Journal of Chemical Ecology (2022) 48:79–88
https://doi.org/10.1007/s10886-021-01321-7

Seasonal Variation in Host Plant Chemistry Drives Sequestration in a Specialist Caterpillar

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Received: 6 August 2021 / Revised: 30 September 2021 / Accepted: 10 October 2021 / Published online: 5 November 2021

Abstract

Sequestration of plant secondary metabolites by herbivores can vary across both host plant phenology and herbivore ontogeny, but few studies have explored how they concurrently change in the field. We explored variation in iridoid glycoside concentration and composition in white turtlehead, *Chelone glabra*, as well as sequestration of iridoid glycosides by its specialist herbivore, the Baltimore checkerspot, *Euphydryas phaeton*, across the development of both herbivore and host plant. In 2012 we sampled plants to describe seasonal variation in the concentrations of two iridoid glycosides, aucubin and catalpol. In 2017, we sampled both host plants and caterpillars over an entire growing season and explored the relationship between plant chemistry and herbivore sequestration. We also compared iridoid glycoside concentrations of plants with and without herbivory to gain insight into whether levels of secondary compounds were impacted by herbivory. We found that total plant iridoid glycosides varied across the season and that total sequestered iridoid glycosides in caterpillars closely mirrored concentration patterns in plants. However, the magnitude of sequestration by caterpillars ranged from 2 to 20 times the concentrations in host plants, with different proportions of aucubin and catalpol. In addition, plants with herbivory had lower iridoid glycoside concentrations than plants without herbivory, although this difference changed over time. These results suggest that while variation in host plant secondary metabolites may be a dominant factor driving sequestration, other ecological factors may mitigate the relationship between host plant chemistry and herbivore sequestration.

Keywords *Chelone glabra* · *Euphydryas phaeton* · Sequestration · Iridoid · Phenology

Introduction

Many insect herbivores are specialists to some degree, exhibiting relatively narrow diet breadths on host plants that express characteristic suites of plant secondary metabolites, hereafter PSMs (Bernays and Chapman 1994; Bernays 2001). Although PSMs are used as defenses against generalist or non-adapted herbivores, they may serve as important cues for oviposition and as feeding stimulants for specialist herbivores that have adapted to tolerate them (e.g., Bowers 1984; Bowers and Puttick 1986; Bowers et al. 1992a; Cheng et al. 2013; Pereyra and Bowers 1988). In addition, some specialist species (and a few generalists) have evolved the ability to sequester PSMs for use in their defense against natural enemies (Opitz and Müller 2009). Given their importance to sequestering herbivores, intraspecific variation in PSMs is likely a dominant factor driving specialist herbivore ecology and evolution and has been highlighted as an area in need of further research (Moore et al. 2014).

Most research has focused on the role of variation in PSMs among different host plant species on sequestering specialist herbivores; however, variation within host plant species is less studied. A classic example of a sequestering specialist is the monarch, *Danaus plexippus* (Nymphalidae), whose larvae sequester cardenolides from different milkweed species, *Asclepias* spp. (Apocynaceae) in defense against predators (Brower et al. 1984). The degree of sequestration by monarchs is driven, in part, by interspecific variation in cardenolide composition and concentrations (Agrawal et al. 2012; Jones et al. 2019). However, studies of the role of intraspecific cardenolide concentrations on sequestration are lacking, despite strong variation across

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plant tissues, genotypes, and populations, and in response to environmental conditions (Agrawal et al. 2012; Vannette and Hunter 2011). The role of seasonality on cardenolide concentrations, which could be driven by phenological, ontogenetic, or environmental change through time, is also little studied. Studies in other plant species have shown that PSMs do vary across the growing season (e.g., Riipi et al. 2002) and between different populations of the same host plant (Jamieson and Bowers 2010), but also substantially within individual host plants through time (Blanchard and Bowers 2020; Palo 1984; Quintero and Bowers 2018). While seasonal changes in environmental conditions can impact variation in PSMs, which in turn has been shown to affect herbivore preference/performance (Huang et al. 2020; Verçosa et al. 2019), how seasonal variation impacts both PSM production and herbivore sequestration remains little studied.

From the perspective of a sequestering insect, variation in PSMs may result in dose-dependent levels of defensive chemicals sequestered, or there may be a threshold above which additional sequestration is not possible, or a combination. Dose-dependent sequestration is well known in monarchs, given that the amounts of cardenolides they sequester increase with increasing hostplant concentrations, although there is a limit to their sequestration, resulting in a saturation effect at higher concentrations (Brower et al. 1984; Jones et al. 2019). Dose-dependent sequestration has also been shown across different host plant species in other sequestering herbivores (e.g., Martins et al. 2015). However, disentangling the impacts of PSM concentrations from other interspecific host plant traits (i.e., nutrients, physical defenses, C:N ratio, etc.) makes comparing the relationship between interspecific variation in PSMs and sequestration difficult, highlighting the importance of studying intraspecific PSM variation. The ability to sequester PSMs can also change across herbivore development (Carper et al. 2019; Jamieson and Bowers 2010); thus, sequestration may be the result of interactions between intraspecific variation in host plant chemistry and sequestration ability (Quintero and Bowers 2018). Still, few studies have assessed sequestration and host plant chemistry across the ontogeny of both herbivore and host in the field.

From the perspective of a plant under attack by a sequestering insect, variation in PSMs could be the result of plant resistance or tolerance to herbivory (reviewed in Kant et al. 2015). For example, theory predicts that sequestering herbivores should perform best at intermediate levels of host PSMs (Ali and Agrawal 2012), and in many plant species, the chain of events triggered by an herbivore's feeding can increase, or induce, PSM production (Howe and Jander 2008; Mithoefer and Boland 2012). Moreover, induction can be systemic (Bakhtiari and Rasmann 2020), suggesting that herbivory can change the allocation of resources to growth or defense after attack. Plants do have mechanisms to tolerate herbivory, such as reallocating resources away from damaged tissues when under herbivore attack (Schultz et al. 2013). While many studies of induction have been done in the field, just how widespread induced defenses or induced susceptibility are in wild growing plants is not well known. Still, given that sequestration can change across herbivore ontogeny and with host plant phenology, studies exploring variation in PSMs and their sequestration in the field are needed to elucidate potential mechanisms driving changes in specialist herbivore-plant interactions through time.

Here, we explore variation in both host plant chemistry and sequestration by a specialist herbivore across the development of the herbivore and its host plant, using plants and caterpillars collected in the field. We focused on a native specialist butterfly, the Baltimore checkerspot (*E. phaeton* Drury, Nymphalidae), and its primary native host plant, *C. glabra* L. (Plantaginaceae). First, we sampled host plants and quantified PSMs over an entire growing season; subsequently, we sampled both host plants and herbivores over a second season, and quantified both PSMs and their sequestration by herbivores. We then explored the relationship between variation in host plant chemistry and herbivore sequestration across the growing season. In addition to sampling plants with herbivory, we also sampled plants without herbivory, to gain insight into whether herbivory changes PSMs and the possible implications for sequestering herbivores. We expected that PSMs would vary over the season and with herbivory. We also hypothesized that if herbivore choice is a dominant factor driving sequestration, concentrations of PSMs in plants with herbivory would be different from plants without, and as such, the amount or composition of sequestered PSMs in herbivores could differ from that within hostplants.

Methods and Materials

Study System

All field research was conducted at a single field site near Montpelier, VT USA (44.283, −72.543), where *E. phaeton* is relatively abundant. The primary host plant of *E. phaeton* at this site is white turtlehead, *C. glabra* (Plantaginaceae), a clonal perennial herb that grows in wetlands across the eastern US (Pennell 1935). Like most members of the Plantaginaceae, *C. glabra* contains iridoid glycosides, with one, catalpol, being the major and sometimes only constituent and a second, aucubin, occurring at much lower levels when it occurs (Bowers et al. 1993). Biosynthetically, aucubin is the precursor of catalpol (Damtoft et al. 1983) and both compounds can be deterrents to generalist herbivores (Bowers and Puttick 1986). Both of these iridoid glycosides are also

sequestered by *E. phaeton* larvae, in amounts as high as 15% dry weight (see Results) and these compounds are retained to the adult stage (Bowers and Puttick 1986; Bowers et al. 1992b), making both larvae and adults unpalatable to predators (Bowers 1980). Plants were sampled across the growing season in 2012 and both plants and caterpillars were sampled across the growing season in 2017.

Like other *Euphydryas* species, Baltimore checkerspots are univoltine. Adults fly in June or July, depending on the location. Egg masses, numbering 100 – 600 eggs (Stamp 1982b), are laid in July to August, and larvae are gregarious, building silken webs in which they develop through the fourth instar (Stamp 1979). Larvae undergo an obligate diapause, over-wintering from September to May as fourth instars. Larvae emerge from diapause in the spring (mid-May at our study site) and feed through June or early July when they pupate. Here we refer to larvae before entering their over-wintering diapause as pre-diapause larvae and those emerged from diapause in May as post-diapause larvae. As a result of this life-history, caterpillars are exposed to *C. glabra* plants at very different phenological stages. Pre-diapause larvae develop in July and August and are feeding on fully developed plants that are often in flower; in contrast, post-diapause larvae are feeding on the newly emerging spring growth of *C. glabra*. It is also in these post-diapause instars that Baltimore checkerspot larvae may feed on other plant species containing iridoid glycosides, such as *Plantago lanceolata* (Plantaginaceae), honeysuckle (*Lonicera spp.*, Caprifoliaceae), *Penstemon digitalis (*Plantaginaceae), and white ash (*Fraxinus americanus,* Oleaceae) (Bowers 1980; Scudder 1889); although at our site, *C. glabra* is the primary host.

Seasonal Variation in Host Plant Chemistry

To explore variation in *C. glabra* chemistry throughout its growing season, we collected undamaged leaf samples from individual ramets across the entire growing season in 2012. We chose undamaged leaves to better understand natural variation across the season, as herbivore damage could impact PSM production (see Secondary Metabolites in Damaged versus Undamaged Host Plants). To sample leaves, we collected two whole undamaged leaves per ramet into coin envelopes from 18 to 20 individual ramets about every 3 weeks throughout the growing season, for a total of 179 leaf samples. Given that Chelone is clonal, we sampled ramets that were at least 10 m apart to help ensure each ramet represented a unique individual. We collected samples seven times across the season: May 23rd, June 12th, June 21st, July 2nd, July 19th, August 3rd, and September 6th. We sampled different ramets at each collection date and all plants exhibited signs of herbivory from *E. phaeton* larvae. We conducted all analyses in R version 3.5.1 (R Core Team 2020). Given that leaf aucubin and catalpol concentrations have been shown to be positively correlated (Richardson et al. 2016), we tested for a correlation between the concentrations of aucubin and catalpol in *C. glabra* leaves in 2012 using Pearson's product moment correlation coefficient. After finding them to be correlated, we used MANOVA to test if the concentrations of aucubin and catalpol changed over the 2012 season. We included aucubin and catalpol concentrations as response variables, and collection date as a fixed effect. A significant effect of date would suggest that iridoid glycoside (IG) concentrations vary over the growing season. Given their correlated nature, we also calculated the proportion of total IGs that was catalpol, by dividing the percent dry weight catalpol by the percent dry weight of total IGs. We used ANOVA to determine if the proportion catalpol changed over the season, and logit-transformed the proportional response. A significant effect of date would suggest that the relative concentrations of aucubin and catalpol change over the season.

Secondary Metabolites in Damaged versus Undamaged Host Plants

To explore variation in IG concentrations between plants with and without herbivory, we sampled intact leaves from 15 ramets that did not show signs of herbivory and from 15 ramets 1-3 m away that did show evidence of herbivory by *E. phaeton* caterpillars. These samples were collected in May and June 2017, at the time that caterpillar samples were also collected (see below). In July and August, we were unable to find ramets that did not have signs of herbivory and thus were unable to make these comparative collections. Given that aucubin and catalpol were correlated in 2012, to explore variation in host plant chemistry in 2017 we analyzed total IG concentrations (combined aucubin and catalpol) and the proportion of IGs that was catalpol (calculated as mg catalpol/mg total IGs). To compare the total IG concentration and proportion of IGs that was catalpol between damaged and undamaged *C. glabra* plants in May and June, we used ANOVAs, with both month and herbivory (present or absent) as main effects. For all analyses, we logit-transformed all percent and proportional responses (Warton and Hui 2011).

Relationship between Host Plant Chemistry and Herbivore Sequestration

To determine how variation in *C. glabra* chemistry impacted sequestration by *E. phaeton* larvae, we collected both plant samples and caterpillars across the growing season in 2017. We simultaneously collected leaves and caterpillars on May 15th, June 18th, July 15th, and August 23rd. For each date, we collected one ramet from each of 15 randomly selected

clones, each separated by >10 m. Given that post-diapause 4th and pre-diapause 2nd-3rd instar larvae are very small (15.67 mg and 1.38 mg on average, respectively), analyzing chemistry on individual caterpillars would make it difficult to detect small quantities of sequestered compounds. Moreover, in these stages larvae tightly aggregate together on host plants (Stamp 1982); thus, variation among individual caterpillars is likely quite low. Therefore, we collected clusters of caterpillars from associated plant ramets in both May (post-diapause 4th instar) and August (pre-diapause 3rd instar). In May, we collected 10 clusters of 5 caterpillars each. In June, we collected clusters of 3-6 caterpillars from each ramet when larvae were much larger (296.36 mg on average for 5th instar caterpillars). In July, larvae were more dispersed and we collected a single large 6th instar caterpillar (370.09 mg on average) per ramet. In August, we collected 15 entire aggregations (between 10 and 51 caterpillars per cluster) to analyze caterpillar chemistry. Given their univoltine life cycle, caterpillars in May – July belong to the same generation (having over-wintered in diapause from the previous September), while those collected in August are from the next generation.

We compared total IG concentrations and the proportion of IGs that was catalpol using ANOVA and Tukey post-hoc tests to test for differences among months in the two IG measures. To determine if variation in *C. glabra* chemistry drove sequestration by *E. phaeton*, we used simple linear regressions with monthly mean *C. glabra* total IGs and proportion catalpol as predictors, and monthly mean *E. phaeton* total IGs and proportion catalpol as responses. We used monthly means since individual caterpillars were not necessarily feeding on the host plant sample that was taken at that time, and thus monthly means represent population scale variation in host plant and caterpillar chemistry.

Iridoid Glycoside Quantification

We quantified iridoid glycoside concentrations using gas chromatography (Knerl and Bowers 2013). All leaf samples were air-dried after initial collection in the field and then frozen at −20 °C until processing. In the lab, each pair of leaves was dried at 50 °C for 48 h and finely ground with a ceramic mortar and pestle. We transferred 25 mg of powdered leaf material from each sample to 15 mL glass test tubes, added 5 mL of MeOH, and allowed samples to extract for 24 h. Caterpillars were shipped overnight from the field in Montpelier, VT to Boulder, CO, and thus starved for 24 h. Upon arrival, their fresh weights were recorded, and then they were immediately frozen in the lab at −20 °C. This transport and processing time prior to IG extraction effectively allowed larvae to clear their gut of plant material so as to not impact the detected amounts of sequestered IGs. We then ground caterpillars (individually in June and July vs. clusters of caterpillars in May and August) with a small amount of sand in 5 mL of MeOH and similarly allowed them to extract for 24 h. For all samples, particulates were filtered, the MeOH evaporated and 1 mL of 0.500 mg/mL phenyl-β-D-glucopyranoside (PBG) was added as an internal standard. To remove lipophilic substances, we added 3 mL of water and 3 mL of ether, vortexed samples, and then centrifuged them for four minutes to separate the water and ether layers, then aspirated and discarded the ether layer, repeating this process two additional times. The remaining water layer, containing the iridoid glycosides, was evaporated. We then added 1 mL of MeOH and allowed samples to dissolve into the solution overnight. We removed a 0.100 mL aliquot from each sample and evaporated the MeOH.

Samples were derivatized by adding 0.100 mL of 1-(trimethylsilyl)imidazole (Sigma-Aldrich) and heating in an oil bath at 75 °C for 20 min (Bowers & Stamp, 1997). We used an Agilent 7890A GC equipped with a DB-1 column (30 m, 0.320 mm, 0.25 μm particle size) and ChemStation B-03-01 software to quantify iridoid glycosides after calibrating with a reference standard of known PBG, aucubin, and catalpol concentrations. Samples were injected at an initial temperature of 200 °C for 1 min, followed by a 3 min increase to 260 °C, held for 8 min, and a 3 min increase to a final temperature of 320 °C for 10 min (30 min total).

We report IG concentrations as percent dry weight because of the variation in water content in plants and caterpillars (Knerl and Bowers 2013). Since caterpillars were processed fresh, we used a dry weight conversion factor that was calculated from separate sets of 5 pre- and post-diapause caterpillars that were weighed fresh, then dried at 50 °C for 48 h and reweighed. The fresh weights of all caterpillars were then multiplied by these conversion factors to estimate dry weight: 0.31 and 0.22 for pre- and post-diapause larvae, respectively.

Results

Seasonal Variation in Host Plant Chemistry

We found that aucubin and catalpol concentrations in *C. glabra* in 2012 were positively correlated ($r = 0.41$, $P < 0.001$). MANOVA showed that both of these compounds varied significantly across the different collection dates (MANOVA: $F_{6,130} = 8.65$, $P < 0.001$), increasing from low concentration in May to much higher concentrations mid-summer (Fig. 1a). Catalpol was by far the major component of the two iridoids, however, the proportion of total IGs that was catalpol varied over the season ($F_{6,131} = 28.73$, $P < 0.001$), declining 16.3% from a high of 0.986 mid-spring in May, to 0.811 in September (Fig. 1b).

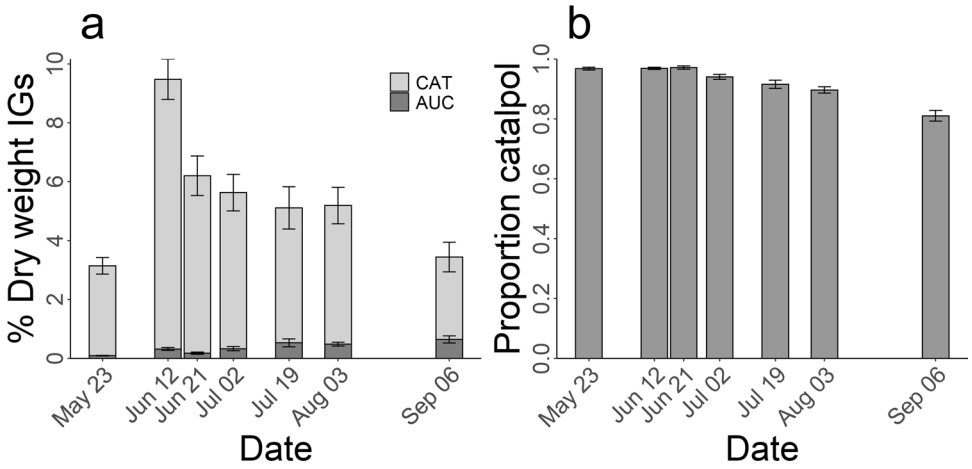

Fig. 1 The total concentration of iridoid glycosides in *Chelone glabra* in 2012 **a** varied across the growing season, with a peak in June. However, the proportion of iridoid glycosides that was catalpol **b** declined over the season and was lowest in September. Bars are mean ± SE for aucubin (AUC) and catalpol (CAT)

Secondary Metabolites in Damaged versus Undamaged Host Plants

In 2017, total IG concentrations in *C. glabra* also varied from May to August ($F_{3,51} = 68.97$, $P < 0.001$), increasing nearly 10 times from May to June, more than doubling from June to July, and finally declining by more than 50% from July to August (Fig. 2a). The proportion of IGs that was catalpol also varied across the season ($F_{3,51} = 20.57$, $P < 0.001$), doubling from May to June, but then remaining high throughout the remainder of the season (Fig. 2b).

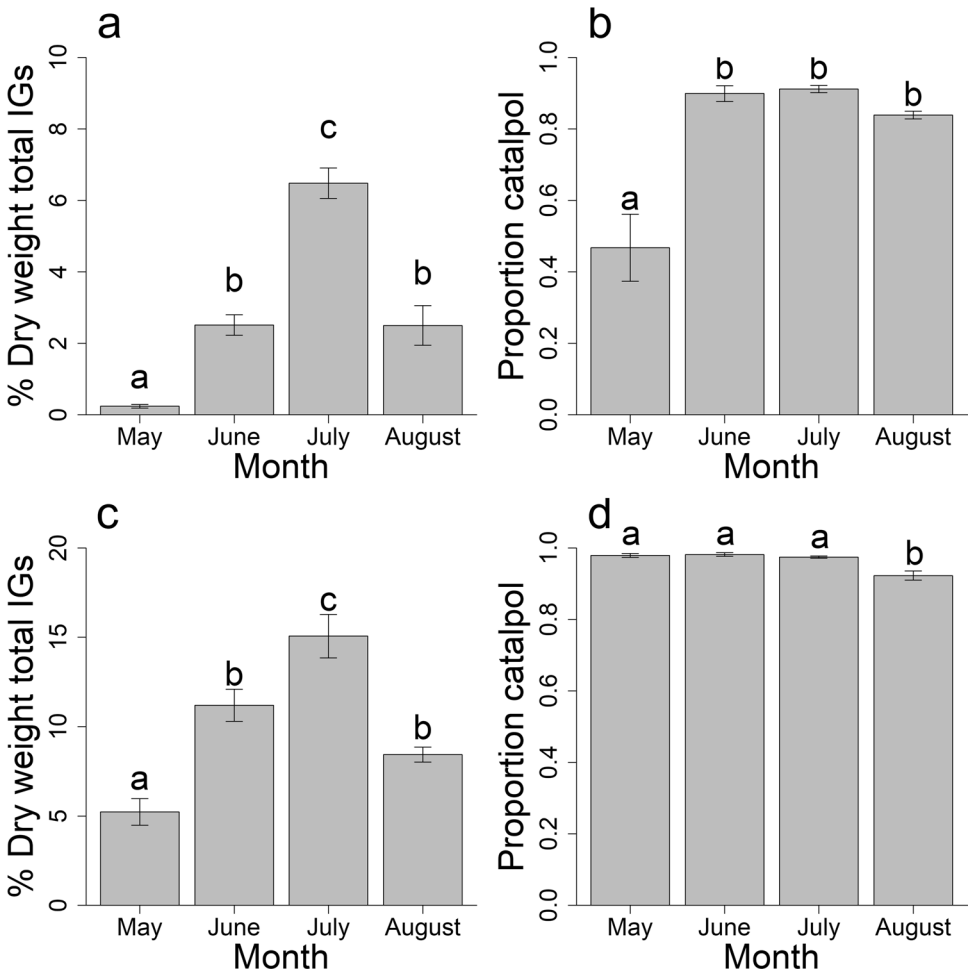

Fig. 2 In 2017, *Chelone glabra* with herbivory had **a** total iridoid glycoside concentrations that varied from May to August, increasing from low levels in May to the highest levels in July. The proportion of IGs that was catalpol **b** also began low in May but remained higher throughout the rest of the season. Caterpillar sequestration of IGs in 2017 mirrored hostplants in terms of **c** the total concentration of IGs, starting low in May and increasing to highest levels in July. However, **d** the proportion of catalpol remained consistently high throughout the season. Bars are mean ± SE; letters denote significantly different months in each panel

We detected significant interactions between month and herbivory on *C. glabra* chemistry during May and June, the months when plants with and without herbivory were collected. As expected, ANOVA revealed significant differences between May and June in total IG concentrations ($F_{1,51} = 39.66$, $P < 0.001$), but also that plants with herbivory had 31% lower IG concentrations on average than plants that had no herbivory ($F_{1,51} = 48.96$, $P < 0.001$). Moreover, there was a significant interaction between month and herbivory ($F_{1,51} = 44.04$, $P < 0.001$), wherein plants with herbivory had 91% lower IG concentrations than plants without herbivory in May, but only 6.5% lower IG concentrations in June (Fig. 3a). The proportion of IGs that was catalpol similarly varied in plants by month ($F_{1,51} = 17.99$, $P < 0.001$). While overall proportion of catalpol was only 5.7% lower in plants with herbivory ($F_{1,51} = 15.31$, $P < 0.001$), we also found a significant interaction between month and herbivory ($F_{1,51} = 11.61$, $P = 0.001$), with plants that had herbivory in May having 45% lower proportions of catalpol than plants without herbivory, compared to just 2.5% lower proportions of catalpol in June (Fig. 3b).

Relationship Between Host Plant Chemistry and Herbivore Sequestration

E. phaeton caterpillars sequestered iridoid glycosides, although the amounts varied substantially among caterpillars of different stages. Larvae sequestered from two (in July) to 20 times (in May) higher total IG concentrations than were found in the host plants on which they were feeding. Total IG concentrations in caterpillars varied across the 2017 season ($F_{3,43} = 25.07$, $P < 0.001$), similarly to host plants, doubling from May to June, increasing 35% from June to July, and then decreasing 44% from July to August (Fig. 2c). However, while the proportion of IGs sequestered by caterpillars that was catalpol also varied across months ($F_{3,43} = 25.07$, $P < 0.001$), the pattern was different from host plants, with caterpillars sequestering almost entirely catalpol in May through July, with a slight, but non-significant decline of 5% from July to August (Fig. 2d).

In 2017, the mean total IG concentration of *C. glabra* in the population was a strong predictor of mean total IG concentrations in *E. phaeton* caterpillars (Fig. 4a), with caterpillars sequestering ~1.53% more IGs for every percent increase of IGs in host plants, suggesting that, while caterpillars do sequester IGs in concentrations higher than that in host plants, the underlying host plant IG concentrations likely limit total IG sequestration. However, we found no relationship between the proportion of IGs that was catalpol in *C. glabra* and *E. phaeton* ($R^2 = -0.43$, $P = 0.786$), suggesting that caterpillars can likely preferentially sequester catalpol over aucubin, since the proportion catalpol in caterpillars could be extremely high, despite much lower proportions in host plants (Fig. 4b).

Discussion

The results of this study suggest that while seasonal variation in host plant chemistry can drive sequestration of PSMs by specialist herbivores, both the magnitude and composition of sequestered PSMs may be driven in part by other factors, such as physiological differences among larvae at different developmental stages, and ecological factors such as caterpillar selection of host plants based on IG content. We found that the relative degree of sequestration of two different IGs differed across the season, and while patterns of total IG sequestration mirrored IGs in host plants, the composition of sequestered IGs was independent of host plant IG composition, suggesting either caterpillar selection for host plant IG composition, or differential sequestration of different IG compounds across larval development. That total IG concentrations were strongly related may not be surprising, given that host plants exhibit tremendous variation in PSMs

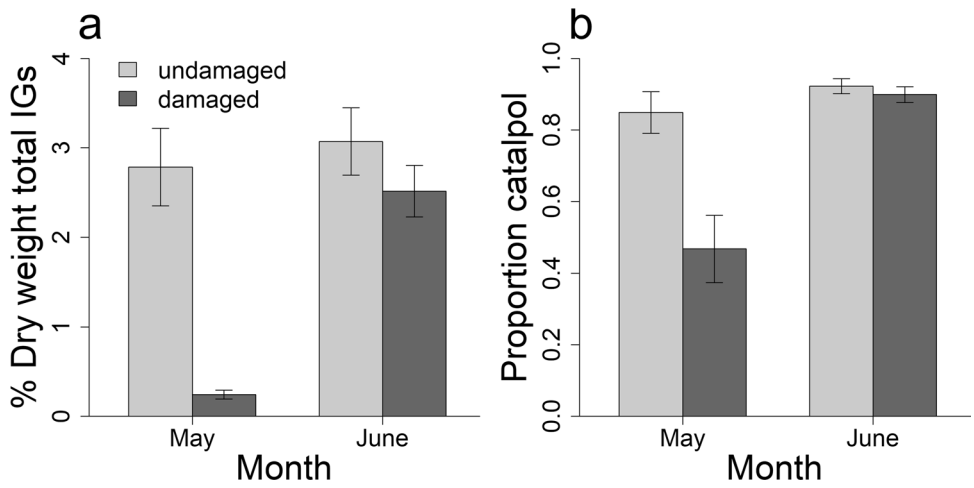

Fig. 3 There was a significant interaction between month and herbivore damage for both **a** total IG concentration and **b** the proportion of IGs that was catalpol in *C. glabra* leaves in 2017. Bars are mean ± SE

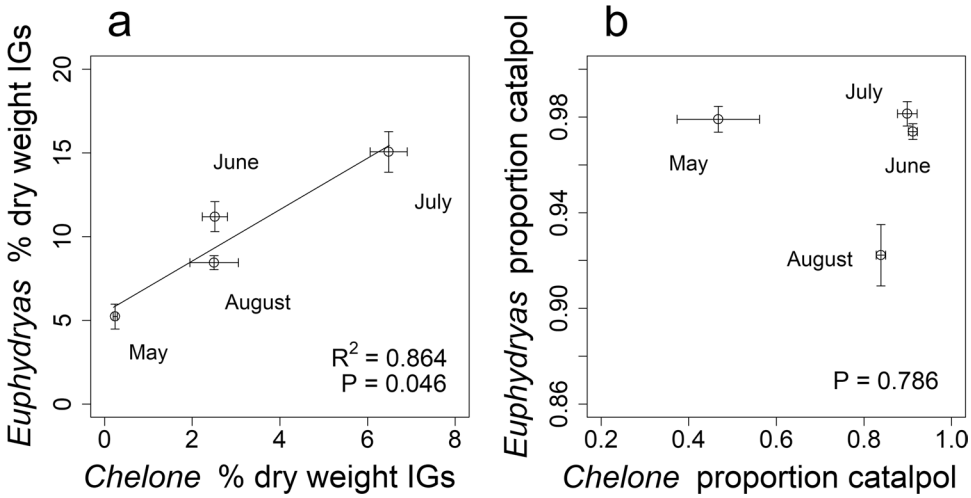

Fig. 4 Regardless of month, **a** total IG concentration in hostplants was a strong predictor of total *Euphydryas phaeton* sequestration. However, **b** here was no relationship between the proportion of IGs that was catalpol in hostplants and the proportion of iridoid glycosides (IGs) that was catalpol in *E. phaeton* larvae. Bars are mean ± SE

among individuals and across development, and that variation ultimately limits caterpillar sequestration. However, that the composition of sequestered IGs differed across herbivore ontogeny and from host plant chemistry in the field, could suggest potential physiological limits or ecological constraints on sequestration across herbivore development. Below we discuss the implications of these findings and explore potential ecological mechanisms driving variation in the sequestration of PSMs from host plants.

For the most part, the seasonal pattern of increasing and then declining total IG concentration in *C. glabra* is similar to those reported in other perennial plants. In birch, for example, changes in the concentrations of PSMs have been linked to a potential tradeoff with investment in growth in early spring and reproduction in late summer, with concentrations of phenolics increasing rapidly in the spring, and slowly declining until senescence (Riipi et al. 2002). Similar patterns have also been seen in herbaceous plants, including those with similar PSMs. For instance, IGs in *Antirrhinum major* vary with plant stage and through time, declining later in the season (Beninger et al. 2007). *P. lanceolata* also produces the same two IGs as *C. glabra* (Darrow and Bowers 1997), is also an alternate host plant for *E. phaeton* in some populations (Bowers et al. 1992b), and also follows a similar developmental pattern in IG concentrations (Quintero and Bowers 2018). Whether or not this common seasonal pattern of host plant PSM concentrations is driven by seasonal variation in trade-offs between growth, defense, and reproduction warrants more study.

Interestingly, the patterns of total IG concentration in both *C. glabra* and *E. phaeton* in our study were strongly positively correlated (Fig. 4a): starting at low levels in the spring, rising in the summer, and falling off again by the end of summer; although, caterpillars sequestered IGs in much higher concentrations than hostplants. Other studies have suggested that there could be concurrent changes in sequestration across the season. For example, in common buckeyes (*Junonia coenia,* Nymphalidae), sequestration of IGs from *P. lanceolata* also increased with the concentration of IGs in host plants, corresponding to incremental periods of host plant development (Quintero and Bowers 2018). Experimental addition of pyrrolizidine alkaloids (PAs) to artificial diets has also been directly related to the sequestration of PAs in the specialist arctiine moth, *Utetheisa ornatrix* (Cogni et al. 2012). That many studies, including this one, have found strong relationships between PSMs and their sequestration by specialists, suggests that hostplant chemistry is indeed one of the most important factors in the sequestration of PSMs.

While total sequestered IG concentrations in caterpillars were positively correlated with host plant concentrations, the relative concentrations of the two sequestered IGs in caterpillars showed little relationship to the concentrations found in host plants. These results suggest that there are limits to the sequestration of PSMs or that herbivores can tailor their sequestration given different physiological or ecological constraints. Richards et al. (2012) experimentally demonstrated limits to sequestration in *J. coenia* caterpillars, another IG sequestering nymphalid; wherein the amount of sequestered IGs from diet plateaued quickly past concentrations of 3% in the diet, with caterpillars excreting higher concentrations of IGs in frass from 6 and 12% IG concentration diets and plateauing more rapidly on mixed IG diets containing both aucubin and catalpol. That, in our study with *E. phaeton*, recently post-diapause larvae (in May) sequestered much higher proportions of catalpol than occurred in host plants could suggest that larvae are preferentially sequestering catalpol over aucubin, although it's unclear why. At least some sequestering species can also compensate for variation in the concentration and/or composition of PSMs in their diet. For example, monarch butterflies sequester more cardenolides from milkweed plants with lower compared to

higher cardenolide concentrations (Jones et al. 2019), though these differences were studied using cardenolide variation between different host plant species and not within a single species. Pipevine swallowtails, *Battus philenor* (Papilionidae), sequester alkaloids from *Aristolochia erecta* (Aristolochiaceae) to deter predators and without apparent costs of sequestering high levels of alkaloids (Fordyce 2001), though 44.3% of variation in larval sequestration was driven by herbivore maternal lineage, suggesting that larval physiology played a larger role in sequestration than host plant variation (Dimarco et al. 2012). Whether or not variation in sequestered IG composition in *E. phaeton* is the result of herbivore choice, host plant response, or an ecological constraint is likely driven in part by the physiological and molecular mechanisms by which they sequester. To date, these mechanisms remain unknown and in general represent an area in need of more study (Erb and Robert 2016).

While seasonality has been shown to drive both PSMs and herbivore sequestration, few studies have tied this variation to the seasonal ecology of the specialist herbivores. In this study, for instance, *E. phaeton* ontogeny spans the entire seasonal development of the host plant, with different generations of herbivores on early and late season plants: i.e., smaller pre-diapause larvae on late season plants, and larger post-diapause larvae on early season plants. Having a different pattern of development from host plants could impact seasonal sequestration, for instance, if sequestration is cumulative and post-diapause caterpillars in May retained sequestered IGs from the previous August when mature plants likely had comparably high concentrations of IGs. This could be evident in the degree to which different developmental instars sequestered total IGs varied, from 20 times higher concentrations than host plants in post-diapause 4th – 5th instars in May to only double the concentrations in fully grown 6th instars in July. This seasonal variation in sequestration could also be the result of stage-specific tradeoffs. Potential tradeoffs between herbivore growth and chemical defense have been studied. For example, nicotine in *Nicotiana* plants has a dose-dependent growth-inhibiting effect on larvae of *Manduca sexta* (Appel and Martin 1992), suggesting that dealing with higher concentrations of PSMs comes at a metabolic cost, which could be especially important during crucial periods of herbivore growth. Reported trade-offs between growth and sequestration vary though, often without measurable fitness consequences. For instance, Cogni et al. (2012) found no impact of sequestration of PAs on male and female longevity, fecundity, or egg viability in the specialist moth *U. ornatrix*. This could suggest that other ecological mechanisms may be more important in driving variation in sequestration across herbivore life-stages.

One additional explanation for differences in IG compositions between hostplants and caterpillars is that variation in sequestered PSMs could be the result of stage-specific sequestration for varying defensive strategies across caterpillar development. For example, common buckeyes also sequester IGs from their host plants and incur an immunological cost of that sequestration (Smilanich et al. 2009). They also exhibit instar-specific relationships between sequestration and immune function (Carper et al. 2019). Given tradeoffs in the costs of sequestration across development (Smilanich et al. 2009), this could indicate adaptation to minimize immunological costs of sequestration in defense against different natural enemy pressure across caterpillar ontogeny. Interestingly, Baltimore checkerspots are parasitized in both pre- and post-diapause life-stages by a specialist braconid wasp, *Cotesia* (*Apanteles*) *euphydryidis*, which can infect up to 40% of caterpillars within a population (Stamp 1981, 1982a). However, parasitism rates differ in pre- and post-diapause larvae and parasitoid presence increases with caterpillar density (Stamp 1982c), suggesting that parasitoids could exhibit differential pressures on caterpillar growth and defense at different life stages. Whether or not this variation in parasitoid pressure is mediated by sequestration of PSMs remains unstudied.

Several non-mutually exclusive ecological mechanisms could in part drive the different patterns we observed for plants with and without herbivory, driven by either herbivore preference or plant response to herbivory. For example, post-diapause larvae could be targeting plants with extremely low IG concentrations in May, especially given their high degree of mobility which has been attributed to larval choice (Mauricio and Bowers 1990). Intuitively, if there are greater costs to sequestering very high concentrations of IGs (Smilanich et al. 2009), especially for small, recently post-diapause caterpillars, then they could benefit from preferentially feeding on low IG host plants. Alternatively, caterpillars could prefer plants higher in catalpol, with high concentrations of IGs in undamaged relative to damaged host plants the result of plant response to herbivory through the reallocating of resources used in defense. Because specialists are predicted to perform best at intermediate PSM levels, plants may only benefit from relatively strong or weak responses to specialist herbivores (Ali and Agrawal 2012). Studies have shown the induction of PSMs can be systemic and can take place across different host plant tissues (Bakhtiari and Rasmann 2020), which can lead to herbivore-induced resource reallocation and subsequently changes in the sequestration of PSMs by herbivores (Orians et al. 2011). Examples of induced susceptibility, in terms of downregulating the production of PSMs in response to herbivory, are comparatively rare (but see Barton 2008; Karban and Niiho 1995). Ultimately, experimental manipulation of herbivory would be needed to determine what mechanism(s) drove the patterns we observed in response to herbivory.

Taken together, our results suggest that intraspecific seasonal variation in host plant chemistry can be a dominant

factor driving sequestered defenses of herbivores in natural populations. However, the degree to which host plant chemistry impacts sequestration likely changes over the course of the season and is likely as a result of stage-specific sequestration by herbivores. Additional studies of ecological mechanisms driving these results in the field would increase our understanding of the factors associated with seasonal variation in plant-herbivore interactions.

Acknowledgments We thank the undergraduates of the Bowers lab for assistance with sample preparation and IG quantification, especially M.E. Zabinski, and the lab in general for comments and suggestions, as well as the helpful comments of two anonymous reviewers. We also extend our appreciation to the local landowner who allowed us to work in the wetland on their farm. This research was supported by the National Science Foundation (award #s: IOS-456338 and DEB-1256817/1638866) and the University of Colorado Undergraduate Research Opportunity Program.

Authors' Contributions L. L. Richardson and M. D. Bowers conceptualized the project. L. L. Richardson collected field samples. A. L. Carper quantified iridoid glycoside concentrations, analyzed data, and prepared the figures and manuscript. L. L. Richardson, M. D. Bowers, and R. E. Irwin revised the manuscript and oversaw the project.

Funding This project was funded by grants from the National Science Foundation (award #s: IOS-456338 and DEB-1256817/1638866) and the University of Colorado Undergraduate Research Opportunity Program.

Data Availability Data will be made available on Dryad pending acceptance.

Code Availability Code will be made available upon request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflicts of Interest/Competing Interests The authors report no conflicts of interest.

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