Targeted predation of extrafloral nectaries by insects despite localized chemical defences

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Extrafloral (EF) nectaries recruit carnivorous arthropods that protect plants from herbivory, but they can also be exploited by nectar thieves. We studied the opportunistic, targeted predation (and destruction) of EF nectaries by insects, and the localized chemical defences that plants presumably use to minimize this effect. In field and laboratory experiments, we identified insects that were possibly responsible for EF nectary predation in Vicia faba (fava bean) and determined the extent and accuracy of the feeding damage done to the EF nectaries by these insects. We also performed biochemical analyses of plant tissue samples in order to detect microscale distribution patterns of chemical defences in the area of the EF nectary. We observed selective, targeted feeding on EF nectaries by several insect species, including some that are otherwise not primarily herbivorous. Biochemical analyses revealed high concentrations of L-3,4-dihydroxyphenylalanine, a non-protein amino acid that is toxic to insects, near and within the EF nectaries. These results suggest that plants allocate defences to the protection of EF nectaries from predation, consistent with expectations of optimal defence theory, and that this may not be entirely effective, as insects limit their exposure to these defences by consuming only the secreting tissue of the nectary.

1. Introduction

In addition to deploying physical and chemical defences that act directly against feeding herbivores, many plants exhibit traits that enhance the recruitment of carnivores and thus function as an ‘indirect’ form of plant defence [1]. Indirect defence traits can include the production of information-bearing cues such as herbivore-induced plant volatiles, which can alert carnivores to the presence of herbivores [2] and even of specific herbivore species [3]. Alternatively, plants can recruit predatory arthropods via the provision of resources, including sheltering structures, food bodies and extrafloral (EF) nectar [1,4,5], and in some cases the provision of such resources may facilitate the evolution of mutualistic interactions between particular plant and insect species [5]. Investment in the production of rewards entails costs for plants and also creates a potential opportunity for exploitation by third parties. For example, while EF nectaries are effective in recruiting herbivore natural enemies such as ants and parasitoids [1,4,6–9], they are also visited by nectar thieves that provide no protective services against antagonists [1,10,11]. In this paper, we explore whether EF nectary tissues may also be targets of opportunistic predation by arthropods.

Present in more than 100 plant families [12], EF nectaries are morphologically diverse and may be found on any above-ground plant parts [4,9,13]. The plant examined in this study, Vicia faba (fava bean), has conspicuous, dark-purple EF nectaries—the coloration perhaps serving as a visual cue for parasitoids [14] or other natural enemies of herbivorous arthropods—which occur on paired stipules at the connection point of the plant stem to each leaf petiole. The abaxial surface of each stipule exhibits a single EF nectary comprising a cluster of hundreds of secretory trichomes [15]. Secretion of EF nectar by plants is frequently induced by herbivory [9], but in the V. faba cultivar with which we worked is at least partially
constitutive, and most (but not all) EF nectaries are regularly covered with a droplet of nectar under both field and (pest-free) greenhouse conditions. As with other EF nectary-bearing plants, major components of V. faba’s EF nectar include water, sugars and small quantities of amino acids [15–17].

This study was initiated following the serendipitous observation of a characteristic damage pattern on V. faba plants under field conditions. Specifically, we noticed that EF nectary tissues were often partially or entirely removed, presumably by insect feeding, while surrounding tissues remained intact—a pattern similar to that observed in controlled insect feeding assays conducted for this study, which are described below (figures 1 and 2). To the best of our knowledge, no previous work has explored the ecological implications of EF nectary predation by insects. A few researchers have provided anecdotal accounts of herbivore damage to EF nectaries in laboratory experiments, for example with hybrid aspen (Populus tremula × Populus tremuloides) [18], or noticed patterns of targeted EF nectary predation in the field similar to that which we observed (M. G. Weber 2014, personal communication; Viburnum spp. plants). But other researchers working with various EF nectary-bearing plants have not noted preferential feeding on EF nectaries (B. Marazzi 2014, personal communication).

To provide a more detailed characterization of targeted predation of V. faba EF nectaries by insects, this study pursued three main objectives: (i) to confirm that herbivore feeding is often targeted non-randomly towards EF nectaries (via mapping and analysis of damage patterns on EF nectary-bearing stipules); (ii) to determine which groups of insects are responsible for EF nectary predation in the field (via field trapping and laboratory feeding assays); and (iii) to explore the underlying reasons why insect feeding should be limited to the EF nectary while the surrounding stipule tissue remains mostly undamaged (via chemical analyses of plant tissue samples).

With respect to the third objective, optimal defence theory suggests that plants should allocate chemical defences in proportion to the threat that herbivory poses to specific plant parts and the corresponding reduction in fitness caused by the damage or loss of that part [19]. Given that EF nectary tissues are important for plant defence and, being rich in both sugar and protein [20], are apparently attractive targets for herbivory, we hypothesized that plant chemical defences should be concentrated in the area of the EF nectary and adjacent tissues. To test this hypothesis, we initiated chemical analyses of small and precise tissue samples from different regions of the EF nectary-bearing stipule and other plant parts. Our initial experiments revealed rapid blackening of stipule extracts, as opposed to slow blackening of leaf extracts (see the electronic supplementary material, S1), caused by the enzymatic oxidation of the toxic, non-protein amino acid L-3,4-dihydroxyphenylalanine (L-DOPA), which is prevalent throughout V. faba plants [21–23]. We therefore focused subsequent chemical analyses on comparing concentrations of L-DOPA in plant tissue samples. We discuss the findings of our study in light of optimal defence theory [19] and draw conclusions about the ecological costs of the EF nectary indirect defence mechanism.

2. Material and methods

(a) Plant and insect rearing
Vicia faba L. cv. Broad Windsor plants were grown from seeds (Territorial Seeds Co., Cottage Grove, OR, USA) in PRO-MIX BX growing medium (Premier Tech Horticulture, Quakertown, PA, USA) with slow-release fertilizer (Osmocote NPK:14-14-14 + Micromax Micronutrients, Scotts, Marysville, OH, USA) under greenhouse conditions (24 C, 16 L : 8 D photoperiod). Specydeptera exigua (Hübner) caterpillars (Benzo research, Carlisle, PA, USA) were reared on artificial diet (Southland Products Inc., Lake Village, AR, USA) in a growth chamber (22 C, 65% RH, 16 L : 8 D photoperiod).

(b) Feeding-experiment damage analyses

(i) Field experiment

In an initial study, we subjected V. faba plants to ambient herbivory in an experimental field plot. Sixty-four three-week-old plants were taken from the greenhouse and planted in a 15 × 15 m chicken-wire fenced plot in an open meadow near State College, Pennsylvania, in August 2013. Plants were placed at random locations within the plot, attached to support stakes and hand watered for the first 3 days. After 8 days, all EF nectary-bearing stipules that showed any damage were mounted between two layers of clear sticky tape and analysed via the leaf-damage data generation method described below. A total of 478 damaged stipules were collected from the 64 experimental plants present in the plot, and a random sample of 100 of these stipules was used for subsequent feeding-damage analyses. Prior to stipule removal, the ratio of damaged stipules to total stipules on the plant and the proportions of damaged stipules that also exhibited damaged or destroyed EF nectaries were calculated for a subset of 37 of the plants in the plot. At the conclusion of the field experiment, sweep nets were used to collect insects from the experimental plot, which were sorted into the following taxonomic groups: grasshoppers, crickets, caterpillars (a few individuals, visibly distinct species), beetles (several visibly distinct species) and earwigs. As a preliminary assessment of the propensity of these groups to damage EF nectaries, representative insects were placed in screen cages containing several V. faba plants for 3 days (under greenhouse conditions).

(ii) Feeding experiment with crickets

Thirty-five ‘ground crickets’ were collected from the experimental plot. Five screen cages with 7 or 8, three-week-old V. faba plants were placed in a growth chamber (23 C day/21 C night, 16 L : 8 D photoperiod) and seven crickets were placed in each cage. A piece of apple was also placed in each cage as a primary food source. After 10 days, all stipules exhibiting damage were collected as above and analysed according to the leaf-damage data generation method described below. Only 24 crickets of the original 35 were recovered at the end of the experiment (the rest were presumably cannibalized).

(iii) Feeding experiment with caterpillars

The initial screening also revealed considerable damage to stipules and EF nectaries by caterpillars. Since there were few caterpillars in the field after the field experiment ended, we chose a laboratory-reared lepidopteran—the generalist S. exigua—for subsequent feeding-damage and weight-gain experiments. Three fourth-instar S. exigua caterpillars were placed on the bottom leaf of individual three-week-old V. faba plants (n = 36). After 48 h, all stipules exhibiting damage were collected as above and analysed via the leaf-damage data generation method described below. Prior to the experiment, caterpillars were transferred from artificial to fresh-plant diet for 3 days.

(c) Generation of leaf-damage data

Mounted stipules from the feeding-damage experiments were digitally scanned at 1200 dpi. Owing to significant variation in
stipule size and shape, and in nectary size, shape and location, it was necessary to project the damage done to each stipule onto a drawing of a schematic stipule (the model stipule on which the schematic stipule drawing was based is shown in figure 3). The picture of the model stipule was transformed into a trichromatic drawing (one colour for the stipule, a second colour for the nectary and a third colour for the background) using an image manipulation program (GIMP 2.8.6). For each damaged stipule, an image was created by manually filling in the damaged areas on a copy of the schematic stipule using a fourth colour. The damage that was drawn on each copy of the schematic stipule was carefully adjusted to compensate for differences in size and shape between the schematic stipule and each scanned stipule. For each of the three feeding-damage experiments, the set of images that was created this way was used to generate a heat map using the Matplotlib Python module [24]. The script calculated the number of times each pixel was hit (eaten) and normalized it by the number of stipules in the dataset, to produce a value in the range of 0–1.

(d) Caterpillar weight-gain experiments

(i) Leaves versus stipules

Third-instar *S. exigua* caterpillars in two treatment groups (*n* = 25 per treatment) were weighed, placed in rearing cups on moist filter paper and allowed to feed ad libitum (fresh leaves or stipules were provided daily. Each group received one type of diet). All caterpillars were weighed at 2 and 4 days after the initiation of the experiment.

(ii) Artificial diet

Second-instar *S. exigua* caterpillars were weighed and placed in rearing cups as above. They were fed five different artificial diets (*n* = 30 caterpillars per treatment group) impregnated with one of the following concentrations of L-DOPA, which were based on the results of the chemical analyses (figure 3): 0% (control), 1.5% (the concentration found in leaves), 3%, 5% (the concentration in EF nectaries) and 5% L-DOPA + sugars [sucrose, glucose, fructose and galactose, in the same concentrations found in the EF nectaries (see Results)]. Diet was refreshed every 2 days. Caterpillars were weighed on days 3, 4, 6 and 8. Average weight was calculated only for live caterpillars. On the 8th day, some caterpillars in the control group had become pupa and therefore only those that had not pupated were weighed.

(e) Tissue samples

Leaf and stipule tissue samples were obtained from plants that were approximately one month old. Because of the low mass of individual nectary samples, groups of four plants were considered as one replication unit (*n* = 20 groups). Stipules and leaves were removed from the plants and rapidly processed. Samples were collected using appropriately sized (see below) metal tubes with sharpened edges. The stipule or leaf being sampled was placed on a thin craft-foam sheet in order to allow the sharp edges of the sampling tubes to pass through the tissue, and the tube was pressed down and slightly rotated to pick up the sample. Once all tissue rings or discs were collected into the sampling tube, the cotton-free end of a long cotton swab was inserted through the upper end of the sampling tube in order to push all the samples from the tube into an Eppendorf vial.

Nectary sample discs were taken from the middle of the nectaries using a 1 mm (inner) diameter sampling tube and contained 100% nectary tissue; EF nectar was wiped from the surface of the stipule prior to sampling. Thirty-five nectary discs were collected from each plant group (henceforth referred to as ‘N’ samples). Nectary-adjacent tissue was sampled by removing all nectary tissue and then using a 2.75 mm diameter sampling tube to remove a thin ring of tissue from around the nectary. Twelve such rings were collected from each plant group (henceforth referred to as ‘A’ samples). A third set of samples comprised of a larger concentric ring surrounding the ‘A’ sample, collected after the ‘A’ sample was taken, using a 6.5 mm diameter sampling tube. Four such rings were collected from each plant group (henceforth referred to as ‘B’ samples). A fourth set of samples was taken from the middle-lower part of the stipule using a 2.75 mm diameter sampling tube. Ten such discs were collected from each plant group (henceforth referred to as ‘C’ samples). (For an illustration of the different samples taken from the stipule, see insert in figure 3.) A fifth set of samples was taken from a young leaflet from the upper half of the plant, using a 2.75 mm sampling tube. Six such rings were collected from each plant group (henceforth referred to as ‘L’ samples). Green pods were sampled in a similar way: large green pods were taken from approximately 2-month-old plants, and a sample was taken from each pod by inserting a 2.75 mm sampling tube through the valve and removing a sample comprising all the layers of the valve. One sample was collected from each plant (henceforth referred to as ‘P’ samples). For each sample type, the number of tissue rings or discs comprising a sample was determined so that the samples weighed approximately 9 mg each. In order to minimize the degradation of analytes (particularly the rapid oxidation of L-DOPA), samples were collected, rapidly weighed, then immediately flash-frozen in liquid nitrogen. EF nectar samples were collected from the experimental plants in the greenhouse before they were processed, using 10 µl glass microcapillaries (10 µl microdispenser replacement tubes, VWR/SP Scientific, West Chester, PA, USA), *n* = 16, sample volume = 10 µl.

(f) Chemical analyses

In order to accurately quantify L-DOPA levels in small tissue samples, samples were analysed according to Lisec et al.’s [25] protocol for metabolite profiling in plants, with slight modifications (see the electronic supplementary material, S2). Gas
chromatography mass spectrometry data were analysed using MassHunter Workstation Software (v. B.06.00 SP01). L-DOPA concentrations were calculated based on a calibration curve. The concentrations of the different sugars were estimated based on the internal standard ribitol.

All chemicals and reagents were purchased from Sigma–Aldrich (Milwaukee, WI, USA), except for the L-DOPA, which was purchased both from Sigma–Aldrich and from Alfa Aesar (Ward Hill, MA, USA).

(g) Effect of L-3,4-dihydroxyphenylalanine on bacteria and fungi

To test the hypothesis that high levels of L-DOPA in and around EF nectaries act as a defence against pathogen invasion through the EF nectary (instead or in addition to the potential role of this compound in deterring herbivory), we grew two plant pathogens on an 1-DOPA-saturated growth medium. We assessed the performance of: (i) the bacteria Erwinia amylovora, the causal agent of fire blight disease in fruit trees, which grows well in nectar and is known to invade plants through floral nectaries [26], and (ii) the fungus Botrytis cinerea, the causal agent of chocolate spot disease in V. faba, whose performance (i.e. mycelium growth and spore germination rates) is enhanced by the addition of sugar to its growth medium [27]. Erwinia amylovora (inoculum obtained from a pear tree) was grown on LB medium. Botrytis cinerea (inoculum obtained from an infected V. faba plant) was grown on 1/2 strength potato dextrose agar. For each test, 10 plates were prepared with a control medium and 10 were prepared by mixing the hot medium with L-DOPA until saturation (approx. 0.5–1%), before autoclaving it and pouring it into the plates. The plates were seeded and settled on a laboratory-bench at room temperature. The development rate of mycelium/bacterial colonies was visually assessed every day for 5 days.

(h) Data analysis

Statistical analyses were done using R, v. 3.1.0 (R Project for Statistical Computing, http://www.r-project.org/). For the caterpillar weight-gain experiment comparing leaves versus stipules, caterpillar weights at the termination of the experiment on the 4th day were analysed using Student’s t-test. Data were ln transformed prior to analysis. For the caterpillar weight-gain experiment with artificial diet, caterpillar weights at the termination of the experiment on the 8th day were analysed using ANOVA test. Data were ln transformed prior to analysis. Data from the chemical analysis of L-DOPA was rank transformed and the differences between samples were analysed using an ANOVA test with a Tukey test for post hoc comparisons.

3. Results

(a) Feeding-damage experiments

In the field experiment, 23% of plant stipules exhibited signs of feeding damage after 8 days. Further examination revealed that EF nectaries were completely (or almost completely) absent from 57% of damaged stipules and exhibited less severe damage in another 24% of cases. Moreover, almost all feeding damage to the EF nectaries was initiated from the abaxial side of the stipule (the side with nectar). A heat map visualization summarizing the localized intensity of herbivore damage to plant stipules confirmed that herbivore feeding was strongly targeted towards EF nectary tissues (figure 2a).

When insects from the field were put in screen cages with V. faba plants, beetles and earwigs (which were observed on V. faba plants in the field) did not damage EF nectaries. Grasshoppers did extensive damage to diverse plant tissues and were also observed to do some apparently selective feeding on EF nectaries. The most extensive and precisely targeted damage to EF nectaries was produced by ground crickets, and we therefore selected these insects for use in more controlled feeding-damage assays in the laboratory. Caterpillars collected from the field plot also did some precise damage to EF nectaries, and we therefore conducted similar
controlled feeding-damage assays with laboratory-reared larvae of the moth *S. exigua* (whose larvae readily feed on *V. faba*). Heat map visualizations summarizing the localized intensity of herbivore feeding in these controlled feeding assays reveal evidence of highly targeted feeding on EF nectary tissues (figure 2b,c), similar to that observed in our field experiment (figure 2a).

(b) Chemical analyses

Chemical analysis of tissue samples from different plant parts (figure 3) revealed that levels of 3,4-DOPA were consistently higher in stipules than in leaves and highest in the immediate vicinity of the EF nectary. No free amino acid other than 3,4-DOPA was detected at meaningful levels. EF nectary itself contained only trace amounts of 3,4-DOPA. Aside from 3,4-DOPA, nectary tissue was rich in sugars (average ± s.e., weight of sugar/fresh weight of tissue sample: 1.38 ± 0.05% sucrose, 0.97 ± 0.07% glucose, 0.63 ± 0.05% fructose and 0.14 ± 0.02% galactose). These sugar concentrations were therefore used in subsequent artificial-diet experiments.

(c) Caterpillar weight-gain experiments

*S. exigua* caterpillars fed on *V. faba* stipules gained significantly less weight than those fed on leaves (figure 4). In the artificial-diet experiment, a clear toxic effect of 3,4-DOPA was evident (figure 5). By the 8th day of the artificial-diet experiment, 63% of the control group had pupated, while no caterpillars from any of the 3,4-DOPA treatment groups had done so. At the concentration of 3,4-DOPA measured in the EF nectaries (5%), caterpillar development was almost completely halted, and 23% of the caterpillars died during the 8 day experiment. When sugars were added to the 5% 3,4-DOPA diet at concentrations measured in the EF nectaries, the caterpillars’ development rate did not change, although none of the caterpillars exposed to this treatment died during the experiment.

4. Discussion

Our results clearly demonstrate that the EF nectaries of *V. faba* are prone to targeted predation by insects. This was apparent from the damage patterns observed in our field assay and the very similar patterns observed in controlled feeding assays with ground crickets and with *S. exigua* caterpillars (figures 1 and 2). The results of the experiment with ground crickets are particularly intriguing as these insects are primarily detritivores and thus are not typically considered to be important antagonists of living plants—indeed, we found only minimal damage to leaves when crickets were caged with *V. faba* plants for 10 days. This suggests that EF nectary production may not only entail the apparent EF nectary theft, as we observed that *S. exigua* caterpillars initially drink nectar and then, almost always, go on to consume EF nectary tissue (M. Gish 2013, personal observation; figure 1).

While such opportunistic antagonists most likely gain only nutritional benefits from EF nectary predation, herbivores that also feed on other tissues of EF nectary-bearing plants might plausibly also benefit via disruption of the indirect defensive function of EF nectaries. Further research is needed to determine whether such benefits are a significant factor favouring EF nectary predation in nature. At a more proximate level of explanation, targeted feeding on EF nectaries may frequently arise as an extension of nectar theft, as we observed that *S. exigua* caterpillars initially drink nectar and then, almost always, go on to consume EF nectary tissue (M. Gish 2013, personal observation; figure 1).

The subsequent feeding pattern, in which EF nectary tissues are consumed—by arthropods that may initially be attracted by the presence of nectar—while adjacent tissues remain mostly undamaged (figures 1 and 2), probably reflects the relatively low palatability of non-nectary tissues.
surrounding the EF nectary, which nevertheless exhibit high levels of l-DOPA (figure 3). In addition to high levels of sugars, EF nectary tissue exhibits high protein content [20] that may outweigh the deleterious effects of l-DOPA on protein synthesis (see below), creating a positive net nutritional value of the EF nectary tissue for herbivores. However, nutritional value probably becomes negative as feeding proceeds from the EF nectary to the surrounding stipule tissue, which contains less sugar and protein. Thus, highly targeted feeding may allow insects to limit their exposure to l-DOPA and other defence compounds that are concentrated in the region of the EF nectary, while still reaping the nutritional rewards of feeding on the sugar- and protein-rich EF nectary tissue.

The likely defensive role of l-DOPA, which is found in all parts of V. faba plants except for the seeds [21–23], is supported by its high concentrations centred in and around the EF nectary in V. faba (figure 3) and its adverse effects on caterpillar development (figure 5). The toxicity of l-DOPA to insects has been documented previously [28,29], and the increasing toxicity of l-DOPA to S. exigua when presented in artificial diet at concentrations approaching 5%—the concentration that we found in EF nectary tissue—is consistent with the findings of a previous study that tested the toxic and repellent effects of l-DOPA present in legume seeds on another Spodoptera species [28]. The mechanisms underlying such toxicity currently remain unclear, although prior work suggests that l-DOPA, which has a structure similar to that of the amino acid tyrosine, may: (i) affect tyrosinase activity, which is essential to the sclerotization of insect cuticles [28]; (ii) interfere with neurotransmission, as it is a precursor for several neurotransmitters [29]; and (iii) interfere with de novo biosynthesis of proteins by competing with tyrosine. The fact that we observed no adverse effects of l-DOPA on the bacterial and fungal pathogens we examined may be interpreted as further support for the inference that high levels of l-DOPA in the area of the EF nectary act primarily as a deterrent against insect herbivores. However, it is likely that other physical and chemical defences (e.g. terpenes) also contribute to EF nectary protection in V. faba and other plant species.

The apparent allocation of constitutive chemical defence to EF nectaries and their surrounding tissues suggests that the destruction of these protective organs by arthropod feeding entails significant costs for plants, potentially exceeding costs associated with the ‘theft’ of nectar by non-protecting organisms, which are thought to be limited given the relatively low physiological cost of EF nectar production [11,30,31]. Such costs may be exacerbated by the inherent vulnerability of EF nectaries which, by design, present valuable nutritional rewards that are readily accessible to potential herbivores—although the EF nectaries of some species are cryptically embedded within a bearing organ or concealed within a gland-like structure [13]—and which must be maintained over a relatively long period of time [6]. Previous studies have documented other costs associated with EF nectary production including the attraction of herbivores that feed on other plant tissues [32] and the potential adverse effects of carnivore recruitment on plant–pollinator interactions [33]. More broadly, previous work has also documented ecological costs associated with other classes of indirect defences. For example, some ants that reside in the domatia (plant structures that provide refuge for arthropods) of myrmecophytic plants castrate their host plant by destroying flowers, thereby manipulating the plant to produce more domatia [34]. Also, herbivore-induced volatiles that recruit natural enemies of foraging herbivores may be exploited by third parties, including herbivores themselves (which in some cases use such cues to facilitate aggregation) and hyperparasitoids that attack the parasitoid natural enemies of feeding herbivores [35,36].

Investment in the chemical defence of EF nectaries can also be interpreted as support for optimal defence theory [19], which predicts that such investment should correspond to the threat that herbivory poses to specific plant parts and the corresponding reduction in fitness caused by the damage or loss of that part. Demonstrations of this principle in plants are uncommon, and the best-known examples are the elevated chemical defences in young leaves [37]. Constitutive secretion of bracteal EF nectar may also exemplify this principle [38]. This study provides a particularly useful test of the predictions of optimal defence theory, as l-DOPA occurs in varying concentrations in diverse plant tissues and its concentration in different tissues may therefore plausibly be interpreted as an indicator of relative defence investment [19,39]. In that regard, the fact that V. faba places higher concentrations of l-DOPA around EF nectaries than seeds (figure 3) might be interpreted as an indicator of the importance of EF nectaries for this plant. On the other hand, the relatively small size of the EF nectary presumably reduces the overall cost of allocating high concentrations of defence compounds to these tissues, and may also dictate that the levels of defence compounds in these tissues must be high in order to ensure that EF nectary-feeding insects experience levels of overall exposure sufficient to act as a feeding deterrent.

Our results also raise questions about the potential interplay of indirect and direct defence strategies and their relative importance in plant protection against herbivory and suggest that indirect plant defences sometimes require direct defences to keep them in an operable condition. It is clear that the allocation of l-DOPA to EF nectaries in V. faba is not entirely

Figure 5. Spodoptera exigua caterpillar development on artificial diet impregnated with different concentrations of l-DOPA. Caterpillars were second instar at day 0. Caterpillar weights on the 8th day were compared using ANOVA (data were ln transformed before analysis. The graph shows the original data): $r^2 = 0.902, F_{4,119} = 283.4, p < 0.001$. Tukey post hoc tests: all pairwise comparisons resulted in significant differences with a $p < 0.001$, except for 5% l-DOPA versus 5% l-DOPA + sugars which had a $p = 0.99$. Error bars indicate s.e.
effective in deterring insect feeding, as we observed moderate or severe damage to nearly 20% of the EF nectaries in our field study. However, it is possible that damage rates would have been higher still if EF nectaries were less well defended, allowing insects that are more susceptible to L-DOPA to also feed on the EF nectaries. It is also likely that the efficacy of L-DOPA or other chemical defences of EF nectaries will vary between environments exposing different assemblages of potential EF nectary-damaging insects and of carnivorous EF nectary-visiting arthropods. Conversely, the identity and relative abundance of particular arthropods from those two groups—as well as the predictability of the interactions between them—probably influences the efficacy of indirect plant defences and hence the need to complement such defences with direct chemical defence of EF nectaries and other plant tissues. Unfortunately, it is difficult to assess how such factors may have shaped the evolution of plant defence strategies in the evolutionarily ancestral environment of V. faba, as the wild progenitor of this plant remains unknown [40].

In this study, the significant predation of EF nectaries observed in our field experiment occurred despite the presence of at least two species of ants, which are among the most common and effective mutualistic predators recruited by plant EF nectaries [5]. Higher ant densities, or different ant species, might have provided more effective protection. However, in contrast to myrmecophytic plants that form intimate mutualistic interactions with particular ant species—typically exhibiting specialized adaptations to facilitate these interactions—the more common non-myrmecophytic plants like V. faba form only facultative mutualisms with opportunistic ants that nest in their vicinity [5-6]. Consequently, patrolling ants may rarely be present on such species in large numbers in the absence of prey. While such facultative interactions might nevertheless be quite effective in preventing the colonization of plants by herbivores that live or feed on the plant for extended periods (e.g. non-myrmecophilic aphids and caterpillars), they are probably less effective against highly mobile insects like crickets and grasshoppers, which probably accounted for much of the EF nectary predation observed in our field experiment.

In conclusion, our results demonstrate that plant EF nectaries can be susceptible to targeted arthropod predation under field conditions, in some cases by species that are not otherwise primarily herbivorous. Furthermore, we documented significant allocation of a compound that probably functions in chemical defence (L-DOPA) to EF nectaries and surrounding tissues in V. faba, suggesting that EF nectary predation can entail significant costs for plants and providing support for the predictions of optimal defence theory [19]. It is likely that the vulnerability of EF nectaries to insect feeding in natural environments, along with the relative importance and interaction of direct and indirect defence strategies, is strongly influenced by ecological factors, including the identity and relative abundance of EF nectary-damaging insects and of carnivorous EF nectary-visiting arthropods. In addition to documenting rates of EF nectary predation in diverse plant species and ecological systems, future research efforts should focus on documenting the mechanisms underlying plant defence of EF nectaries and the role of ecological factors in shaping the structure, biochemistry and functioning of these important defensive organs.

Data accessibility. Additional methods and image supporting this article have been uploaded as part of the electronic supplementary material.

Authors’ contributions. All authors designed the study. M.G. performed the experiments and the analyses. M.G. wrote the first draft of the manuscript, and M.C.M. and C.M.D. contributed substantially to revisions.

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