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Running head: Pre-dispersal seed predators alter plant nectar production

Seed predators can increase nectar volumes in an alpine daisy: but do the insects benefit?

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Abstract

Pollinators and pre-dispersal seed predators can interact via the plants they share. We examined how pre-dispersal seed predators modify nectar quality and quantity and thereby influence pollinator behavior. Working in a Tibetan alpine meadow, we hypothesized that increasing levels of pre-dispersal seed predation by larvae of tephritid flies would reduce nectar quantity and quality in *Saussurea nigrescens* (Asteraceae), and that this would make the flowers less attractive to honeybees, the most frequent floral visitors. Our field experiments showed that floret nectar volumes responded differently to high and low densities of fly larvae, with significant increases when there was one larva present, but decreases when two or more larvae were present in a capitulum. Experimental manipulations of fly larvae yielded the same result. The increases in nectar volume generated by a single larva are likely to be beneficial for these insects in locations where pollinators preferentially visit those *S. nigrescens* that produce more nectar. At our study sites, honeybees were the main pollinators and visitation rates were unaffected by the changes in nectar volumes, but they are introduced to the area, and native pollinators may be more selective.

Key words *Apis mellifera* · Asteraceae · Insect-plant interactions · nectar · *Saussurea, Tephritis* · Tibetan Plateau
**Introduction**

Pollinators and flowering plants have contributed significantly to our understanding of the evolution of mutualisms involving both generalist and specialist pollination systems (Kjellberg et al. 2001; Lunau 2004; Mitchell et al. 2009; Bronstein 2015).

Co-evolution is most readily apparent in relation to the accessibility of rewards, with pollinators exerting selective pressures on floral traits such as spur length (Nilsson 1988) and plants selecting for pollinator tongue length (Whittall and Hodges 2007; Miller-Struttmann et al. 2015). The quantity of accessible rewards thereby influences which pollinators are attracted, and of which species, and can also influence how long individual pollinators remain on each flower (Kalinganire 2001).

Plants and pollinators rarely if ever interact in isolation from other organisms, many of which are likely to have antagonistic rather than mutualistic relationships with the plants (Galen 1999). They include antagonists that can directly or indirectly influence various traits through consumption of plant parts, such as decrease flower size (Barber et al. 2012), modify nectar odour (Press and Phoenix 2005), reduce the quality of floral displays (McCall and Irwin 2006), and reduce nectar production (McDade and Kinsman 1980), all of which may influence pollinator behavior (Rodríguez-Rodríguez et al. 2015).

Seed predatory insects often have a strong influence on plant reproductive success and can generate significant selection pressures on their host plants (Kolb and Eriksson...
Their impact on their hosts varies in relation to plant reproductive traits such as flowering phenology, flower number and seed size and these attributes are also significant for the insects’ own reproductive success, thereby facilitating co-adaptation and co-evolution (Thompson 2005). Pre-dispersal seed predators require seeds to feed their larvae, but often oviposit early in floral development, before ovules have been pollinated. Their reproductive success is therefore influenced by the quality and quantity of subsequent pollination events (Strauss and Irwin 2004). The adults of some seed predators such as fig wasps and yucca moths ensure that seeds are available for their larvae by routinely pollinating the flowers themselves (Pellmyr and Huth 1994), but for the majority of species this is achieved by the selection of oviposition sites in flowers or inflorescences that have a high probability of being pollinated. Many pre-dispersal seed predators have a limited range of host plants, and often just a single host species (Collin and Shykoff 2010). Within each plant species, the likelihood and quality of pollination can depend on a wide range of environmental and biological variables, but phenotypic characteristics of individual plants are also significant. For insect-pollinated species, the size and quality of floral displays and the quality of rewards they offer influence the extent of seed set (Thomson 1988; Vaughton and Ramsey 1998). Consequently, the flowers that are most rewarding for oviposition by seed predators are likely to be those that are also most favourable to the plant’s pollinators (Cariveau et al. 2004).

Seeds are nitrogen-rich and often contain high concentrations of energetically-
expensive defensive compounds (Janzen et al. 1977; Birch et al. 1986). Damage
generated by seed predators during oviposition or early larval feeding, together with
adaptive responses by the plants to the presence of the insects, can result in flowers
containing seed predators receiving less investment, with resources being switched to
undamaged flowers or retained elsewhere (Kudoh and Whigham 1998; Westerbergh
and Westerbergh 2001; Cariveau et al. 2004). Consequently, flowers containing seed
predators may be less attractive to pollinators and set fewer seeds (Cariveau et al.
2004).

Floral nectar is the most widely-provided reward for insect visitors to flowers (Scaven
and Rafferty 2013) and plants with more and higher quality nectar can attract more
Nectar is composed mainly of sugars derived from photosynthesis and forms part of a
plant’s overall carbohydrate content. Carbohydrates are stored in both reproductive
and vegetative organs (Pacini and Nepi 2007) and linkage has been recorded between
carbohydrate storage and nectar production, with more nectar produced at times of
day when overall carbohydrate content is higher (Mu et al. 2015). Herbivory often
reduces the quantity of reserves stored within plants (Machado et al. 2013), which
suggests it may also reduce nectar production, and indirectly influence pollinator
behavior to the further detriment of the plant.

Here, we describe the interplay between a pre-dispersal seed predator, the quantity and
quality of nectar in flowers of its host plant, the carbohydrates stored throughout the
plant and the willingness of bees to visit its flowers. We hypothesized that

(1) Pre-dispersal seed predators reduce the quantity and quality of nectar
in the flowers where they were feeding,

(2) Increasing numbers of seed predators have a progressively greater
impact on nectar production,

(3) Seed predators reduce the proportion of the plant’s carbohydrates
stored in the flowers, relative to other parts of the plant, and

(4) Any changes in nectar rewards as a result of the seed predators
reduce flower visitation by the pollinators of the plant.

Methods

Natural History

*Saussurea nigrescens* (Asteraceae section Compositae, ECCAS 1999) is a
widespread high-altitude perennial distributed 2000-4300m on the Tibetan Plateau. Its
growing season is short, with plants reviving in mid-May and senescing by mid-
September. Mature plants vary in height about 15-45cm. Flowering occurs from July
to August and seeds mature and disperse in late-September. Each plant produces 2-5
dull-purple capitula, each of which contains 20-55 florets. The florets have an annular
bowl-shaped nectary between the ovary and anthers (bowl diameter is 1.5-2.0mm).
The flowers are monoclinous (with stamens and pistils in the same flower) but
outcrossing is favoured by protandry (stamens mature before the ovaries), see Mu et
Saussurea nigrescens is the most economically important nectar-producing plant in parts of the Tibetan plateau and contributes approximately 80% of the annual honey harvest in Hongyuan County, China (Mu et al. 2014). The plant is allogamous and requires cross-pollination, which is usually by honeybees (Mu et al. 2014).

The only recorded pre-dispersal seed predators of S. nigrescens are larvae of several tephritid flies belonging to the genera Campiglossa, Tephritis and Urophora (Xi et al. 2016). Tephritid females oviposit into the flower buds of the host plant in the middle of July. Their larvae develop within the capitula, where they consume developing seeds and damage the receptacles (Xi et al. 2016). Between one and three larvae share a capitulum. No obvious gall formation takes place. The tephritid larvae do not feed on floral nectar, nor do they directly damage the nectaries. They pupate inside the capitula, where they remain until the adults emerge the following spring.

Study sites

The study was conducted from 2014 to 2015 at the Hongyuan Alpine Meadow Ecosystem Research Station of the Chinese Academy of Sciences, located in Hongyuan County, Sichuan Province, China (32°48’-32°52’N, 102°01’-102°33’E). This area of the eastern Qinghai-Tibetan Plateau is at an altitude of about 3500m. With short and cool spring, summer and autumn seasons and a long cold winter. Liu et al. (2012) and Mu et al. (2015) provided details of the local climate, soil conditions
and vegetation. The meadows are sometimes used for cattle grazing during the winter, but are otherwise undisturbed. *Tephritis femoralis* Chen is the common tephritid seed predator of *S. nigrescens* locally.

In late July 2014 we selected three sites (sites 1-3) located about 5 km apart that had similar soil moisture and physical and chemical properties (Mu et al. 2014). Their plant communities were also similar. *Saussurea nigrescens* was the most abundant species at the sites, contributing 33-46% of total plant cover during its flowering period. *Elymus nutans* Griseb, *Anemone rivularis* Buch.-Ham. ex DC. and *Potentilla anserina* L. were also common. The three sites were at roughly similar distances from the nearest apiaries and similar numbers of bees and adult tephritid flies were recorded visiting the flowers of *S. nigrescens* (Mu et al. 2014). *Saussurea nigrescens* was the major source of nectar for bees at all three study sites (Mu et al. 2014). Native pollinators of *S. nigrescens* include an Asian honeybee (*Apis cerana*) and the bumblebees *Bombus filchnerae*, *B. humilis*, and *B. supremus* (Macior et al. 2001). The introduced *Apis mellifera* has been tended by beekeepers in the area since 1981 (Sun et al. 2013).

Field records of tephritid numbers and nectar variables

In July 2014 we tagged 50 healthy *S. nigrescens* with undamaged leaves at each of the three sites. After the tephritids had an opportunity to oviposit, but before the flowers had opened, we enclosed all the capitula on each plant within fine mesh
netting to exclude further insect visitors (Real and Rathcke 1991). This plant produces its maximum nectar volumes when the anthers are white and emerging from the capitula (Mu et al. 2014). When they reached this stage, we selected five florets at random from each of the capitula and on sunny days at 10:00-15:00Hrs we measured their nectar volumes and concentrations. Nectar volumes were measured using 1 µl or 5 µl micropipettes (Hirschmann Laborgeräte, Germany). Nectar concentrations were measured concurrently with a hand-held refractometer (Eclipse, Stanley Company, England Bellingham, UK) following the methods of Johnson et al. (2006). The numbers of tephritid larvae inside each capitulum were then counted using a binocular microscope. A total of 472 capitula were selected to monitor nectar volume and concentration.

Plant responses to experimental manipulations

In July 2015 we selected 120 vigorous and undamaged *S. nigrescens* growing at site 2 and enclosed groups of six plants within cylindrical steel netting enclosures (100cm diameter × 100cm high, mesh size 0.1 mm × 0.1 mm). When flower buds were at the stage suitable for tephritid oviposition, all but one randomly chosen capitulum on each plant were removed. One, two or three *Tephritis femoralis* (Tephritidae) larvae (about 2mm in length) were then inserted into the remaining capitula. The control group was physically manipulated in the same way as the other groups, but no larvae were inserted. The larvae were obtained as described by Xi et al. (2016). The netting remained in place until the capitula reached peak nectar
production, when floret nectar volumes and concentrations were recorded as before. A total of 120 capitula were selected to monitor nectar volume and concentration.

After nectar production had been monitored, each plant was removed and the lengths and fresh weights of capitula, leaves, stems and roots were weighed in the field using a 0.001g capacity balance. The plant components were then stored immediately in a portable icebox cooled with dry ice and their soluble sugar and starch contents were measured later following the procedures of Hansen et al. (1975) and Yoshida et al. (1976). The relative amounts of carbohydrates (soluble sugars and starch, mg/g) in the capitula, leaves, stems and roots were expressed as a percentage of the total carbohydrates in each plant. Carbohydrate contents were calculated as in Rivera-Solís et al. (2012). A total of 120 capitula were selected to monitor the carbohydrate contents.

Honeybee responses to experimental manipulations

Twelve 2 × 2m × 1m high exclosures covered in the steel netting described above were erected at site 2 to enclose groups of S. nigrescens with recently-developed flower buds. Once they reached the stage suitable for tephritid oviposition 30 vigorous, undamaged plants in each plot had all but one of their capitula removed and zero, one, two or three tephritid larvae were inserted as before. Any additional plants in the enclosures had all their capitula removed, leaving a consistent 30 capitula within each 4 m² plot. To monitor honeybee visitation, we first removed the netting
screens. Six operatives then simultaneously recorded honeybee visits to individual capitula in pairs of adjacent plots every hour (each plot was observed for 30 minutes per hour) at 9:00 - 17:00 on three sunny days in July 2015. Observers were located about 3m from each plot, which allowed for clear observation of pollinator behavior. Honeybee visitation rates to each capitulum per hour were calculated following the protocol of Arroyo et al. (1985). We then harvested the plants and measured fresh weights as before. The plant parts were then dried to constant mass and re-weighed to the nearest 0.001g.

Statistical analyses

All statistical analyses were performed in R (R Development Core Team 2013, URL http://www.R-project.org). Plant traits were first tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene’s test. Floret nectar volume was log_{10}-transformed and the proportion of flowering plants per subplot were arcsine-transformed to achieve normality. Correlation analyses were used to determine the relationship between floret nectar volume and capitula carbohydrate contents. Pollinator visitation rates were assessed using one-way ANOVA followed by Tukey’s test.

We used general linear-mixed models (GLMMs) to assess the effects of pre-dispersal seed predator numbers and capitula mass on variation in floret nectar volume and concentration, capitulum carbohydrates and total carbohydrates storage as percentages.
of dry and wet weights. To account for interaction effects between pre-dispersal seed predator and capitulum size, we used a model with fly numbers and capitulum mass as fixed factors, and site and above-ground vegetative mass as random factors. For the mixed models, we used the *lme* function in the *lme4* package in R (Bates et al. 2011).

**Results**

*Saussurea nigrescens* colonized naturally by tephritid larvae produced different volumes of nectar depending on how many larvae were present, but there was no simple relationship between numbers of these seed predators and nectar volume (Fig. 1, Table 1, Appendix A). Capitula containing a single tephritid larva produced more nectar than controls, whereas capitula containing two or three larvae produced significantly less nectar. The presence of one larva increase nectar volumes by an average of 15.1%, but two or three larvae led to decreases of 76.9% and 83.1% respectively (Fig. 1). In contrast to nectar volume, the tephritids had no effect on nectar concentration (Appendix A). Very similar results were obtained when tephritid numbers were manipulated experimentally (Fig. 1, Table 1). With one larva added, nectar increased by 17.0% relative to controls, and when two or three larvae were added nectar volumes fell by 77.8% and 79.4% respectively. Nectar concentrations were again unchanged (Appendix A).

The starch and soluble sugar contents of *S. nigrescens* capitula showed the same response to tephritid larvae as nectar volume, with elevated carbohydrates compared with controls in capitula containing one larva and significantly lower concentrations
of carbohydrates when two or three larvae were present (Fig. 2). Across treatments there was a significant positive correlation between capitulum carbohydrate concentrations and average nectar volume, with indications that the same pattern was also present within capitula that contained one larva (Fig. 3). The changes in capitulum carbohydrate concentrations in response to the tephritids reflected changes in the relative distributions of carbohydrates within the plants (Fig. 4). A single larva was associated with an increase in the percentage of the plant’s total carbohydrates stored within the capitula, relative to controls, but two or three larvae were associated with declines in the capitula carbohydrates (Fig. 4; \( t = -2.44, P < 0.05 \)). Corresponding changes were present in the proportion of carbohydrates stored in the stems, with declines when one larva was present, and increases when there were more larvae (Fig. 4, \( t = 1.99, P < 0.05 \)). There were no treatment effects on the proportion of the plants’ carbohydrates in the leaves and roots (Fig. 4, \( t = -0.56, P = 0.58 \) and \( t = 0.86, P = 0.39 \), respectively).

Despite the changes in nectar volumes associated with the tephritid larvae, they had no significant impact on the visitation rates of honeybees to the capitula (\( F = 0.903, P = 0.388 \), Appendix B).

**Discussion**

We hypothesized that pre-dispersal seed predators would reduce floret nectar production and nectar concentrations in proportion to their densities, and that this
would lead to a decline in pollinator visitation rates. These hypotheses were not supported by the data from both field records and experimental manipulations. Floret nectar volumes did decline in capitula that contained two or three tephritid larvae, but volumes increased significantly in capitula with one larva. Furthermore, nectar concentrations were consistently independent of seed predator numbers, as were visitation rates by honeybees. Although carbohydrate storage in the capitula was influenced by the presence of the tephritid larvae, it was a non-linear response that mirrored nectar production, with capitula containing a single larva having a higher proportion of the plant’s carbohydrates than controls with no seed predators.

Rates of nectar production and secretion are related to the quantities of soluble sugars and starch available and thereby to carbohydrate reserves and rates of photosynthesis (Búrquez and Corbet 1991; Pacini et al. 2003). Physical conditions such as elevated night-time temperatures, and damage to leaves and flowers can all reduce nectar production (Wäckers et al. 2001; Mu et al. 2015). In our study system the positive relationship between local carbohydrate storage and floret nectar volume was particularly clear, because they increased and decreased together in response to varying densities of tephritid larvae. Relative to un-occupied capitula, carbohydrate storage increased by 19.4% and floret nectar volumes increase by 17.0% in capitula with one larva, but were reduced by 30.3% and 32.6% respectively when more larvae were present. Carbohydrate storage elsewhere on the plants reflected these changes.
The non-linear carbohydrate storage and nectar volume responses by the plants to the presence of insects feeding in their capitula can be interpreted in terms of adaptive responses by the plants in the face of varying levels of damage, but also as reflecting manipulations by the insects. The increase in capitula carbohydrate storage and nectar volumes when a single larva is present could be a plant compensation effect (Rivera-Solís et al. 2012). Plants attacked by herbivores often allocate more resources to their reproductive organs (reviewed by Trumble et al. 1993), but increased allocation to reproduction, including elevation of nectar quality, is most evident when vegetative structures are damaged (Inouye 1982; Lanza 1988; Smith et al. 1990).

An alternative explanation for the increase in nectar volumes produced by capitula occupied by a single tephritid larva is that the insects are actively inducing this effect, and that increased nectar volumes favour their own reproductive success, rather than that of their host plants. The tephritid larvae feed on developing seeds, and depend on capitula being visited by pollinators after their eggs have been laid. Any increase in the likelihood of those capitula being adequately pollinated, such as might be the case by increasing nectar volumes, would therefore be to the advantage of the insect. Many of the species of tephritids with larvae that develop in the capitula of Asteraceae are gall formers (Varley 1947; Straw 1989). This includes congeners of the Tephritis species from S. nigrescens (Goeden et al. 1988). Gall forming insects routinely generate nutrient sinks that draw in resources from elsewhere on their host.
plants (Price et al. 1987). Although *T. femoralis* does not generate clearly-defined
galls in the capitula of *S. nigrescens*, its larvae may be capable of initiating some of
the plant physiological effects associated with galling, including stimulation of local
carbohydrate storage. This may have direct beneficial effects in terms of food quality
for developing larvae, and indirect benefits via larger nectar volumes leading to
increased numbers of seed in the capitula it occupies.

Quite different carbohydrate storage and nectar volume responses were recorded
in capitula where more than one tephritid larva was present. The declines seen in
capitula containing multiple larvae are likely to reflect the damage they cause to the
receptacles, through which nutrients and metabolites are transported (Teuber et al.
1983; Rivera-Solís et al. 2012). Damage to the receptacles was more frequent and
much more extensive when two or more larvae were sharing a capitulum, with more
than 90% of the receptacles damaged in capitula with two or three larvae, compared
with only around 10% of the receptacles when only a single larva was present (J Mu
unpublished data). The increased feeding on the receptacles in capitula with several
larvae may be the result of antagonistic behavior among the fly larvae and a shortage
of seeds remaining to be eaten (Averill and Prokopy 1987).

Seed predators are expected to preferentially oviposit on flowers that are more
attractive to pollinators, because these flowers are most likely to set seed (Strauss and
Irwin 2004). Competition is often intense among seed predators sharing what are
usually finite and spatially-limited resources. Only 15% of the *S. nigrescens* capitula at our study site that contained tephritid larvae had more than one larva present (X XI unpublished data), suggesting that females typically lay a single egg on each plant. This oviposition pattern not only reduces the likelihood of competition, but also means that most of the larvae are developing in capitula that are producing more nectar than adjacent capitula that lack the insects.

Contrary to expectations that the insects would gain from developing in capitula with higher nectar volumes, capitula with one larva present did not attract more honeybees than unoccupied capitula. This result was contrary to much of the literature, which suggests that floral nectar volumes have a positive relationship with pollinator visitation (Schemske and Bradshaw 1999; Pyke 1982, 2016). Different bee species may respond differently to aspects of nectar rewards, such as nectar volume and concentration. For example, honeybees have been shown to prefer flowers that have high sugar concentrations (Scheiner et al. 1999, 2001; Vaudo et al. 2015), whereas bumblebees are sensitive to nectar volume (Harder and Real 1987). In this study, we found no changes in nectar concentration (sugar content) resulting from the pre-dispersal seed predators, and this may have led to the similar honeybee visitation rates. We did not examine visitation rates by native bees such as bumblebees and they may be more responsive to nectar volumes than honeybees. Our future investigations will examine whether pre-dispersal seed predators have different effects on the behavior of honeybees and native bees.
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Figure 1. Variation in *Saussurea nigrescens* nectar volumes in relation to the numbers of tephritid larvae in their capitula (Means ± SEs per floret). Figures 1A-1C plants growing at sites 1-3 with natural variation in larval numbers, Figure 1D experimentally introduced tephritid larvae (site 2 only). Different letters above columns indicate differences within sites at $P<0.05$ (one-way analysis of variance).
Figure 2. Capitulum carbohydrate concentrations of *S. nigrescens* that had contained varying numbers of tephritid larvae (Means ± 1 SE). Different letters above columns indicate differences at $P<0.05$ (one-way analysis of variance).
Figure 3. The relationship between *Saussurea nigrescens* capitulum carbohydrate concentrations and mean floret nectar volume in capitula where different numbers of tephritid larvae had been introduced. N = 30 florets for each larval density.
Figure 4. Percentage of total carbohydrates (soluble sugars and starch, mg/g) in capitula, leaves, stems and roots of *S. nigrescens* (means ± 1 SE). Different letters above columns indicate differences between parts of the plants at \( P<0.05 \) (one-way analysis of variance).
Table 1. Summary of linear mixed effect models comparing the effects of pre-dispersal seed predators and capitulum size on floret nectar volume, nectar concentration, and capitulum carbohydrate concentrations. AIC = Akaike Information Criterion and BIC = Bayesian Information Criterion. The numbers after Floret nectar volume and Nectar concentration are AIC and BIC, respectively.

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Appendix list

Appendix A

*S. nigrescens* nectar concentrations at sites 1 (A), 2 (B), and 3 (C) under natural condition and after experimental introductions (D).

Appendix B

*Apis mellifera* visitation rates per capitulum of *Saussurea nigrescens* in relation to tephritid larval numbers.
Appendix A

*S. nigrescens* nectar concentrations with natural variation in numbers of tephritid larvae at sites 1-3 (A-C) and in the larval-addition experiment at site 2 (D). Similar letters above columns indicate no differences within sites at $P<0.05$ (one-way analysis of variance).
Appendix B

Apis mellifera visits to capitula of Saussurea nigrescens that contained different numbers of tephritid larvae (means ± 1 SE). Capitulum visitation rates were compared by one-way ANOVAs followed by Tukey's tests. Similar letters above columns indicate no difference in visitation rates at $P<0.05$ (one-way analysis of variance).