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Limited ability of Palestine Sunbirds *Nectarinia osea* to cope with pyridine alkaloids in nectar of Tree Tobacco *Nicotiana glauca*

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Summary

1. Secondary compounds are common in floral nectar but their relative effects on nectar consumption and utilization in nectarivorous birds are unclear.
2. We studied the effect of two pyridine alkaloids, nicotine and anabasine, present in Tree Tobacco (*Nicotiana glauca*) nectar, on food consumption, gut transit time and sugar assimilation efficiency of the Palestine Sunbird (*Nectarinia osea*), a pollinator of *N. glauca* in east Mediterranean ecosystems.
3. Sunbirds demonstrated dose-dependent deterrence; they were not deterred by the lowest natural concentrations of these alkaloids in nectar (0·1 ppm nicotine and 0·6 ppm anabasine) but they were significantly deterred by the average concentrations detected in nectar (0·5 ppm nicotine and 5 ppm anabasine).
4. The two pyridine alkaloids reduced gut transit time (by 30–42%) and sugar assimilation efficiency (by 9–17%) compared with the control alkaloid-free diet.
5. Sunbirds are able to cope with low, but not average, concentrations of nicotine and anabasine in *N. glauca* nectar. If sunbirds are efficient pollinators of *N. glauca* they may induce selection on it to reduce pyridine alkaloid production in the nectar. Alternatively, high concentrations in some *N. glauca* plants may lead the birds to visit more plants with lower alkaloid concentrations. Hence, they will be more efficient pollinators, especially if other nectar-producing plants are scarce.

Key-words: Food preference, gut transit time, plant–animal interactions, secondary compounds, sugar assimilation

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Introduction

Animal-pollinated plants attract their pollinators by rewarding them with nectar which is usually rich in carbohydrates (about 90% by dry weight, Lüttge 1977) and also contains amino acids, lipids, antioxidants and mineral ions. However, floral nectar of 55% of the species tested in the tropics and 36% of the species tested worldwide also contains secondary compounds, such as non-protein amino acids, phenolics and alkaloids (Baker 1977; Bernardello *et al*. 1994).

Plant secondary compounds are recognized as deterrents and toxins for a variety of organisms (Wink 1999). Although many nectarivores are clearly exposed to secondary compounds, the impact of these on their physiology and foraging behaviour has been largely overlooked. This is not surprising as most of the effort in understanding the functions of secondary compounds on plant–animal relationships has focused on antagonistic interactions. Perhaps because many of these components are highly toxic, and in some cases their concentration rises dramatically after plants have been wounded, the most common assumption is that they play a critical role in the plant defence against various attacks such as herbivores and microbes, and against other plants in the competition for space, nutrients and light (Baldwin 1996; Karban & Baldwin 1997; Wink 1999). Hence, the presence of toxic substances in nectar seems non-adaptive and incompatible with its reward function by either reducing its nutritional value or by direct deterrence of pollinators from the flower.

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Alternatively, secondary compounds may play an adaptive role as mediators of plant–animal interactions such as pollination and seed dispersal, and thus may increase plant fitness (Adler 2000; Cipollini 2000; Tewksbury & Nabhan 2001; Izhaki 2002; Tsahar, Fridman & Izhaki 2002). A few adaptive hypotheses, based on the assumption that secondary compounds present in the nectar are more beneficial than detrimental, have been proposed to explain their evolution in nectar, but empirical verification has generally been neglected (Adler 2000).

The ability of nectarivores to cope with secondary compounds in nectar may be associated with their adaptive role for the plant. For example, secondary compounds may efficiently screen against non-specialized pollinators and nectar robbers, while specialized pollinators are either tolerant of or even attracted by the same concentrations (Janzen 1977). It is well established that many organisms detoxify and rapidly excrete secondary compounds in limited dosages, whereas others encounter various physiological malfunctions, some fatal (Wink 1999). By contrast, some metabolites may stimulate birds' food intake and metabolism (Bairlein 1996). Nevertheless, currently no study has evaluated the impact of secondary compounds in nectar on nectarivorous birds such as hummingbirds and sunbirds, although their role as legitimate pollinators is well recognized (e.g. Faegri & van der Pijl 1979).

In this study we explored the behavioural and physiological effects of secondary compounds in the nectar of Tree Tobacco (*Nicotiana glauca*, Solanaceae) on the Palestine Sunbird (*Nectarinia osea*) in east Mediterranean ecosystems. *N. glauca* is a fast growing shrub or small tree that blooms with long tubular yellow flowers almost year round in warm climates. Although it is native to southern Bolivia and northern Argentina, during the last century it invaded many warm areas around the world from North America to Africa, Europe and Asia, including the Middle East (Hernandez 1981). Pollination vectors are essential for *N. glauca* because its stamens are shorter than the stigma (Galetto & Bernardello 1993). Because it has a relatively long corolla, its pollination mainly depends on birds with long bills such as hummingbirds in the tropics and sunbirds in the Palaearctic (Hernandez 1981; Galetto & Bernardello 1993).

The Palestine Sunbird is the only nectarivorous bird in the east Mediterranean region (Harrison 1975). It is a small passerine (6–7 g) with a long, slender, decurved bill (1·4–2·0 cm long) and an even longer tongue, which allows it to feed mainly on floral nectar and arthropods (Collins & Paton 1989; Markman, Pinshow & Wright 1999; Markman *et al*. 2004), and to act as a legitimate pollinator of flowers with relatively long corollas (Vaknin, Yom Tov & Eisikowitch 1996). We frequently observed that sunbirds feed on the flowers of *N. glauca* but their actual role in its pollination, although apparently important, has not yet been documented. Like other nectarivorous birds, sunbirds select low-protein, high-carbohydrate foods (carbohydrate specialist) while feeding on dilute sugar solutions (Klasing 1998). To gain energy from their diet, sunbirds and hummingbirds have high sugar absorption efficiencies although their gut passage is rapid (Karasov *et al*. 1986; Downs 1997; Roxburgh & Pinshow 2002).

As in other *Nicotiana* species, alkaloids are the major group of constitutive secondary compounds in *N. glauca*, but in contrast to most *Nicotiana* species, the dominant alkaloid in *N. glauca* tissues (roots, leaves, seeds, fruits and corollas) is anabasine rather than nicotine (Bush & Crow 1989). Anabasine and nicotine are highly toxic to all heterotrophs with neuromuscular junctions because they are strong agonists at the nicotinic acetylcholine receptor (nAChR), which affects nerve activity (Wink, Schmeller & Lotz-Brüning 1998). LDL_0 (lowest published lethal concentration) of anabasine is 10 mg kg^{-1} (rat, oral) and of nicotine it is 1 mg kg^{-1} (human, oral) (Golob *et al*. 1999). Anabasine, but not nicotine, is also a teratogenic compound in mammals (Keeler & Crow 1984; Panter *et al*. 1998). Both alkaloids have been used as insecticides in some parts of the world (Wink 1993; Dewick 1997). The toxic effect of anabasine and nicotine is dose dependent and can cause various physiological effects. These effects, in humans for example, are shivering, nausea, vomiting and diarrhoea in low concentrations, up to respiratory compromise, paralysis and death in high concentrations (Schmeller & Wink 1998; Mellick 1999; Wink 2000).

We investigated whether anabasine and nicotine, in concentrations occurring naturally in the nectar of *N. glauca*, affect nectar consumption of Palestine Sunbirds. To study the effect of the pyridine alkaloids, we exposed birds in captivity to an artificial nectar diet containing various levels of these alkaloids, and we measured food consumption. To further explore the physiological effects of these alkaloids on sunbirds we also determined gut transit time and sugar assimilation coefficient.

Materials and methods

BIRD MAINTENANCE

Palestine sunbirds (*Nectarinia osea*) were mist netted in July 2002–March 2003 in northern Israel. Mean body mass (± SE) was 6·6 ± 0·2 g (n = 12). Birds were kept individually in indoor metal cages ($60 \times 40 \times 80$ cm^3) in a climate room with constant temperature (25 ± 3 °C) and a 14L:10D photoperiod of artificial light. Maintenance diet consisted of sucrose (20%) and sucrose (30%)–protein (1%) (Isomil, Abbott Laboratories, Netherlands) solutions that were presented to the birds in commercial feeders (40 ml) *ad libitum*. In addition, the birds were offered fruit flies (*Drosophila* sp.) twice a week.

NECTAR SAMPLING AND SUGAR ANALYSIS

Nectar of *N. glauca* was sampled for pyridine alkaloids in October 2002 (n = 14 plants) and in August–September 2003 (n = 37). The mean (± SE) nicotine and anabasine concentrations (wet mass) were 0·50 ± 0·12 ppm and 5·0 ± 0·8 ppm, respectively. The average nicotine and

anabasine of the lowest quartile were 0·10 ± 0·06 ppm and 0·58 ± 0·05 ppm, respectively. We also analysed nectar of *N. glauca* for sugars using a temperature-compensated refractometer (Atago ATC-1E, Tokyo, Japan, 0–32%). The mean (± SE) sugar equivalent concentration was 20% ± 0·3% (n = 10 plants, 66 flowers).

HPLC DERIVATIZATION AND ANALYTICAL PROCEDURES

We analysed the nicotine and anabasine concentrations in *N. glauca* nectar. Nectar samples were dried by a speedvac (VR-Maxi, Heto, Allerod, Denmark) and then kept at –20 °C. Methanol (150 µl) was added to each of the dried samples, and after vortexing, the samples were centrifuged at 13 000 rpm for 5 min. Some 50 µl of the supernanant was derivatized, and the following solutions were sequentially added: 25 µl 4 M acetate buffer (pH 4·7); 10 µl 1·5 M potassium cyanide in water; 10 µl 0·4 M chloramine-T in water; 50 µl 50 mmol l^{-1} thiobarbituric acid in water–acetone (50:50 v/v). The contents were mixed and incubated for 5 min; the reaction was stopped by the addition of 10 µl 0·1 M sodium metabisulphite in water. High-performance liquid chromatography (HPLC) analysis was performed exactly 3 min after the reaction had stopped.

The HPLC configuration (HPLC, Beckmann system gold, Beckmann, Fullerton, USA) for determination of anabasine and nicotine consisted of a HPLC pump (Beckmann 125P) connected to a photodiode array detector (Beckmann 168; wavelength: 505 nm). The mobile phase–linear gradient was water–acetonitrile from 0 to 100% acetonitrile in 15 min. The column used was Merck LiChroCART RP-18 (5 µm, 250 mm × 4 mm) (Merck, Darmstadt, Germany). Injection volume was 20 µl and the flow-rate was 1 ml min^{-1}. Before the next injection, the column was equilibrated for 3 min. Concentrations of nicotine and anabasine were determined by calibration curves using standards at concentrations between 0·3 and 50 ng μl^{-1}.

ARTIFICIAL DIET FORMULATION

To keep nutritional values as close to nature as possible, we chose 20% sucrose solution as the maintenance solution for the birds and as the control diet in the experiments. Hence, trials were conducted with four alkaloid-containing diets, made of sucrose solution (20%, wet mass) laced with: (1) 0·6 ppm (wet mass) anabasine (Cat. No. A-5656 Sigma Ltd, Munich, Germany), (2) 5·0 ppm anabasine, (3) 0·1 ppm (wet mass) nicotine (Cat. No. 18 637–4 Aldrich Ltd, Munich, Germany), (4) 0·5 ppm nicotine, and a control diet made of sucrose solution (20%).

FOOD PREFERENCE TRIALS

Two syringes were filled either with a control diet (20% sucrose solution) and one of the four alkaloid-containing diets. These two syringes were presented to each of the captive sunbirds (n = 11–12) at 09:00 for half an hour on two consecutive days. The side location of each syringe in the cage (right or left) was randomly rotated on experimental days to control side-bias in case any bird preferred one side to the other (see Jackson, Nicolson & Lotz 1998a). The amount consumed was measured by weighing the syringes before and after the experiment. Food intake of each bird for each experimental diet was averaged over the two experimental days, as there were no significant differences between days. After each experiment, the birds were fed on maintenance diet for 5 days, until the next experimental diet was offered. The first feeding trial was conducted with anabasine 0·6 ppm, then anabasine 5 ppm, nicotine 0·1 ppm and nicotine 0·5 ppm. We preferred the paired-choice feeding trial design, as it is a compromise between multiple-choice tests (which exacerbate preferences and may suffer from lack of independence but simulate the natural situation) and single-choice tests (which often underestimate preferences, as the animals need to meet their energy requirements, and do not have a choice) (Manly 1993).

GUT TRANSIT TIME

The experiments of gut transit time were conducted 2 weeks after the food preference experiments. An hour after sunrise the feeders were replaced by 5-ml syringes containing either one of the four alkaloid-containing diets or the control diet (20% sucrose). The syringes were left in the cages for an hour before being removed, and the birds (n = 11) were food deprived for 30 min. Then, the syringe with the experimental diets laced with 0·0015 g red dye powder (E122) per 1-ml solution was placed in the cage. After 10 min the dyed solution was replaced by the clear experimental diet. The syringes with the experimental diets (dyed and clear) were weighed to determine food intake. The transit time was measured as the time (min) passed from the first feeding bout on the dyed solution to the first appearance of a coloured bird secretion on a white paper placed on the bottom of the cages (see also Downs 1997). The procedure was repeated twice on the same day, in the morning and after a 3-h period in which the birds were fed the control diet. We averaged transit time for each bird for each diet.

SUGAR ASSIMILATION EFFICIENCY

The sugar assimilation trials were conducted a day after the gut transit measurement for each experimental diet, and there were 5 days between them. Excreta of each bird (n = 11) from each of the four alkaloid-containing and control diets were collected over a period of 2 h into trays with mineral oil placed beneath the cages. The trays were emptied and the excreta separated from the oil by centrifuge (Sorvall RC 5B PLUS, Dupont, Wilmington, Delaware, USA). The sugar concentration

in the nectar and the excreta was calculated by a temperature-compensated refractometer (Atago ATC-1E, 0–32%). The sugar assimilation efficiencies (AEs) were calculated according to Roxburgh (2000) as follows:

$$\mathrm{AEs} = \frac{S_{nectar} - [S_{\mathrm{excreta}} \times (V_{\mathrm{excreta}}/V_{\mathrm{nectar}})]}{S_{\mathrm{nectar}}} \times 100,$$

where S is sugar concentration and V is volume. Although the refractometry obtained from excreta may reflect not only the sugars but also non-sugar solutes in the cloacal fluid, it underestimates the actual sugar content by only about 1% in nectarivores when they maintain high AE values (Jackson, Nicolson & van Wyk 1998b). The volume of nectar consumed by the birds was measured by weighing the feeders before and after the experiment. Evaporation from the feeders during trials was recorded and found to be insignificant.

STATISTICAL ANALYSIS

Differences in intake between control and diets containing alkaloids were calculated for each food preference trial. These differences were analysed by a two-tailed one-sample *t*-test. Repeated measures ANOVAS were performed to detect differences in transit times and assimilation efficiencies between food types; these were followed by Bonferroni pairwise comparisons ($P < 0{\cdot}05$). Body mass and food intake were used as covariates in an analysis of gut transit time. All proportions were arcsin square root transformed prior to statistical analyses.

Results

FOOD PREFERENCE

Sunbirds consumed a total of 1·11–1·43 ml per 30-min trial. They significantly discriminated against the average natural concentrations of alkaloid diets and preferred the control (20% sucrose) (Fig. 1). The higher the alkaloid concentration in the food, the sharper the discrimination (Fig. 1). Food intake of the low alkaloid concentrations decreased by 33% compared with the control diets and by 77–88% in the high alkaloid concentrations compared with the control diets (Fig. 1).

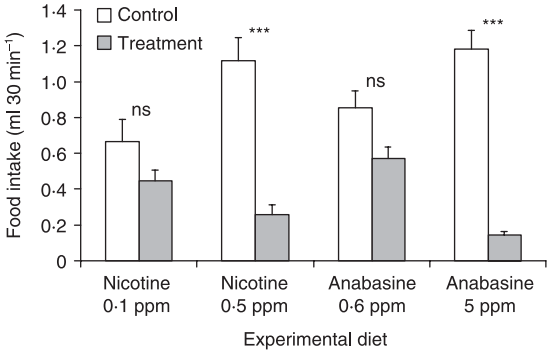

Fig. 1. Food intake (mean + SE, ml per 30 min) by Palestine Sunbirds ($n = 11$–12) fed on four diets containing 20% sucrose and nicotine (0·1 and 0·5 ppm) or anabasine (0·6 and 5 ppm). In each trial, any of the birds was exposed to two feeders: one with experimental diet and one with a control diet (20% sucrose). The results of one-sample *t*-test on the differences between control and experimental intakes appear above bars (***$P < 0{\cdot}001$, ns = not significant).

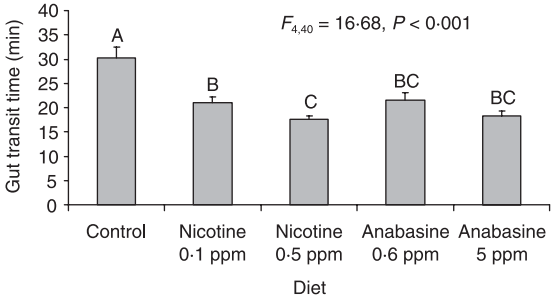

Fig. 2. Gut transit time (mean + SE, min) of Palestine Sunbirds ($n = 11$) fed on control diet containing 20% sucrose and on four experimental diets containing 20% sucrose and nicotine (0·1 and 0·5 ppm) or anabasine (0·6 and 5 ppm). Different letters above bars indicate significant difference in transit times with different diets (Bonferroni pairwise comparison, $P < 0{\cdot}05$).

GUT TRANSIT TIME

Gut transit times, controlled for body mass and food intake, were significantly different with each of the five diets offered to the birds (one-way repeated measures ANOVA, $F_{4,40} = 16{\cdot}68$, $P < 0{\cdot}001$). The average gut transit time of sunbirds fed on 20% sucrose was 30 min. When fed on alkaloid-containing diets the average transit times were 30–42% shorter than controls (Fig. 2) for both alkaloids. The mean transit time on 0·1 ppm nicotine diet was significantly shorter than that of the control diet and significantly longer than that of 0·5 ppm nicotine. No dose effects on transit time were found between the lower (0·6 ppm) and higher (5 ppm) anabasine concentrations (Fig. 2).

SUGAR ASSIMILATION EFFICIENCY

Assimilation efficiencies were significantly different between the control and the four alkaloid-containing diets offered to the birds (one-way repeated measures ANOVA, $F_{4,40} = 23{\cdot}0$, $P < 0{\cdot}001$). Sunbirds completely assimilated the sugar in the control diet (20% sucrose) whereas the sugar assimilation efficiency significantly decreased by 9–17% on the artificial diet containing pyridine alkaloids (Fig. 3). No differences in sugar assimilation were detected among the four pyridine alkaloid-containing diets.

Discussion

THE EFFECT OF PYRIDINE ALKALOIDS ON SUNBIRD DIGESTION

We found that Palestine Sunbirds were sensitive to the presence of secondary compounds in artificial nectar.

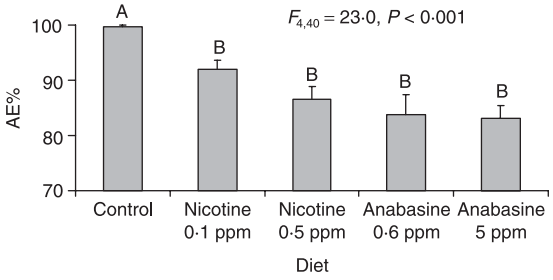

Fig. 3. Sugar assimilation efficiency (mean + SE) of Palestine Sunbirds (n = 11) fed on control diet containing 20% sucrose and on four experimental diets containing 20% sucrose and nicotine (0·1 and 0·5 ppm) or anabasine (0·6 and 5 ppm). Different letters above bars indicate significant difference in assimilation efficiencies with different diets (Bonferroni pairwise comparison, $P < 0{\cdot}05$).

They were markedly deterred by concentrations of nicotine (0·5 ppm) and anabasine (5 ppm), which are the average natural concentrations in *N. glauca* nectar. As similar effects on consumption were obtained by 0·5 ppm nicotine and 5·0 ppm anabasine, the latter can be considered less of a deterrent to sunbirds. However, although they showed deterrence to some extent when offered the lower concentrations (0.1 ppm nicotine and 0·6 anabasine), the results were not significant, so it seems that sunbirds tolerate low alkaloid concentrations to some degree or can not detect them. Hence, if sunbirds are important pollinators of *N. glauca* in the Mediterranean region, they may currently practise selection on *N. glauca* to produce nectar with low alkaloid concentration. Because nectar contains a mixture of chemicals it is still unknown if sunbirds are able to discriminate between these concentrations in the field.

Several studies have suggested that frugivorous birds tolerate certain secondary compounds more efficiently than non-frugivores (Struempf, Schondube & Martínez del Rio 1999). Furthermore, frugivorous birds may be attracted to fruits that contain certain secondary compounds (Bairlein 1996). Although it is known that birds have detoxifying enzymes in the liver and kidneys (Schmidt-Nielsen 1990), it is unclear which mechanisms enable frugivores to detoxify or avoid detrimental effects of secondary compounds (Levey & Cipollini 1999). For both nicotine and anabasine, alkaloid intake (volume × concentration) was twice as high in the high *vs* low alkaloid treatment. Still, the ability of sunbirds to tolerate pyridine alkaloids seems fairly limited as they significantly decreased their consumption of the high alkaloid diet while increasing that of the alkaloid-free diet, probably to dilute the toxic effect and/or to ensure a nutritionally sufficient diet. Nevertheless, sunbirds in Israel are probably the main pollinators of *N. glauca* flowers in the wild (Tadmor-Melamed 2004). This discrepancy may be explained either by their ability to discriminate between *N. glauca* plants on the basis of pyridine alkaloid content, selecting those with relatively low alkaloid concentrations, or by their ability to dilute the effect of these alkaloids by mixing their diet with nectar of other plant species. Both explanations were shown to be possible in captive sunbirds in the present study.

The sunbirds' deterrence from an alkaloid-containing diet may be associated with the significant reduction in the efficiency of sugar assimilation. Although sugar assimilation was reduced by less than 20% when the birds were fed an alkaloid diet, this may well be a major disadvantage for such a small passerine with a high metabolic rate that usually digests the dietary sugars entirely (Roxburgh & Pinshow 2002). The relatively short gut transit time of birds exposed to a pyridine alkaloid diet, compared with those on an alkaloid-free diet, indicates that these alkaloids induce a laxative effect on sunbirds and therefore probably result in impaired digestion. Pyridine alkaloids in mammals increase tone and motor activity of the smooth muscle of the intestine (Bruneton 1999), leading to accelerated intestinal transit. A similar mechanism responsible for such gastrointestinal hyperactivity in mammals probably exists in birds, but has yet to be demonstrated. Nevertheless, the decreased gut transit time could account for the lower assimilation efficiencies observed with the alkaloid-containing diet.

THE POTENTIAL RELATIONSHIP BETWEEN ALKALOIDS IN NECTAR AND PLANT FITNESS

The reproductive success of *N. glauca* is currently overwhelming, as indicated by its massive worldwide invasion (Hernandez 1981). A possible explanation might be a speedy transition from cross-pollination to self-pollination in the newly colonized regions (Schueller 2004). It was suggested that the evolution of shorter anther–stigma distance (herkogamy) facilitates self-pollination in regions where efficient pollinators such as hummingbirds are scarce (Schueller 2004). However, only 6% of the flowers of *N. glauca* in Israel are self-pollinated (Tadmor-Melamed 2004), so the plant depends on a pollinator for reproduction. In addition to sunbirds, four other species (Honey-bees, *Apis mellifera*; Carpenter Bees, *Xylocopa pubescens*; and ants *Crematogaster mosis* and *Acantholepis bipartita*) visit *N. galuca* in Israel but all of them are nectar robbers (Tadmor-Melamed 2004). Another explanation is that such remarkable reproductive success in diverse ecosystems may indicate the plant's general ability to cope with various arrays of pollinators, nectar robbers and pathogens. Secondary compounds in its nectar still may serve several adaptive functions that promote reproductive success.

First, the pronounced dose-dependent deterrence of sunbirds by *N. glauca* nectar does not necessarily impart a fitness disadvantage to the plant as the birds never entirely avoided the four alkaloid-laced sucrose solutions even though they were offered a nearby sucrose solution *ad libitum*. Pronounced variation in

alkaloid concentrations among *N. glauca* plants may force the birds to spend only a short time on those that produce high alkaloid concentrations before moving to others that produce low concentrations to dilute the toxic effect, thereby ensuring high levels of cross-pollination. This should be the case particularly in summer, when *N. glauca* is among the few flowering plants in our study area. Second, the alkaloid concentrations we documented may reflect a successful trade-off between deterrence of potential nectar robbers and microbes that may depredate it and its attractiveness to legitimate pollinators in our ecosystem. The benefits of producing these alkaloid concentrations in nectar may compensate the costs of the reduced attractiveness to legitimate pollinators such as sunbirds. The sunbirds were in fact willing to consume these alkaloids, albeit a small amount, and therefore still serve as *N. glauca* pollinators.

Nevertheless, the production of pyridine alkaloids in nectar may be non-adaptive. According to this hypothesis, the distribution of secondary compounds within organs may be roughly equivalent to the distribution of the primary metabolic pathways responsible for the production of the secondary compounds (as by-products) and they do not necessarily have an adaptive role in each organ (Eriksson & Ehrlén 1998). However, because *N. glauca* was introduced into the east Mediterranean region only a century ago (Bornmuller 1898), one may claim that insufficient time has elapsed to allow coevolution between *N. glauca* and sunbirds to produce notable adaptations in both partners. This might be true for the plant (but see Schueller 2004), but not for the birds. The sunbird's generation time is shorter than that of the plant and may contribute to the bird's relatively rapid adaptations, so an asymmetrical evolutionary response of the two partners is expected. Recent studies have documented rapid adaptations in various behavioural and morphological characteristics of passerines such as migration habits and bill size (Berthold *et al*. 1992; Grant & Grant 1995; Pennisi 2002). We suggest that the relationship between sunbirds and *N. glauca* should constitute an interesting case study of rapid coevolution. A relevant and testable hypothesis is that hummingbirds, the native pollinators of *N. glauca* in the New World (Hernandez 1981), are physiologically and behaviourally better adapted to cope with the pyridine alkaloids in nectar than sunbirds.

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