and digestibility, and/or detoxification changes over insect development (Scriber and Slansky 1981; Hochuli 2001; Maino and Kearney 2015).

Adding further complexity to this already intricate web of interactions between plant and herbivore ontogeny is the herbivores' natural enemies. On the one hand, temporal variation in the strength of top-down control strongly depends on ontogenetic changes in plant traits attracting and supporting predators and parasitoids, such as volatile organic compounds, shelter and food rewards (i.e. indirect defenses, reviewed in Quintero et al. 2013) and/or simply due to plant size and architectural complexity (e.g. Van Bael et al. 2003; Langellotto and Denno 2004; Boege 2005). On the other hand, top-down control can also change throughout herbivore ontogeny due to size-dependent parasitism (Murphy et al. 2014) and predation (Remmel et al. 2011) or due to size-dependent behavioral changes in herbivore diet and feeding times in the presence of natural enemies (Thaler and Griffin 2008; Barton 2010; Thaler et al. 2012). Finally, palatability or suitability of herbivores as food for natural enemies may change as a function of both plant age (Quintero et al. 2014) and herbivore age, especially in the case of specialist herbivores consuming and sequestering plant allelochemicals (e.g. Bowers and Collinge 1992; Jamieson and Bowers 2010).

Here, we assess the extent to which ontogenetic variation in plant defenses and nutritional quality, as well as herbivore development, affect caterpillar feeding choice, performance, and sequestration rate of plant allelochemicals as a proxy of larval vulnerability to natural enemies. We predict a significant plant by herbivore ontogeny interaction, with younger stages (i.e. instars) being more susceptible to ontogenetic trajectories in plant traits than older herbivore stages. This prediction is based on the fact that early instars have higher metabolic rates, lower detoxification enzyme activity, and differential nutritional needs than older ones (Stockoff 1993; Zalucki et al. 2002) and are often more vulnerable to predation risk (Remmel et al. 2011). Hence, small changes in plant quality might have stronger consequences at early than later stages. Experiments were conducted across four distinct ontogenetic stages of the model system, *Plantago lanceolata* L. (Plantaginaceae), and three to five instars of its specialist herbivore, 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). Our recent work has shown that the transition towards less nutritious but better chemically and physically defended plants from seedling to reproductive stages of *P. lanceolata* alter *J. coenia* butterfly host selection as well as 5th instar feeding efficiency and sequestration of plant allelochemicals (Quintero et al. 2014). Yet, how ontogenetic trajectories in herbivore body size, consumption rate, host plant digestibility, and vulnerability to natural enemies may interact with ontogenetic trajectories in plant traits shaping herbivore behavior and physiology over time has been much less studied.

Materials and methods

Study system

Plantago lanceolata is a common short-lived weed (annual or facultative perennial) introduced to North America from Eurasia ca. 200 years ago (Cavers et al. 1980). It produces iridoid glycosides (hereafter IGs) as its primary allelochemicals influencing both 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 (reviewed in Dobler et al. 2011). The two major IGs produced by *P. lanceolata* are aucubin and catalpol, which vary from less than 1% up to 10–12% dry weight, depending on plant ontogeny, genotype, and nutrient availability, among other factors (Bowers and Stamp 1993; Prudic et al. 2005; Barton 2007; Quintero and Bowers 2011). Besides IGs (Ronsted et al. 2000), *P. lanceolata* contains a number of bioactive phenolic compounds

such as flavonoids and phenylethanoid glycosides such as verbascoside and plantamajoside (Chiang et al. 2003; Sutter and Müller 2011; Beara et al. 2012; Pankoke et al. 2013), which may also vary across ontogeny as they have shown significant variation with leaf age (Pankoke et al. 2013). Furthermore, *P. lanceolata* also invests in physical defenses such as leaf toughness (Schippers and Olff 2000) and trichomes (de la Fuente 2002).

Junonia coenia (Nymphalidae), the common buckeye butterfly, is a New World butterfly that can have one to three broods per year in temperate regions (Brock and Kaufman 2003). *Junonia coenia* is a specialist on plants containing IGs, being commonly associated with *P. lanceolata* in the USA (Graves and Shapiro 2003). 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). Furthermore, buckeye caterpillars not only use IGs as feeding stimulants, but are also able to sequester aucubin and catalpol in their hemolymph (Bowers and Collinge 1992). Levels of IGs in buckeye caterpillars, which are positively correlated with levels of IGs in their diet, vary normally from less than 5% to over 20% dry weight (Camara 1997). Caterpillars storing higher levels of IGs in their tissues benefit from decreased mortality from generalist invertebrate predators, including wasps (Stamp 2001), 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. 2009; Richards et al. 2012; Quintero et al. 2014). 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), various developmental stages of herbivores are likely to be exposed to a wide diversity of host age classes. 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 which was frequently supplied with individuals collected in the wild to maintain genetic diversity. Our laboratory colony was started from field collected individuals a year before the experiment started, with no evidence of domestication to lab conditions.

Experimental design

Plants used in these experiments were grown at the University of Colorado greenhouse during spring–summer 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® (i.e. calcined clay) in 4.5 L pots. As in Quintero et al. (2014), four distinct plant developmental stages were used: J1 which represents juvenile plants soon after they ended their seedling stage (i.e. containing from 5 to 15 young leaves, and averaging 0.40 g dry mass), J2 juvenile plants that have reached a complete rosette with young, middle-aged and old leaves but have not yet developed any reproductive structures (~ 1.60 to 4.30 g dry mass), FL flowering mature rosette plants that had few to many scapes with buds or open flowers (~ 6 to 11.5 g dry mass), and FR fruiting mature rosette plants with many scapes ranging from fruit development to seed release (~ 14.20 g dry mass). 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) mixed in a ratio of 15–5–15 N–P–K with trace micronutrients was supplied to all plants every 3 to 4 days throughout the duration of the experiment.

All caterpillar stages used were exposed simultaneously to all host plant developmental stages, with the plant stages synchronized by germinating seeds at intervals of 30 days from March to June. In this way, although average environmental conditions (e.g., daylength and temperature) from sowing to harvest varied among age classes even in the greenhouse, we ensured that all individual plants were exposed to the same environmental conditions prior to exposure to herbivores or harvest (Quintero and Bowers 2012, Quintero et al. 2014). All larval experiments were conducted under controlled growth chamber conditions with a photoperiod of 14 h day: 10 h night, and day–night temperatures of 27/22 °C. Individual plants reared in the greenhouse were harvested every 2 to 3 days to supply caterpillars with a constant source of fresh, previously undamaged leaves. From this harvested material, subsets of mixed young and middle-aged leaves were saved (N = 20–35 sets per age class) to measure leaf IGs, nitrogen and water content, and leaf toughness as described below.

Leaf traits across plant ontogeny

Combined young and middle-aged leaves from each plant stage were weighed fresh, oven-dried at 50 °C for 48 h, 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 weight –dry weight)/wet weight] × 100, while nitrogen content was quantified by Micro-Dumas combustion on a NA1500 C/H/N

analyzer, using approx. 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, leaf toughness was measured as specific leaf area, SLA, 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 ten leaves per age class were used. SLA has been shown to inversely correlate with fiber, such that lower SLA indicates higher concentrations of fiber and thus, higher leaf toughness (e.g. Choong 1996).

Developmental variation in combined young and middle-aged leaf biomass, toughness, and water and nitrogen content were analyzed using one-way analyses of variance (ANOVAs), 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 percent water, nitrogen, and IG concentrations were arcsine square-root transformed to improve normality and homogeneity of variance. Leaf trait data across plant ontogeny were reported previously (Quintero et al. 2014), but are given here for use in interpretation of the insect data.

Caterpillar feeding choice assays

To evaluate buckeye caterpillar feeding preferences as a function of host plant developmental stage and larval development, three sets of pairwise choice tests were performed from June 30th to July 8th 2010, using the following pairs of host plant stages: J1–J2, J1–FL, and J2–FL, with 20 replicates per test. Host plant preference in these three sets of pairwise choice tests was assessed using newly hatched 1st, newly molted 3rd, and newly molted 5th instar caterpillars. The experimental arena consisted of a Petri dish (2 cm × 7 cm) with a piece of moist filter paper on the bottom and a leaf disk (diameter = 1.5 cm) of each of the two host plant stages that had been punched from fresh leaves (avoiding major leaf veins where possible), and separated 1.5 cm apart from each other. Larvae were positioned midway between the two leaf disks at the beginning of every feeding trial. Since the leaf area offered was always constant but larval instars are known to differ widely in their consumption rate, we varied the timing of the test and number of larvae used per replicate. In the case of 1st instar caterpillars, we used groups of six individuals and the larvae were allowed to feed for 24 h, whereas for 3rd and 5th instar caterpillars we used a single individual and the larvae were allowed to feed for 24 or 1 h, respectively. This variation among instars allowed us to provide sufficient time for less mobile younger life stages to make choices, while insuring that no leaf disk was exhausted before the end of the trial. At the end of each trial, the larvae were removed and the leaf disks were scanned. Leaf consumption in all experiments was quantified by measuring the leaf area consumed (total area − area remaining after the trial, mm^2) using a digital scanner and Adobe Photoshop 5. Preferences were defined as consumption of one type of leaf disk (the youngest plant stage of the two offered) divided by the area removed in both disks (Blüthgen and Metzner 2007). A one-sample *t* test compared the distribution of the preferences to the expected value for equal feeding on both types (0.5). Preferences were arcsine square-root transformed, to improve normality and homogeneity of variance.

Caterpillar performance and feeding efficiency

In a separate experiment, data from newly molted 1st, 2nd, 3rd, 4th and 5th instar caterpillars, previously reared on leaves of J1, J2, FL and FR plants from hatching, were used to calculate relative growth rate (RGR). In addition, another set of 3rd, 4th and 5th instar caterpillars were used to evaluate four nutritional indices, according to standard gravimetric methods (Waldbauer 1968): consumption index (CI), approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD). Nutritional indices data for 5th instar larvae were presented in Quintero et al. (2014). RGR, defined as 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 newly molted to the day before they molted to the next instar or pupated. Larvae in groups of ten individuals per Petri dish were monitored every day, counted, weighed as a group to the nearest 0.01 mg, and a constant supply of fresh young and middle-aged leaves was provided. Twelve replicates per age class ($N = 48$) were performed for each of these non-choice tests. Mean individual weight per petri dish (i.e. unit of replication) per instar was used to account for changes in the number of surviving caterpillars among treatments over time. In the case of the nutritional indices, all measurements were gathered over a 24 h interval starting with 20 newly molted larvae per instar and treatment diet ($N = 240$), placed individually in small sealed, plastic containers (160 mm^2), and provided with sufficient leaf material. Five measurements were collected per replicate: initial and final food mass (fresh weight), initial and final larval mass (fresh weight),

and final fecal mass (dry weight). Prior to this 24 h period as well as after it, caterpillars were starved for four to 8 h to ensure an empty gut. A separate subset of larvae and leaves from each treatment combination was dried and weighed at the beginning and the end of the experiment to obtain dry weight conversion factors. Variation in larval RGR was tested by a two-way ANOVA followed by Bonferroni post hoc tests. To avoid problems with the statistical analysis of ratios, all nutritional indices were analyzed using two-way ANCOVAs (Raubenheimer and Simpson 1992). Host plant age and larval instar were included as fixed factors. The numerator of the formula used to calculate each nutritional index was the dependent variable, while the denominator was used as a covariate; all square-root transformed. In the case of CI, initial larval mass was used as the covariate.

Caterpillar IG sequestration

Following the nutritional indices experiment, all 3rd, 4th and 5th instar larvae per treatment diet, starved for 4 to 8 h to ensure an empty gut, were freeze killed and later processed for extraction of sequestered IGs. Sample size per instar and diet treatment varied between 15 and 20 individuals. Data for 5th instar larvae were presented in Quintero et al. (2014). To measure sequestration of IGs, whole caterpillars were ground with sand in 5 ml MEOH, prepared for IG quantification by gas chromatography, and IGs quantified as described in Richards et al. (2012). Variation in the concentration of IGs sequestered, arcsine square root transformed, was assessed using a two-way MANOVA with percent dry weight aucubin and catalpol as dependent variables, and host plant age and larval instar as fixed factors. When a significant effect was detected, univariate ANOVAs for each compound separately were used.

Results

Plant quality and defenses

Combined young and middle-aged leaves used to feed *J. coenia* caterpillars varied in all measured traits as a function of host plant age (Quintero et al. 2014, Table 1). Specifically, while overall nutritional quality measured as water and nitrogen content decreased by ~ 10 and 70%, respectively, from young juvenile to post-reproductive stages, physical (SLA) and chemical defenses (IGs: MANOVA Wilks' $\lambda = 0.54$, $F_{6,280} = 16.72$, $P < 0.0001$) increased ~ 3× to 4.5×, respectively, over the same period of time. It is interesting to note that leaves of J1 and J2 stages did not differ except for SLA values, suggesting that an increase in physical defenses might be the major difference between young and middle-aged leaves in these two juvenile stages.

Caterpillar feeding choice assays

Larval preferences among host plant developmental stages varied as a function of the caterpillar's own developmental stage (i.e. instar) (Fig. 1). Recently hatched 1st instar caterpillars significantly preferred leaves of J1 over J2 plant stages ($t = 3.86$, $P = 0.001$) but showed no preference between leaves of J1-FL or J2-FL ($t = 1.31$, $P = 0.21$ and $t = 0.22$, $P = 0.83$, respectively); however, older 3rd and 5th instar caterpillars showed the opposite trend (Fig. 1). Specifically, 3rd and 5th instar caterpillars showed no preference between leaves of the two youngest host plant stages J1–J2 ($t = 1.01$, $P = 0.32$ and $t = 0.79$, $P = 0.44$; respectively) but strongly preferred leaves of J1 or J2 stages over leaves of the reproductive FL stage (3^{rd} instar J1-FL $t = 7.84$,

Table 1 Ontogenetic variation in leaf traits of combined young and middle-aged leaves of *Plantago lanceolata* used in all *J. coenia* larval experiments (Mean ± 1SE)

Plant traits	J1	J2	FL	FR	*df*	*F*	*P*
Water (%)	88.06 ± 0.41 (a)	88.15 ± 0.26 (a)	84.39 ± 0.52 (b)	80.17 ± 0.50 (c)	3141	79.97	0.0001
Nitrogen (%)	4.26 ± 0.20 (a)	4.21 ± 0.16 (a)	1.95 ± 0.10 (b)	1.25 ± 0.05 (c)	3139	141.19	0.0001
SLA ($cm^2\ mg^{-1}$)	0.47 ± 0.05 (a)	0.24 ± 0.02 (b)	0.21 ± 0.02 (c)	0.17 ± 0.02 (d)	376	417.44	0.0001
Total IGs (%)	0.99 ± 0.16 (a)	0.87 ± 0.09 (a)	2.78 ± 0.46 (b)	3.86 ± 0.47 (c)	3141	32.15	0.0001
Aucubin (%)	0.73 ± 0.13 (a)	0.72 ± 0.08 (a)	1.98 ± 0.31 (b)	2.83 ± 0.36 (c)	3141	29.47	0.0001
Catalpol (%)	0.26 ± 0.05 (a)	0.15 ± 0.04 (a)	0.80 ± 0.16 (b)	1.03 ± 0.14 (b)	3141	28.71	0.0001

Leaf traits measured were: nutritional quality (water and nitrogen content), physical defenses (i.e. SLA, with high values representing low leaf toughness), and chemical defenses (percent dry weight iridoid glycosides: aucubin and catalpol). Original data were square root or arcsine square-root transformed for statistical analyses, actual values are shown for illustrative purposes only. For each variable, overall one-way ANOVA results are presented in the last three columns and letters in parenthesis indicate mean group differences among plant age treatments as tested by Bonferroni post hoc tests ($P < 0.05$). These data are shown in Fig. 2 in Quintero et al. (2014)

The four plant ontogenetic stages used were *J1* young juvenile plants soon after they ended their seedling stage, 5–15 young leaves; *J2* juvenile plants that have reached a complete rosette with young, middle-aged and old leaves but have not yet reproduce; *FL* mature rosettes with buds or open flowers scapes; *FR* mature rosette with many scapes ranging from fruit development to seed release ($N = 20$–35 per age class)

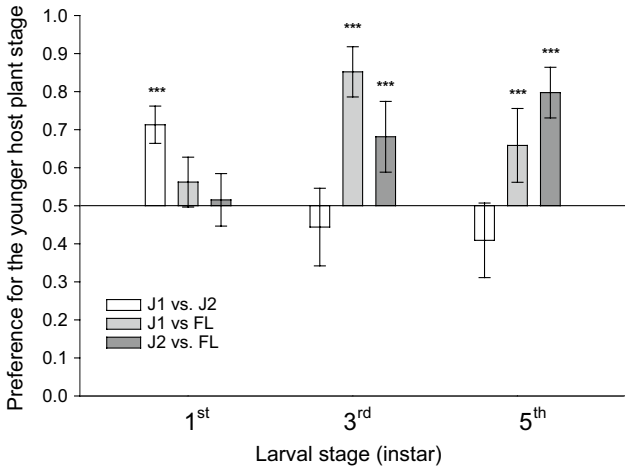

Fig. 1 *Junonia coenia* feeding preferences among *Plantago lanceolata* developmental stages (Mean ± 1 S.E.). Each bar shows the result of a dual choice test. Three choice tests were assessed by 1st, 3rd and 5th instar caterpillars: (1) J1 versus J2, (2) J1 versus FL, and (3) J2 versus FL. Preferences were defined as consumption of one type of leaf disk (the youngest plant stages of the two) divided by the area removed in both disks. Hence, a value of 0.5 indicates equal feeding from both disks. Sample size was always 20 for all comparisons and all larval stages. Significance levels for the one-sample t-test are given (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

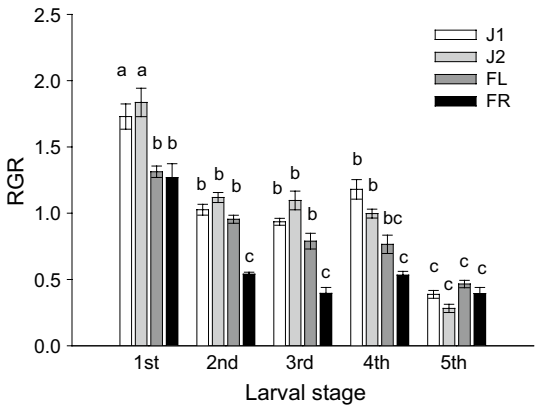

Fig. 2 *Junonia coenia* larval relative growth rate, RGR, (Mean ± 1 SE) for all instars feeding on young and middle-aged leaves of four host plant developmental stages (J1, J2, FL and FR). Sample sizes varied between 8 and 12 for each larval and host plant stage combination (total $N = 219$) and letters indicate mean group differences as tested by Bonferroni post hoc tests ($P < 0.05$), based on previous significance of overall two-way ANOVA tests (Table 2)

$P = 0.0001$; J2-FL $t = 3.39$, $P = 0.003$; 5th instar J1-FL $t = 3.31$, $P = 0.004$; J2-FL $t = 7.11$, $P = 0.0001$) (Fig. 1).

Caterpillar performance and feeding efficiency

Changes in plant quality and defenses across ontogeny strongly impacted caterpillar RGR, showing a general decrease in larval RGR as host plants aged, except for 5th instar larvae (Fig. 2). In addition, RGR also decreased as caterpillars developed, with 1st instars showing the highest RGR followed by 2nd, 3rd and 4th instar larvae with an average RGR two to almost four times higher than 5th instar caterpillars (Fig. 2). Furthermore, there was a significant interaction between plant and caterpillar stage, highlighting that the difference among diet treatments was larger for younger instars than for the last 5th instar (Table 2, Fig. 2). Feeding efficiency data showed that, in general, the ability of a larva to process leaf tissue, varying in nutritional quality and defenses as a function of host plant age, significantly varied throughout larval development (see significant age × instar interactions in Table 2), with 3rd instar larvae being the most susceptible to ontogenetic trajectories in host plant leaf quality (Fig. 3). The consumption index (CI) showed a consistent trend across larval stages, with a significant increase of two to four times more leaf material consumed as host plant age increased (Fig. 3a). Postingestive efficiency, measured as approximate digestibility (AD), did not significantly vary across larval instars (Table 2) and showed little variation across host plant stages (Fig. 3b). In contrast, post-digestive measures of efficiency of food conversion (ECD and ECI) were in general significantly higher for larvae feeding on juvenile plants as compared to those feeding on mature plants (Fig. 3c, d).

Caterpillar IG sequestration

Host plant developmental stage and larval instar had significant effects on caterpillar sequestration ability, showing an overall increase in caterpillar IG concentration as host plants aged and as larvae developed (Fig. 4). In general, caterpillars feeding on leaves of immature plant stages (i.e. J1 and J2) did not differ in their levels of sequestered IGs across instars but caterpillars feeding on leaves of mature reproductive stages acquired 30% to almost 70% more defenses in their tissues than those feeding on leaves of J1 and J2 plants, mirroring the changes seen among their diets (Table 1). In fact, mean plant IG concentration was generally correlated with mean caterpillar sequestered IGs for most larval stages (3rd instar: $R^2 = 0.79$, $P = 0.07$; 4th instar: $R^2 = 0.64$, $P = 0.13$; 5th instar: $R^2 = 0.97$, $P = 0.01$). In addition, MANOVA and ANOVA results both showed not only a significant effect of host plant age and caterpillar instar, but also significant interactions (Table 3). Therefore, variation in sequestered IGs throughout larval development changed as a function of host plant developmental stage. For instance, caterpillars feeding on leaves of older FR stages always sequestered high levels of IGs without substantial variation from 3rd to 5th instars (i.e. total IGs, aucubin + catalpol, from 3rd to 5th instar varied from 11 to 11.6% dry wt., respectively), while caterpillars reared on leaves of younger host plant

Table 2 Summary of two-way ANCOVAs comparing larval nutritional indices and two-way ANOVA for larval RGR as a function of host plant developmental stage (caterpillar diet treatment: combined young and middle-aged leaves of J1, J2, FL and FR), larval instar (3rd to 5th or 1st to 5th instar, respectively) and their interaction

Source of variation	CI			ECI			AD			ECD			RGR		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Covariate	1	70.63	**0.0001**	1	9.99	**0.002**	1	280.12	**0.0001**	1	6.64	**0.011**			
Plant age	3	63.83	**0.0001**	3	1.30	**0.033**	3	2.99	**0.032**	3	2.33	0.075	3	17.99	**0.0001**
Instar	2	14.03	0.066	2	24.86	**0.0001**	2	0.99	0.373	2	129.88	**0.0001**	4	56.82	**0.0001**
Age × instar	6	16.14	**0.0001**	6	8.12	**0.0001**	6	4.9	**0.0001**	6	10.92	**0.0001**	12	2.07	**0.021**
Error	209			209			209			209			218		

See text for description of covariate in the case of nutritional indices and heading abbreviations. Significant effects are indicated in bold

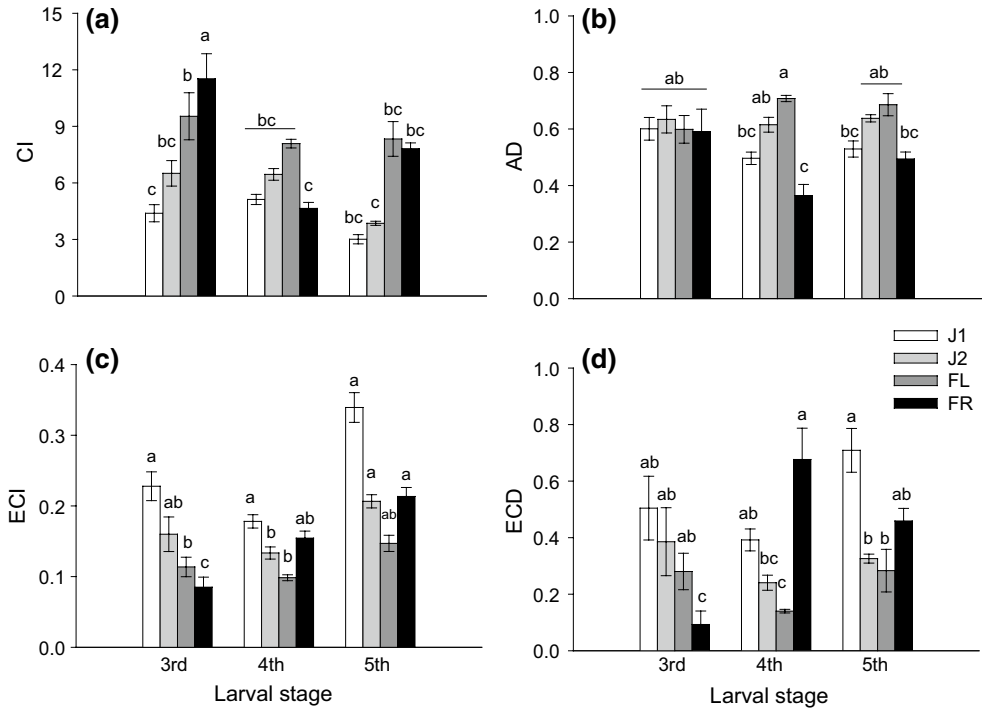

Fig. 3 *Junonia coenia* nutritional indices (Mean ± 1 SE) for 3rd, 4th and 5th instar feeding on young and middle-aged leaves of four host plant developmental stages (J1, J2, FL and FR): **a** Consumption Index (CI), **b** approximate digestibility (AD), **c** efficiency of conversion of ingested food (ECI), and **d** efficiency of conversion of digested food (ECD). Sample sizes varied between 11 and 27 for each larval and host plant stage combination (total $N = 222$) and letters indicate mean group differences as tested by Bonferroni post hoc tests ($P < 0.05$), based on previous significance of overall two-way ANCOVA tests (Table 2)

developmental stages, J1 to FL, more than doubled their levels of sequestered IGs in the same period (Fig. 4).

Discussion

Ontogeny is a ubiquitous process certainly shaping plants' and herbivores' traits and, as a result, temporal variation in population dynamics (Campos et al. 2016) and community structure of higher trophic levels. Nonetheless, our knowledge of how plant and herbivore ontogeny interact remains scarce. As expected, here we show that the magnitude in which plant ontogenetic trajectories altered the behavior and physiology of a specialist herbivore changed throughout immature insect development. In particular, the consequences of feeding on different host plant ontogenetic stages were more pronounced in younger compared to older immature herbivore stages. Given that most assessments of plant ontogenetic trajectories on herbivore performance under natural and lab conditions often use older instars (Hochuli 2001) for practical reasons (but see e.g. Clarke and Zalucki 2000; Zalucki et al. 2002; Johnson and Zalucki 2005, 2007;

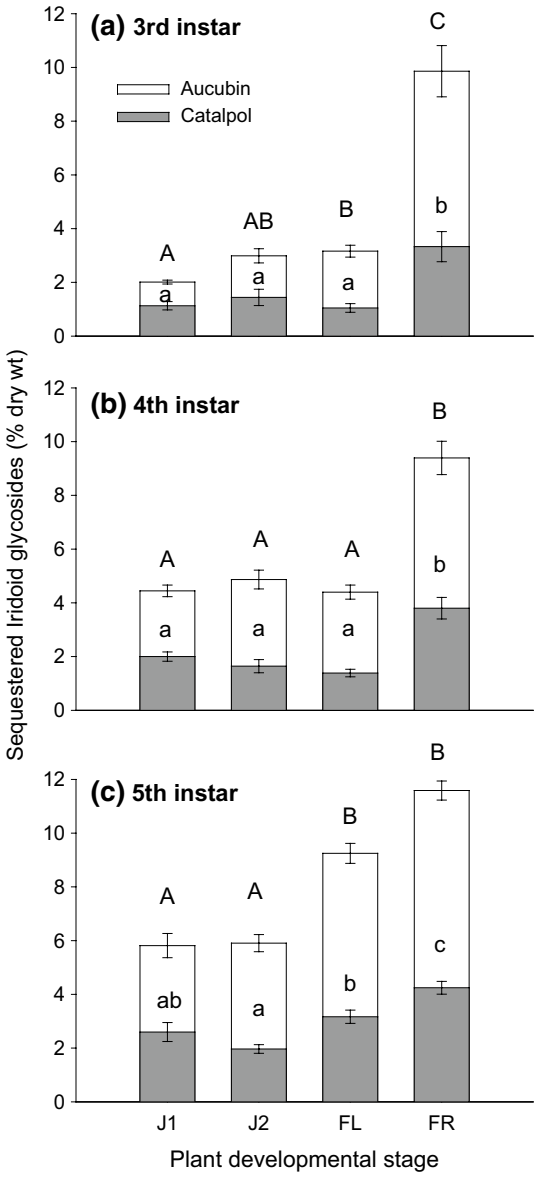

Fig. 4 *Junonia coenia* IG sequestration (Mean ± 1 SE) for 3rd, 4th and 5th instar caterpillars, feeding from neonate on young and middle-aged leaves of four host plant developmental stages (J1, J2, FL and FR). Percent dry weight aucubin and catalpol were assessed when caterpillars just molted to the **a** 3rd instar, **b** 4th instar and **c** 5th instar. Original data were arcsine square-root transformed for statistical analyses; actual values are shown for illustrative purposes only. Sample sizes varied between 11 and 27 for each larval and host plant stage combination (total $N = 222$) and letters indicate mean group differences as tested by Bonferroni post hoc tests ($P < 0.05$), based on previous significance of overall two-way ANOVA tests (Table 3). Capital letters were used to represent mean group differences for aucubin and lower-case letters were used to represent group mean differences for catalpol concentrations

Table 3 Summary of two-way MANOVA and ANOVAs comparing larval sequestration rate as a function of host plant developmental stage (caterpillar diet treatment: combined young and middle-aged leaves of J1, J2, FL and FR), larval instar (3rd, 4th and 5th instar) and their interaction

	Source of variation	λ	*df*	*F*	*P*
Aucubin and catalpol	Plant age	0.44	6418	34.7	0.0001
	Instar	0.74	4418	17.3	0.0001
	Age × instar	0.85	12418	2.9	0.001
Aucubin	Plant age		3210	60.86	0.0001
	Instar		2210	35.95	0.0001
	Age × instar		6,210	3.56	0.002
Catalpol	Plant age		3210	28.1	0.0001
	Instar		2210	15.37	0.0001
	Age × instar		6210	2.0	0.066

Bukovinszky et al. 2009; Santana and Zucoloto 2011; Saastamoinen et al. 2013), it is critical to realize that we might be underestimating the actual role of plant phenological and ontogenetic trajectories on herbivore community dynamics in the field.

The amount and quality of food consumed in the course of an herbivore's ontogeny have wide-ranging effects on numerous life-history characteristics such as survival, growth rate, developmental time, adult size, and fitness (Llandres et al. 2015). Hence, selection of suitable host plant species and developmental stages should be critical. For holometabolous herbivorous insects, where adult females select the feeding site of often non-mobile immature stages, adults' oviposition and early instars' feeding preferences should correlate. In our system, Buckeye butterflies showed a stronger oviposition preference for younger *P. lanceolata* stages, laying on average 60% more eggs on juvenile (J1 or J2 plants) than on reproductive plants (FL or FR plants) (Quintero et al. 2014). Yet, newly hatched 1st instar larvae showed little preference between leaves of juvenile and reproductive stages, except for a significant preference towards leaves of early (J1 over J2) juvenile plants, while more mature 3rd and 5th instar larvae significantly preferred leaves of juvenile over leaves of reproductive host plant stages with no distinction between leaves of the two juvenile host plant stages (see Fig. 1). This discrepancy in feeding choice among immature larvae can be related to caterpillars' mobility, previous experience, mandible structure, and nutritional needs.

Early instar lepidopteran larvae, with restricted mobility and no previous experience with their host plant, might be less selective. Indeed, they may initiate feeding without searching for other choices within the arena (i.e. more similar to a non-choice experiment). Thus, in our case, first instars' selection might have been driven by the number of caterpillars that ended up at each leaf disk, as selection did not correlate with larval RGR (Fig. 2). In fact, on average, differences in the number of 1st instar caterpillars on each leaf disk at the end of the trial were seen only in J1–J2 trials (data not shown), potentially explaining the observed

differences in consumption. In contrast, newly molted 3rd and 5th instars, highly mobile and previously reared on a mix of *P. lanceolata* leaves, not only inspected and consumed from both disks, but also their previous experience in tissue quality variation might have increased their selectivity, as seen in other systems (e.g. Stockoff 1993; Santana and Zucoloto 2011).

Caterpillars undergo considerable morphological changes in mandible structure as they develop, going from smaller heads with toothed cutting edge mandibles to larger heads containing more muscles and smooth edged mandibles with a retinaculum on the oral surface (Bernays 1991; Hochuli 2001). Based on these morphological changes, it is expected that younger caterpillars with their toothed mandibles may have an advantage, being able to efficiently handle tough leaves or selectively consume portions of the leaf that are low in fiber and easily digestible (e.g. skeletonized leaf damage). In contrast, older instars with their tearing, crushing action mandibles may more efficiently manage softer, more flaccid leaves (but see Clarke and Zalucki 2000), being able to cut through the full leaf thickness and break the leaf material into smaller pieces, enhancing nutrient extraction (Hochuli 1996, 2001). Indeed, 1st instar *J. coenia* larvae scraped the surface of the leaf disks randomly, without consuming them from the cut edge as was more commonly seen for older instars. This change in feeding strategy as larvae develop may help to explain the differences seen in both selection and consumption. Interestingly, these results also highlight the fact that divergence in adult and larval host selection may vary over herbivores' ontogeny, potentially explaining the numerous reported mismatches between female oviposition choice and larval preference and/or performance (Gripenberg et al. 2010).

Nutritional needs may significantly vary as larvae age (Stockoff 1993; Pinault et al. 2009) shaping not only host selection but also postingestive regulation to achieve optimal growth rate (Behmer 2009). In this study, larval relative growth rate (RGR) decreased as larvae aged and the age of host plant increased. This trend is not surprising since early instars often have higher metabolic activities and growth rates (Zalucki et al. 2002) and younger host plant stages often provide higher nutritional quality with lower levels of defenses to consumers (Boege and Marquis 2005; Hanley et al. 2007; Barton and Koricheva 2010). But interestingly, the effect of host plant stage on larval performance was stronger for younger than older instars (Figs. 2, 3). This can be related to the 70% decrease in nitrogen content seen between J1 and FR *P. lanceolata* stages (Table 1). Given the higher RGR and metabolic activity of early instar larvae, nitrogen availability can be more crucial at early developmental stages (Zalucki et al. 2002), whereas older instars can better compensate for nitrogen and protein deficits by prolonged growth (Raubenheimer and Simpson 1999). In addition, the decrease in nitrogen content with the concomitant increase of three times more physical and 4.5 times more chemical defenses as host plant ages (Table 1) may well explain the increased consumption index and decreased digestive efficiency observed at all instars when host plant age increases. Increased consumption rate (CI) may compensate for potential nutritional deficits, but probably more so when physical defenses are low or absent (Travers-Martin and Müller 2008). As physical defenses increase when *P. lanceolata* ages, leaf toughness may limit the size of meals being eaten, slow gut passage rates, and reduce nutrient supply (Hochuli 1996; Clissold et al. 2009). Since 3rd instar caterpillars have already lost their toothed cutting edge mandibles but have yet smaller head sizes and muscles than the 5th instar (Hochuli 2001), increased fiber may have more of an impact on digestive efficiency at earlier compared to later instars.

As a consequence of increased consumption and prolonged feeding by larvae growing on older host plant stages, carbohydrates and secondary defenses may become overconsumed, diverting energy towards sequestration instead of growth and towards minimizing the potential negative effects of high IG content even for this specialist herbivore (e.g. Adler et al. 1995; Camara 1997). For IGs, the negative effects on specialist herbivores may arise when the compounds are activated by plant hydrolytic enzymes, β-glucosidases (Dobler et al. 2011; Pankoke et al. 2013). The resulting iridoid aglycones can denature amino acids, proteins, and nucleic acids, or act as enzyme inhibitors, rendering proteins undigestible (Dobler et al. 2011), all of which may affect earlier larval stages more than later ones due to higher metabolic rate and lower detoxification enzyme activity (Zalucki et al. 2002). In addition to IGs, *P. lanceolata* contains other allelochemicals, which are likely also affected by plant ontogeny (Pankoke et al. 2013) and may contribute to the reduced larval performance and plant digestibility observed with increasing host plant age; but their relative contribution to *J. coenia* performance need further investigation. Lastly, increasing the concentration of sequestered compounds for this specialist herbivore has direct implications in their relationship with higher trophic levels.

Vulnerability to natural enemies can strongly vary throughout insect development as some predators and parasitoids might be limited by prey size (Remmel et al. 2011; Murphy et al. 2014), prey toxicity/quality (Dyer and Floyd 1993) and/or prey immunocompetence as insects develop (e.g. Rantala and Roff 2005; Bukovinszky et al. 2009). In this study, while body size obviously increased throughout larval development, body size and mean individual weight negatively correlated with host plant age (Figure S1). On the other hand, the ability to sequester IGs increased with larval and host plant age, but the major difference in IG sequestration due to diet (i.e. host plant age) occurred early rather than

later during larval development (4.5 × vs. 2 × maximum difference within instars, see Fig. 3). Yet, changes in mortality rates as caterpillars age based on host plant ontogeny could be hard to predict. This unpredictability relies on previous evidence that higher sequestered IG content is associated with lower risk of predation by several invertebrate predators (e.g. de la Fuente et al. 1995; Strohmeyer et al. 1998; Theodoratus and Bowers 1999; Reudler et al. 2015); but higher susceptibility to parasitoid attack (Smilanich et al. 2009; Richards et al. 2012; Quintero et al. 2014; Lampert and Bowers 2015; but see Laurentz et al. 2012). Nonetheless, although we have not tested here larval predation risk or immunocompetence throughout *J. coenia* development, we predict that early instars should be more susceptible to predation/parasitism than older instars. This prediction is based on the fact that susceptibility to invertebrate predators, especially parasitoids (Hawkins et al. 1997), is often stronger at earlier life stages of larval development (Costa 1993; Remmel et al. 2011) and immunocompetence in other lepidopteran species has shown to increase as larvae develop (Bukovinszky et al. 2009; Saastamoinen et al. 2013). Nonetheless, these predictions should be further tested, ideally under natural field conditions.

Our results highlight that early herbivore instars are very susceptible to host plant ontogenetic changes. It has been commonly recognized that environmental, and especially dietary, conditions experienced early in life during insect development have strong consequences in adult phenotype and fitness (Taborsky 2006; Barrett et al. 2009; Dmitriew and Rowe 2011), sometimes even having a carryover effect into the next generation (Saastamoinen et al. 2013). In the last decade, it has been broadly recognized that plant ontogeny strongly shape the outcome of plant–insect interactions (Boege and Marquis 2005; Barton and Koricheva 2010; Quintero et al. 2013; Ochoa-Lopez et al. 2015). Now, it is time to acknowledge that insect ontogeny might have the same (or greater) strength in driving host selection, plant-induced defenses, and plant damage (Hochuli 2001); and thus, the potential synergy of plant- and herbivore-ontogeny could be hard to predict. The consequences of mismatches between plant and herbivore phenology have long being demonstrated, altering herbivore survival, development, growth, fitness, habitat range, and population dynamics (Jones and Despland 2006; Schwartzberg et al. 2014). Hence, the expected increase in the rate of asynchronies between plant and herbivore phenology/ontogeny due to climate change (Yang and Rudolf 2010) highlights the need to assess the consequences of unpredictable temporal interactions across trophic levels, for both natural and agricultural systems.

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Author contribution statement CQ and MDB conceived and designed the experiments. CQ performed the experiments, analyzed the data, and wrote the manuscript; MDB provided editorial advice.

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