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Plant Secondary Metabolites in Nectar: Impacts on Pollinators and Ecological Functions

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Summary

1. The ecological function of secondary metabolites in plant defence against herbivores is well established but their role in plant-pollinator interactions is less obvious. Nectar is the major floral reward for pollinators, so the occurrence of defence compounds in the nectar of many species is unexpected. However, increasing evidence supports a variety of potential benefits for both plant and pollinator from these components.

2. Secondary metabolites in nectar can be toxic or repellent to flower visitors, but they can also make nectar attractive or go undetected. For example, caffeine in nectar improves pollinator memory for cues associated with food rewards and enhances pollen transfer. Nectar secondary metabolites alter microbial communities in nectar and reduce parasite loads in bees. All of these effects depend on the concentration of nectar metabolites so should be evaluated experimentally at a range of ecologically relevant doses.
3. The emergence of evidence for nectar chemicals that tip the benefits of plant-pollinator mutualisms in favour of the plant or pollinator has accelerated over the past 15 years. Beneficial effects include the following: a) increasing specialization in plant-pollinator interactions, b) protecting nectar from robbery or larceny, and c) preservation of nutrients in nectar and reducing disease levels in flower visitors.
4. This review synthesises evidence from recent literature that supports selection for secondary metabolites in floral nectar as an adaptation that drives the co-evolution between plants and their pollinators. However, heir presence in nectar could simply be a consequence of their occurrence elsewhere in the plant for defence (pleiotropy). Among other knowledge gaps on nectar chemistry we draw attention to a need for evidence demonstrating benefits to the plant, greater consideration of the importance of levels of exposure and broadening target species beyond the current emphasis on alkaloids in bee pollinated species.

1. Toxic nectar: adaptive function or pleiotropy

Plants produce secondary metabolites that accumulate in plant tissues for a variety of functions but primarily as a means of defence against herbivores, fungi, and bacteria and as plant signals

46 (Schoonhoven et al. 2005). The term secondary metabolite describes natural chemicals produced
47 by plants, fungi and other organisms that are not used in primary metabolic pathways (Pichersky
48 and Gang 2000). However, their roles in other plant functions are specific, variable and
49 numerous, with perhaps 100,000 or more structures likely (Verpoorte 2000). Typically
50 characterised as low molecular weight organic compounds with great structural diversity, they
51 often have restricted distribution to just a few species or genera, suggesting specific adaptations
52 to particular functions. Their importance as regulated defence systems in their interactions with
53 insect herbivores, especially as toxins or repellents, has been well established through decades of
54 research (Fraenkel 1959; Whittaker and Feeny, 1971; Berenbaum,1995; Agrawal and Weber
55 2015).

56 Optimal defence theory predicts a correlation between the value of tissue and the level of
57 defence such that the distribution of defensive chemicals within a plant may be restricted to
58 critical tissues (McCall and Fordyce, 2010; Cook et al., 2013). However, secondary metabolites
59 including those that are toxic or repellent also occur in floral nectar where their role is less
60 obvious because nectar is a reward for pollinating animals (Table 1) (Pacini and Nepi 2007;
61 Detzel and Wink 1993; Manson et al. 2013; Tiedeken et al. 2016; Irwin et al. 2014). It is possible
62 that their occurrence in nectar is regulated for ecological functions: to enhance pollination
63 service or protect the flower and/or pollinator rewards (Table 1) (Adler 2001; Irwin et al., 2014;
64 Manson et al. 2012).

65

66 Alternatively, defence compounds are under selection by plant antagonists and may occur in
67 nectar during nectar production (Adler, 2001). Selection in this case might favour plants that
68 keep secondary metabolites out of nectar; recent work suggests their concentrations in nectar are

lower than in other plant parts (Cook et al., 2013). At present, there is little evidence supporting the idea that the presence of secondary metabolites in nectar has co-evolved via interaction with pollinators. Instead, it is more parsimonious to suppose that adaptive functions may arise after plants have been selected for the production of toxins as defences against plant antagonists. Rather than imposing selection for the production of novel secondary metabolites, pollinators are more likely to impose selection on the concentrations of defensive metabolites that wind up in nectar and pollen. Pollinators impose selection pressure on plants, especially on floral traits, so it is reasonable to expect that pollination and chemical defence may not necessarily have evolved independently in all cases (Campbell et al. 2015; Adler 2001). For example, outcrossing species of *Nicotiana* sp. produce lower levels of nicotine in nectar, flower and leaf tissue than self-compatible species, suggesting that selection against flower toxins also affects the use of this compound as a defence. In this case, selection by mutualists for nicotine-free nectar outweighs selection for nicotine-laced leaves by antagonists (Adler et al. 2012).

Pollination in most angiosperms requires the services of pollinators for which they are typically rewarded (Kevan and Baker 1983; Raguso and Willis 2005). Attraction and fidelity to a particular plant species, however, is enhanced by the co-occurrence of nectar and floral traits such as odours (Wright and Schiestl 2009; Kessler et al. 2015a). So, it is conceivable that non-volatile nectar secondary metabolites might also act as attractants and cues for pollinators or enhance pollination behaviours (Couvillon et al. 2015).

We propose a revised framework that categorises effects on pollinators and possible ecological roles of secondary metabolites in nectar around broad biological activities and functions, particularly in the light of recent research. After discussing the occurrence of secondary metabolites in nectar, we review research on their effects under two broad themes: (1) impact on

92 the behaviour of pollinators, which has consequences for pollinator specialization and filtering
93 (including protection against nectar robbery or larceny); and (2) antimicrobial activities that may
94 maintain nectar quality or ameliorate diseases. We also consider how pollinators cope with toxic
95 secondary compounds after ingestion. Finally, we identify areas of focus for future research.

96

97 **2. Occurrence of secondary metabolites in nectar**

98 How secondary metabolites arrive in nectar is unclear (Heil 2011). They could be transported
99 from phloem or xylem through nectary cells in a similar way to carbohydrates. In buckwheat,
100 trichomes in nectary glands secrete sugars into nectaries from phloem via nectary parenchyma
101 (Cawoy et al. 2008). Secondary metabolites biosynthesised elsewhere in the plant could be
102 secreted into nectar in a similar way. In irises, Lohaus & Schwerdtfeger (2014) found the same
103 iridoid glycosides in the nectar and phloem sap of two different species, suggesting that iridoids
104 may indeed leak passively into nectar. Furthermore, the nectar, anthers, corollas, stems and
105 pollen of *Delphinium sp*. contain similar alkaloids differing only in their concentration,
106 suggesting a similar origin (Cook et al. 2013). More recently, Anton and Kaminska (2015) have
107 proposed two mechanisms in Ranunculaceae, after microscopic examination of nectaries. Nectar
108 in *Consolida regalis* and *Delphinium elatum* is exuded through micro-channels in the nectary
109 cuticle, whereas in *Aconitum lycoctonum* and *Aquilegia vulgaris* nectar results from rupturing of
110 nectary cell walls and the release of the entire cytoplasmic content of the cell into the nectary
111 cavity. This may explain instances where the corolla and nectar chemistry are similar, as
112 reported by Lohaus and Schwerdtfeger (2014) and Cook et al. (2013). While there are few
113 examples of studies that have addressed this issue, it is clear that the phloem and xylem
114 contribution to nectar varies across taxa (Nepi 2007) so the source of secondary metabolites

115 ultimately found in nectar is also likely to vary across plant species. Until further work on this
116 subject is done, we will not know how phloem and nectar are related and what mechanisms exist
117 for transport of metabolites into nectar or their exclusion from it (Pate et al. 1985).
118
119 The assumption that the content of nectar arises directly from phloem may, however, be too
120 simple (Orona-Tamayo et al. (2013).. Studies where differences in the chemistry of nectar,
121 pollen and floral parts have been found imply that plants can regulate these compounds in
122 specific tissues (Irwin et al. 2014; Manson et al. 2012). This may not be surprising since
123 examples of tissue specific accumulation of defensive secondary metabolites are known (McCall
124 and Fordyce 2010). However, Adler et al. (2006) reported that herbivory by a moth caterpillar
125 increased concentrations of the defence compound, anabasine, in the nectar but not in the leaves.
126 This could be a result of tissue specific regulation in roots and transport to nectar via phloem that
127 does not impact anabasine expression in leaves. Adler et al. (2012) reported that nicotine
128 concentrations in nectar and other tissues of *Nicotiana* species were correlated but lower across
129 all plant parts in outcrossing species. However, in *N. africanum*, the concentrations of
130 nornicotine in leaves did not predict the concentrations in nectar where nicotine and its
131 derivatives were not recorded (Marlin et al. 2014). On the other hand, exclusion of compounds
132 from phloem is also reported and illustrated by the limonoids found in *Citrus.* Nomilin is
133 biosynthesised in the phloem region of stem tissues (Ou et al. 1988) and then translocated to
134 leaves, fruit and seeds, where it is further modified (Hasegawa et al., 1986). In contrast,
135 limonoids are not found in *Citrus* nectar (Wright et al. 2013; Fig 1). Furthermore, Wright et al.
136 (2013) report that in nectar of *Coffea* species caffeine occurs as the only secondary metabolite
137 whereas numerous other compounds occur in other floral tissues (Fig 2). In another example, the

138 corollas and other flower parts of *Rhododendron ponticum* contain a variety of compound classes
139 (Egan 2015) whereas the nectar contains primarily grayanotoxins (Egan et al. 2016). While there
140 is some evidence supporting nectar specific regulation much more work is needed in this area.
141
142 Variability in the presence and concentration of nectar secondary metabolites is also an
143 important but often overlooked parameter. Nectar secondary metabolites vary across time,
144 space, phenotype and climate and even within a plant (e.g. Kessler et al. 2012; Irwin et al. 2014;
145 Cook et al. 2013). Ecological explanations concerning nectar compounds may be less certain
146 where the expression of the chemicals is so variable. Kaczorowski et al. (2014) measured nectar
147 alkaloids of *N. glauca*, finding that the average concentrations of anabasine and nicotine were
148 two orders of magnitude lower than in a previous study (Tadmor-Melamed et al. 2004). Kessler
149 et al. (2012) also reported high variability in nicotine concentrations in a species of *Nicotiana* but
150 provided evidence that variation in nectar nicotine was itself the underlying cause of the
151 pollinator behaviour modifying effect. High variation in caffeine concentrations was reported in
152 *Coffea* and *Citrus* flower nectars but experimental protocols covered all ecologically relevant
153 concentrations and natural quantities were always within the concentration range for behaviour
154 modifying effects on honeybees (Wright et al. 2013). Some studies investigate effects of only a
155 single concentration which could provide misleading outcomes depending on how ecologically
156 relevant that concentration is. Studies on dose-response relationships, such as that of Manson et
157 al. (2013), would provide more robust evidence for effects or a lack thereof.
158
159 Future studies need to focus on chemistry of the whole plant, particularly considering how nectar
160 chemistry may be influenced by herbivory (e.g., Adler et al., 2012). A better understanding of

161 spatial and temporal variation of nectar secondary metabolites could help inform mechanisms of
162 selection on this trait (Egan et al., 2016).
163

164 ## 3. Nectar chemicals mediating behaviour of pollinators

165 The impact on pollinator behaviour is likely to be the main source of selection against the
166 occurrence of secondary metabolites in nectar. Pollinatorslearn to find flowers with high quality
167 or abundant nectar, and they can also learn to avoid visiting flowers with nectar containing toxic
168 secondary compounds (Gegear et al. 2007; Wright et al. 2010). Their mechanisms for doing this
169 include association of floral traits with the taste of secondary metabolites in nectar or with the
170 post-ingestive consequences of accidentally ingesting such compounds if they are toxic (Wright
171 et al. 2010).

172 The best examples of pollinators learning to reject flowers with the taste of toxins are from bees.
173 For example, bumblebees (*Bombus impatiens* and *B. terrestris*) given a choice of sucrose
174 solution associated with a yellow flower or a blue flower associated with sucrose solution
175 containing the alkaloids gelsemine or quinine, choose the yellow flowers (Gegear et al. 2007;
176 Avargues-Weber et al. 2010). In a proboscis extension assay for associative learning, restrained
177 honeybees trained to associate a food reward with an odour learn to avoid extending their
178 proboscis to the odour when the sucrose reward contains quinine (Wright et al. 2010). Likewise,
179 free-flying honeybees learn to avoid taking food from feeders treated with high concentrations of
180 alkaloids or other toxins (Singaravelen et al. 2005). Moths (*Heliothis virescens*) can also learn to
181 avoid odours associated with quinine in food (Jorgensen et al. 2007).

When a pollinator visits a flower, its proboscis is often the first body part to contact nectar. Insect pollinators detect chemical compounds using contact chemosensilla that house neurons that respond to sugars, salts, acids, water and to non-nutrient compounds (Inoue et al. 2009; Omura et al. 2008; Wright et al. 2010). When neurons in this location detect toxic or bitter substances such such as quinine, this leads bees to reject food (Wright et al., 2010). Rejection of foods containing alkaloids is clearly seen when the feeding behaviour itself is assayed; restrained honeybees retract the proboscis when quinine laced sucrose is placed at its tip (Wright et al. 2010). This response depends on the toxin, the pollinator species, and on whether the animal is hungry (Wright et al. 2010). The responses to toxins in nectar are attenuated by the concentration of sugars; solutions high in carbohydrates are less likely to be rejected even when toxins are present (Gegear et al. 2007, Köhler et al. 2012, Lerch-Henning & Nicolson 2013).

Secondary metabolites can also be phagostimulatory to insect pollinators, but this seems to be limited to insects that specialise on feeding on toxic plants as larvae. Adult hawkmoths (*Manduca sexta*) find low concentrations of caffeine or lobelline phagostimulatory when they are presented to the mouthparts (Reiter et al. 2015). Other lepidopteran adults that specialise on consuming plants with highly toxic alkaloids, such as the danaid butterfly, *Euploea mulciber*, also find alkaloids from their host plants phagostimulatory (Honda et al. 2006).

Toxicity of secondary compounds is typically a function of their concentration but it is important to note that they may not always be detected even at toxic levels. An example is the cyanogenic glycoside amygdalin, found in the nectar of almond flowers (London-Shafir et al. 2003). Honeybees do not detect this compound in sucrose solutions, and in fact will drink concentrations high enough to kill them (Wright et al. 2010; Sanchez et al., 2010). Another study of honeybees found that they unwittingly share solutions containing pyrrolizidine alkaloids with

other members of their colony via trophallaxis (Reinhard et al. 2009). Detzel and Wink (1993) found that there was no relationship between the ability of bees to detect compounds and their lethality, but some compounds, like alkaloids, had lower thresholds of detection and were also more lethal than others (e.g. glycosides). The threshold for detection of most of these compounds was between 100-1000 ppm. Bumblebees are also reported to have a low detection threshold for some secondary metabolites in sucrose solutions, including cardenolides or cardiac glycosides (Manson et al. 2012), diterpenoids (Tiedeken et al. 2014), alkaloids (Baracchi et al. 2015), and even pesticides present in nectar (Kessler et al. 2015b). All these studies illustrate that bees encountering secondary metabolites in nectar may be exposed to potential harm that could have consequences for individuals or colonies.

In situations where insect pollinators have difficulty initially detecting secondary metabolites in nectar, they may learn to associate floral cues such as odours with the post-ingestive consequences of consuming toxins (Wright et al. 2010). This form of learning takes time, however, and requires bees to forage repeatedly to experience the same cues in association with nectar containing the toxin. In circumstances where bees are trained to associate one odour with a sucrose solution and another odour with sucrose containing a toxic secondary metabolite (e.g. amygdalin), they will generalise the symptoms of malaise caused by ingesting the toxin to both odours (Wright et al. 2010).

The consequence of nectar being repellent or having negative post-ingestive consequence for the pollinator would also be negative for a plant species particularly if pollinators learned to avoid its flowers. This effect would likely be selected against so where present nectar compounds are likely to be at concentrations that are undetectable (Tiedeken et al., 2014) or do not have immediate negative consequences for pollinators. They might also play other roles in plant-

228 pollinator interactions that benefit pollinators and the plant. Grayanotoxin I, for example, may
229 benefit bumblebees by reducing the competition from other pollinating species that are
230 intoxicated or repelled by grayanotoxins in *Rhododendron ponticum* nectar and enhance
231 polloantion efficiency of the host (Tiedeken et al. 2016).

232 Alkaloids provide examples of secondary metabolites that may optimise pollination service
233 although they may not necessarily benefit the pollinators. Caffeine can act as a drug that affects
234 the insect nervous system to alter behaviour (Wright et al. 2013). When honeybees consume
235 nectar-relevant doses of caffeine in a sucrose solution during olfactory learning, they are more
236 apt to remember the odour associated with reward than when given sucrose alone (Wright et al.
237 2013). Caffeine in food also affects the fidelity and persistence of bees returning to food sources
238 containing the compound (Couvillon et al. 2015; Thomson et al. 2015). However, they may
239 continue to return to the source of caffeinated food after the food has been removed (Couvillon et
240 al. 2015) suggesting memory of the location of food remains strong, potentially to the
241 disadvantage of the pollinator.

242 Nicotine could also amplify the rewarding properties of nectar because it is an agonist of
243 nicotinergic acetylcholine receptors (nAChRs) involved in fast neurotransmission between
244 neurons. In particular, nAChRs are located in dopamine neurons governing reward in the
245 vertebrate (Hyman et al. 2006) and insect brain (Barnstedt et al., 2016). Indeed, several studies
246 have shown that nicotine and nicotine-like compounds have pharmacological effects on
247 pollinator behaviour. Free-flying honeybees and bumblebees show preferences for sucrose
248 solutions containing concentrations of nicotine <15 μM (Singaravelen et al. 2005; Baracchi et al.
249 2015), but they can detect and are repelled by concentrations greater than this (Singaravelen et
250 al. 2005, Köhler et al. 2012, Tiedeken et al., 2014).

In summary, Paracelsus' proclamation that "Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy" highlights how important levels of exposure are to the effects of secondary metabolites on pollinators. Caffeine in floral nectar is a good example of this. One study showed that free-flying honeybees choose to consume sucrose solutions containing caffeine only when the concentration is ~0.1 mM or less (Singaravelen et al. 2005). Subsequent experiments using proboscis extension assays showed that honeybees can detect concentrations of caffeine greater than 10 mM (Mustard et al. 2012; Wright et al. 2013). Interestingly, the amount of caffeine found in the floral nectar of *Coffea* and *Citrus* species is on average less than 0.3 mM (Wright et al. 2013). Thus, the responses to secondary metabolites in nectar are highly dependent on the concentrations (Manson et al. 2013) and depend on the pollinator species (Tiedeken et al. 2016). Broad generalizations about the biological activities of plant compounds on large taxonomic groups may, therefore, be inaccurate (Baker & Baker 1975; Rhoades & Bergdhal, 1981).

4. Role of secondary metabolites in maintaining nectar quality and ameliorating bee diseases

Besides their pharmacological effects on the brain and other pollination enhancing effects, secondary metabolites in nectar may provide other benefits to pollinators . Effects on pollinator performance could also include biotic interactions such as plant-pollinator-microbe interactions (Adler, 2001; Forbey & Hunter 2012). Secondary metabolites may prevent spoilage of nectar by microbes (an indirect benefit through the maintenance of nectar quality) or may reduce the impact of pathogens (a direct benefit for pollinator health). However, the evidence so far is not strong for either of these.

Nectar is a rich medium for microbial growth, and is easily contaminated with yeasts and bacteria, transferred on the bodies of bees and other floral visitors (Herrera et al. 2009; Fridman et al. 2011). Microbial enzymes hydrolyze sucrose and may also preferentially metabolise glucose or fructose, leading to imbalance in the carbohydrate ratio and reduced carbohydrate reward (Herrera et al. 2008; Vannette et al. 2013). Microbial contamination also alters amino acid composition (Peay et al. 2012). These changes in nectar chemistry may affect pollinator attraction and ultimately plant fitness (see for example Schaeffer and Irwin 2014). However, these possible effects are complicated by the presence of secondary metabolites in nectar, which are expected to make it a less hospitable environment for microbes. Possible antimicrobial effects were tested by Fridman et al. (2011) who examined the nectars of three plant species *Amygdalus communis*, *Citrus paradisi* and *Nicotiana glauca*. Although these three species showed distinct nectar bacterial communities there was no effect of added amygdalin, caffeine and nicotine on the growth of bacterial isolates. The yeast *Candida gelsemii* was isolated from the toxic nectar of *Gelsemium sempervirens* which contains the alkaloid gelsemine (Manson et al. 2007). Recently, Vanette & Fukami (2016) tested the effects of five compounds (catalpol, aucubin, caffeine, nicotine and ouabain) in synthetic nectar and found that microbes reduced the concentration of some compounds in nectar. Pyridine alkaloids in *Nicotiana* nectar affect the richness and composition of its bacterial communities, with nicotine having the strongest antimicrobial effect (Aizenberg-Gershtein et al. 2015). Interactions with microbes add a new level of complexity to the potential ecological functions of secondary metabolites in nectar.

296 There are other defence chemicals in nectar with protective functions (Heil 2011; Seo et al.
297 2013). Nectar proteins or nectarins in the nectar of ornamental tobacco plants protect plant tissue
298 from invasion by pathogens (Carter & Thornburg 2004). These antimicrobial enzymes produce
299 strong oxidants, such as hydrogen peroxide at levels up to 4 mM, via the nectar redox cycle
300 (Park & Thornburg 2009). Seo et al. (2013) recently characterised the nectar proteome of
301 *Nicotiana attenuata*, showing natural variation across the plant's native habitat. Other defence-
302 related proteins, including a lectin, have been identified in leek nectar (Peumans et al. 1997).
303 Non-protein amino acids are also widespread in nectar and their functions are generally
304 unknown, but they could play a similar role (Nepi, 2014). The extrafloral nectar of *Acacia*
305 species, especially when ant protection is involved, contains an assortment of proteins with
306 antimicrobial activity (Gonzalez-Teuber et al. 2009). Most work in this area is limited to tobacco
307 plants but a broader study of nectar proteins could reveal enzyme activities that influence the
308 ecological function of nectar for pollinators and even regulate chemical content through local
309 biosynthetic modifications.
310
311 There is some evidence that secondary metabolites in nectar could benefit pollinators by
312 increasing their resistance to parasite and pathogen infection. Almost all of these studies have
313 involved bumblebees infected with *Crithidia bombi*, a trypanosomatid gut parasite. Manson et al
314 (2010) assessed the effect on this interaction of gelsemine from *G. sempervirens*: consumption of
315 gelsemine in artificial nectar by *Bombus impatiens* reduced pathogen loads after infection, but
316 pre-exposure of the pathogen to gelsemine did not significantly reduce infection. In contrast,
317 grayanotoxins did not protect *B. terrestris audax* against *C. bombi* (Tiedeken et al. 2016) and
318 nicotine had only weak effects against the same pathogen in the same host (Baracchi et al. 2015);

dietary nicotine did not clear the infection, and pre-exposure of the pathogen to nicotine did not affect its viability. Richardson et al. (2015) tested eight naturally occurring nectar chemicals, also in the *B. impatiens*-*Crithidia* system, of which half reduced the parasite load of bees that were inoculated and kept individually. Anabasine had the strongest effect but microcolonies provisioned with it did not respond to infection by increasing their consumption of this alkaloid. In a subsequent study (Richardson et al. 2016), *B. impatiens* infected with *C. bombi* foraged for longer at flowers of *Chelone glabra* with high iridoid glycoside concentrations in their nectar, resulting in benefits for plant reproduction.

The review by McArt et al. (2014) of floral traits and the transmission of plant and animal pathogens shows how much more is known on the plant pathology side. There is a need for studies that examine the role of nectar toxins in pathogen transmission and infection intensity in systems other than *Bombus*-*Crithidia*, particularly for emerging diseases and in diseases that are crossing between species and which could have serious impacts on pollinator health at landscape scales (Fuerst et al. 2014).

Activity of plant toxins against disease agents suggests the possibility of self-medication behaviour (de Roode et al. 2013): parasitised pollinators may consume more alkaloids or other toxins in nectar or pollen. There are rigorous criteria for establishing that a behaviour is a form of self-medication (Singer et al. 2009): it should improve the fitness of infected animals, whereas in the absence of infection it should decrease fitness, and infection should trigger this behaviour (also see de Roode et al. 2013). Even in herbivores, there are few studies that meet these criteria (Forbey & Hunter 2012). Among pollinators, Baracchi et al. (2015) looked for self-medication

342 behaviour in bumblebees parasitised with *C. bombi* and provided with nicotine, but found
343 contradictory results. In more general terms, collection of resins to make propolis contributes to
344 the 'social immunity' of honey bees and has been shown to decrease the investment of individual
345 bees in immune function (Simone et al. 2009). Colonies challenged with a fungal parasite
346 (chalkbrood) increased their resin foraging rates (Simone-Finstrom & Spivak 2012). It is likely
347 that pollinators achieve some level of protection by selecting a mixed pollen diet that includes
348 both the correct blend of nutrients and also secondary compounds that confer disease resistance.
349 This also applies to collecting diverse nectar sources: Erler et al. (2014) showed strong
350 antimicrobial activity of polyfloral honey compared to monofloral honeys. Erler and Moritz
351 (2015) point out the advantage of eusociality in enabling storage of antimicrobial substances in
352 floral resources for times when the appropriate plants are not in flower.
353
354 **5. Metabolic resistance to nectar toxins**
355
356 Animals have several biochemical and physiological mechanisms for dealing with xenobiotics
357 after ingestion (Foley & Moore 2005; Despres et al. 2007; Irwin et al. 2014). For some of these
358 potential mechanisms there is little information available for pollinators compared to herbivores
359 (Irwin et al. 2014). For example, we could find only one example of a pollinator sequestering
360 nectar toxins: the adult danaid butterflies that feed on milkweeds as larvae sequester
361 pyrrolizidine alkaloids from nectar (de Oliveira et al. 2015). Biotransformation processes used to
362 avoid self-poisoning by pyrrolizidine alkaloids are summarised by Hartmann (2004). Gosselin et
363 al. (2013) looked for possible sequestration of alkaloids from *Aconitum* (Ranunculaceae) in
364 tissues of bumblebees, but found only trace levels in the specialist species and none in the

365 generalist. Another area of ignorance is the modulation of absorption of secondary metabolites
366 by efflux transporters in the gut (Sorensen & Dearing 2006). The importance of this is only
367 beginning to be realised in studies of vertebrate herbivory. As far as insects are concerned,
368 Gaertner et al. (1998) provided evidence for a P-glycoprotein-like mechanism in nicotine
369 transport in the tobacco hornworm *Manduca sexta*.
370
371 The most important mechanisms behind the tolerance of animals to toxins involve metabolism
372 and elimination of these compounds. Excretion of toxic compounds frequently requires their
373 prior enzymatic conversion to less toxic and more water-soluble forms. Detoxification pathways
374 in honey bees, as in other animals, can be divided into phase I (functionalization, often involving
375 oxidation to render the toxin more soluble), phase II (conjugation to a carrier molecule) and
376 phase III (excretion): these are reviewed by Berenbaum & Johnson (2015). The most prominent
377 enzyme superfamilies that are responsible for the metabolism of toxins are the cytochrome P450
378 monooxygenases (P450s), glutathione transferases (GSTs) and carboxylesterases (Li et al. 2007).
379 Sequencing of the honey bee genome demonstrated the relative paucity of genes associated with
380 xenobiotic metabolism (Claudianos et al. 2006). Cytochrome P450 enzymes in honey bees are
381 induced by pesticide treatment (Johnson et al. 2012) and by constituents of honey (Mao et al.
382 2013): addition of p-coumaric acid (a component of pollen grains, found in honey) to a sucrose
383 diet enhanced the detoxification of coumaphos.
384
385 The molecular basis of nicotine detoxification has been investigated in adult and larval honey
386 bees (du Rand et al. 2015; du Rand 2015). Proteomic and metabolomic analysis showed active
387 detoxification of nicotine (three days exposure to 300 μM) in adults and larvae, associated with

388 increased energetic investment and antioxidant and general stress responses. In larvae, two P450s
389 were upregulated, and growth and development pathways were also affected. The metabolic fate
390 of nicotine in adults was explored using radiolabelled nicotine and LC-MS analysis to identify its
391 known catabolites (du Rand 2015). Unlike larvae of the tobacco specialist *Manduca sexta* in
392 which most ingested nicotine is rapidly excreted unmodified (Snyder et al. 1994), honey bees
393 convert nicotine into several phase I metabolites with 2'C-oxidation of nicotine being the main
394 catabolic pathway. Young workers in cages (or in the hive) do not defaecate and these nicotine-
395 derived metabolites accumulate in the rectum. The energetic costs associated with nicotine
396 detoxification in honey bees may well apply to other pollinators and the processing of other
397 xenobiotics. Nicotine is of special interest because of the structural similarity with synthetic
398 neonicotinoids, the most widely used insecticides worldwide. Cross-resistance to plant toxins
399 and insecticides (specifically nicotine and neonicotinoids) is apparent in nicotine-tolerant strains
400 of two insect pests: the peach-potato aphid *Myzus persicae* (Bass et al. 2013) and the tobacco
401 whitefly *Bemisia tabaci* (Kliot et al. 2014). On a broader scale, Despres et al. (2007) discuss the
402 ecological and evolutionary significance of such cross-resistance.
403
404 Pollinators may also have enzymes in saliva, the crop or the gut that break down secondary
405 metabolites. One would predict that conversion of nectar to honey by honeybees should lead to
406 increased concentrations of any secondary metabolites present provided the compounds do not
407 undergo natural degradation. Lectin in leek nectar is lost during processing into honey (Peumans
408 et al. 1997), as are phenolics in nectar of *Aloe littoralis* (Liu et al. 2007), amygdalin in almond
409 nectar (London-Shafir et al. 2003), and nicotine when included in artificial nectar for bees
410 (Singaravelan et al. 2006). The mechanisms are unknown, but a simple explanation is that these

411 compounds have been metabolised or are labile in these conditions. To verify this, researchers
412 should check for breakdown products in honey as well as the original compounds. Whether this
413 detoxification occurs in the bees or the hive environment (Naef et al. 2004; Liu et al. 2007) is not
414 clear. In a recent review, Berenbaum & Johnson (2015) use the term 'social detoxification' for
415 various honey bee behaviours that may reduce the need for enzymatic detoxification. These
416 include collecting from diverse pollen and nectar sources to dilute particular toxins, and
417 subsequent food processing in the colony, where the production of honey and bee bread may lead
418 to degradation of toxins.
419

420 **6. Future directions.**

421 Pollen was outside the scope of this review largely because the ecological function or detrimental
422 effects of pollen secondary metabolites have been overlooked save for a few examples (Arnold et
423 al. 2014). This is despite evidence suggesting potentially important negative ecological
424 consequences for pollinators consuming toxic pollen (Haider et al. 2013). Concentrations of
425 potential toxins are often higher in pollen than nectar (London-Shafir et al. 2003, Cook et al.
426 2013; Irwin et al. 2014); therefore, since the biological effects of secondary metabolites are
427 typically dose dependent, the detrimental effects of pollen toxins on pollinators could be more
428 severe than those of nectar toxins. The protection of pollen makes sense since it is the male
429 gamete. Because pollen is also the primary source of protein and other nutrients for many
430 pollinating insects but especially bees and their larvae (Michener 2007), its toxicity could impose
431 colony level effects as suggested by Arnold et al. (2014). Oligolectic species may specialise on
432 pollen of a few species or genera while other species are generalists (polylectic) (Cane and Sipes
433 2006) but the role of plant chemicals in pollen in mediating these behaviours is still unclear and

434 needs attention. Ultimately pollen is a source of large quantities of plant secondary metabolites
435 for pollinators and any long term colony or population benefits such as in ameliorating disease or
436 toxic effects may be as important as those reported for nectar secondary metabolites.

437 Most new studies on the secondary metabolites encountered by pollinators in floral rewards have
438 concerned nectar and consequently this has been the focus of the present paper, particularly the
439 evidence that supports adaptive functions for these metabolites in nectar. Most research to date
440 is focused on alkaloids of bee-pollinated species (Elliott et al. 2008; Koehler et al. 2012; Cook et
441 al. 2013; Gosselin et al. 2013; Irwin et al. 2014; Tiedeken et al. 2014) which could be because
442 these compounds are used by humans (Ott 1998). High profile plant species with well
443 documented toxicities to humans make compelling targets for study but may not necessarily be
444 the most informative examples. Less than 10% of plant nectars studied contain alkaloids while
445 more than 30% contain phenolics and 50% reportedly contain non-protein amino acids (Baker,
446 1977). Phenolics include stilbenes, isoflavonoids and benzofurans which have proven antifungal
447 and trypanocidal activities (Aslam et al. 2009; Getti et al. 2006); so they may also have greater
448 potential than alkaloids in the search for compounds that reduce levels of infection by *Crithidia*
449 and *Nosema* (Baracchi et al., 2015; Richardson et al., 2015).

450 While there is now evidence demonstrating that nectar chemicals modulate pollinator behaviour
451 for the benefit of the plant (Wright et al. 2015; Couvillon et al. 2015, Thomson et al. 2015),
452 whether these effects improve plant fitness is still largely unsubstantiated and needs attention.
453 Recent evidence from *Rhododendron ponticum* suggests that diterpenoid grayanotoxins in nectar
454 that are selectively toxic to bees may filter out generalists (Tiedeken et al. 2016). *Rhododendron*
455 *ponticum* is an invasive species in the British Isles but native to the Iberian Peninsula. The
456 concentration of these nectar toxins is either significantly lower or absent in established

457 populations in the invasive range (Egan, 2015). Poorly adapted pollinators in the invasive range
458 may select for reduced nectar toxins. This hypothesis is supported by a positive correlation
459 between plant fitness as a measure of seed set with toxin concentration in the native population
460 and a negative correlation in the invasive population (Egan 2015; Egan et al. 2016).
461 Furthermore, correlations between nectar chemistry and pollination syndromes across the whole
462 *Rhododendron* genus provide support for toxic nectar selection (Egan 2015). Elsewhere,
463 evidence from *N. attenuata* suggests that repellence of nicotine to hummingbirds increases
464 flower visits but the unpredictable variation in nicotine among flowers within populations and
465 even on the same plant prevents complete deterrence. Plants with nicotine in their nectar had a
466 greater number of genetically different sires, compared to plants in which nicotine production
467 had been 'switched off' (Kessler et al. 2012). Where nectaries are concealed in specialist
468 syndrome flowers for all but a few pollinators, nectar robbery or larceny may occur. The
469 evidence in some examples that nectar alkaloids protect nectar from robbery is weak. For
470 example, Gosselin et al. (2013) use the occurrence of trace amounts of aconitine type alkaloids in
471 the body of a specialist long-tongued bumblebee and its absence from a short-tongued generalist
472 and potential larcenist as evidence for the role of these compounds in conserving nectar for the
473 specialist. This work did not evaluate field visitation of the two target species of bees to the
474 plant and, in the absence of behavioural data, evidence that two species differ in their response to
475 the toxin or its repellent effects is also required. While Adler and Irwin (2005) report that
476 artificial variation in nectar alkaloids is positively correlated with nectar robbery, elsewhere there
477 is little evidence of relationships between nectar secondary metabolites and robbing in natural
478 systems and in the case of *Aconitum* it is not clear from Gosselin et al. (2013) if the protection of
479 nectaries is afforded by components in the nectar or components in the corolla.

480

481 The impact of secondary metabolites on pollinator species other than honeybees and one or two
482 other model bee species (e.g., *Bombus impatiens* or *B. terrestris*) is a limiting factor, particularly
483 since wild pollinators contribute significantly to food production and resilience of ecosystems
484 (Garibaldi et al. 2013). For example, Tiedeken et al. (2016) showed that grayanotoxin I in *R.*
485 *ponticum* nectar is highly toxic to honeybees from the British Isles but not toxic to *Bombus*
486 *terrestris audax*, the UK subspecies of *B. terrestris*. However, importantly they also evaluated
487 the toxicity against a mining bee species (*Andrena scotica*) and showed potent negative but sub-
488 lethal effects that suggest impacts of toxic nectars in invasive species could have wider
489 implications for pollinators.

490

491 One additionally overlooked area is the role of the bee microbiome, particularly considering the
492 importance of a healthy microbiome underpinning stronger disease tolerance (Koch and Schmid-
493 Hempel 2012). We therefore predict that understanding the role of bacteria and yeasts in
494 modifying secondary metabolites from nectar and pollen or alternatively being compromised by
495 them (Vannette and Fukami 2016) could shed more light on how secondary metabolites
496 influence pollinator behaviour or health. For example, Ceja-Navarro et al. (2015) report that
497 caffeine is detoxified by the gut bacteria of the coffee berry borer, which enables this serious pest
498 insect to otherwise colonise coffee beans. It is possible that bacterial symbionts in bees and other
499 pollinators may provide mechanisms to detoxify plant metabolites and enable pollinators to
500 tolerate nectar toxins. Further, the role of nectar secondary metabolites as a component of multi-
501 modal signals, including volatiles and flower colour, constitutes a largely overlooked area of
502 research (Parachnowitsch and Manson 2015).

The multi-organismal reality of interactions mediated by secondary metabolites and the multitude of chemicals encountered in nectar add layers of complexity to this research domain. Moreover, variation in concentrations of compounds across time and space and even on the same plant is largely unexplained and makes predictions of ecological function more difficult. We predict an emerging prominence of phylogenetics and comparative genomics systems biology, metabolic engineering and neuroscience for studying pollinator plant interactions and the genetics underlying the chemistry of nectar and pollen and how secondary metabolite occurrence in nectar is regulated (Kang and Baldwin 2008; Lin et al. 2014; Manson et al. 2012). This last point is particularly pertinent. Evidence that nectar secondary metabolites are regulated by the plant and are associated with plant fitness is required to demonstrate that all these fascinating potential effects are not simply the consequence of chemical biosynthesis elsewhere in the plant. Gene silencing techniques have been used to demonstrate how nectar chemicals optimise outcrossing (Kessler et al., 2012) and a stronger understanding of the chemistry and interactions with pollinators will ultimately provide more evidence for ecological functions in nectar and pollen. This will require large scale analysis of species across landscapes and time to fully understand the phenology of floral chemistry (Egan et al., 2016).

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