

Effects of nicotine on the digestive performance of nectar-feeding birds reflect their relative tolerance to this alkaloid

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Abstract

The paradox of secondary metabolites, toxic defence compounds produced by plants, in nectar and fruits is well known. Deterrence of feeding by nectarivorous and frugivorous birds is better understood than the effect of these chemicals on the digestive performance of birds. Digestive parameters such as transit time and sugar assimilation are important in assessing nutrient utilization and deterrence may be related to post-ingestive effects involving these parameters. Nectar and many fruits contain mainly sugars and water, and avian consumers compensate for low sugar content in their diet by increasing food intake: this may also increase their intake of secondary metabolites. We investigated how the alkaloid nicotine, naturally present in nectar of *Nicotiana* species, influences compensatory feeding and digestive performance of nectar-feeding birds. High nicotine concentration negatively affected compensatory feeding and apparent assimilation efficiency of white-bellied sunbirds *Cinnyris talatala* and Cape white-eyes *Zosterops virens*; but nicotine slowed gut transit time only in the latter species. In contrast, food intake and digestive performance of dark-capped bulbuls *Pycnonotus tricolor* was unaffected by nicotine up to a concentration of 50 μ M. Bulbuls are primarily frugivorous, hence they are more exposed to secondary metabolites than sunbirds and possibly white-eyes. Because their diet is richer in toxins, frugivorous birds may have evolved more efficient detoxification strategies than those of specialist nectar-feeding birds.

Keywords –apparent assimilation efficiency, bird pollinators, foraging behaviour, gut transit time, and secondary metabolites

Introduction

Despite the clear role of floral nectar and fruit pulp as a nutritional reward to attract pollinators and seed dispersing animals, little is known about the adaptive significance, if any, of secondary metabolites in nectar and fruits. Several hypotheses, not all mutually exclusive, have been put forward to explain the puzzling presence of those compounds in attractive substances (Adler 2000; Cipollini and Levey 1997a; Herrera 1982). It is still debated if the presence of these toxic compounds is a pleiotropic consequence of plant chemical defence strategy (Eriksson and Ehrlé 1998; Manson et al. 2012; Strauss et al. 2002). Alternatively, secondary metabolites could attract effective pollinators or seed dispersers while repelling nectar and pulp robbers (Cipollini and Levey 1997a; Johnson et al. 2006; Stephenson 1982). It has been shown that secondary metabolites in nectar could benefit plant reproduction if pollinators move more frequently between flowers (Kessler et al. 2008; Thomson et al. 2015). Similarly, secondary metabolites in fruits could increase passage time of seeds through the digestive system of frugivores and hence the distance of seed dispersal (Cipollini and Levey 1997a; Murray et al. 1994; Wahaj et al. 1998).

Alkaloids are one of the major groups of secondary metabolites in plants, distributed widely in angiosperm roots, leaves and fruits, and many are toxic and bitter-tasting (De Luca and St Pierre 2000; Schoonhoven et al. 2005; Wink 2003). Deterrent effects of alkaloids on nectar and fruit consumers are well documented. Nicotine, naturally present in *Nicotiana* nectar, deters hummingbirds, sunbirds and white-eyes (Kessler et al. 2012; Lerch-Henning and Nicolson 2013; Tadmor-Melamed et al. 2004). Steroid alkaloids, occurring as glycoalkaloids in ripe *Solanum* spp. fruits (Heftmann 1983), are toxic to many frugivorous birds (Cipollini and Levey 1997b; Levey and Cipollini 1998). However, some birds are surprisingly tolerant to the presence of alkaloids. Capsaicin, an alkaloid-like compound found in chilli, reduces food consumption in mammals but not in birds; curve-billed thrashers *Toxostoma curvirostre* are not deterred by the presence of capsaicin in artificial fruits (Tewksbury and Nabhan 2001). Mealworms injected with quinine deter European starlings *Sturnus vulgaris*, but the proportion of these prey eaten depends on factors such as variability in the injected dose and the size of undefended prey, demonstrating that birds are able to manage the ratio of toxin to nutrients ingested (Barnett et al. 2014; Halpin et al. 2013).

Compared to these deterrent effects of alkaloids on foraging behaviour, there is little information concerning the post-ingestive effects of these toxins on nectar and fruit consumers. Studies on frugivorous birds have used different time scales, methods of exposure, species and secondary metabolites, thus making it difficult to draw general conclusions. In addition, frugivorous birds consume different diets and their digestive traits vary correspondingly (Witmer and Van Soest 1998). This can be illustrated by examples of studies in which retention time was measured. Murray et al. (1994) found that fruits of the family Solanaceae contained laxative chemicals that reduced seed retention time, while in contrast glycoalkaloids had a significant constipative effect on cedar waxwings *Bombycilla cedrorum*, increasing seed retention time (Wahaj et al. 1998). Emodin, an anthraquinone present in fruits of the family Rhamnaceae, was found to increase gut retention time and food assimilation of yellow-vented bulbuls *Pycnonotus xanthopygos* (Tsahar et al. 2002; Tsahar et al. 2003); thus, the naturally low emodin concentration in fruits increases digestibility for these frugivorous birds (Tsahar et al. 2003).

Sugars such as sucrose, glucose and fructose are the main nutrients in nectar and fruits (Baker et al. 1998; Martínez del Rio et al. 1992) and their efficient digestion depends in part on passive absorption by the paracellular pathway, especially in small birds and bats (Caviedes-Vidal et al. 2007; Karasov et al. 2012; Napier et al. 2008). This could be disadvantageous for consumers if fruits and nectar contain hydrophilic secondary metabolites, because they will be easily absorbed by the paracellular route (Diamond 1991; Karasov et al. 2012). In addition, nectar sugar concentration is highly variable (Martínez del Rio et al. 2001; Nicolson 2002) and specialist nectar-feeding birds are able to accurately regulate daily energy intake by varying their food intake according to the sugar content of nectar (Nicolson and Fleming 2003). However, if diluted nectar contains secondary metabolites it imposes an additional challenge to specialist nectarivores, since their increased nectar intake means the inevitable ingestion of a greater amount of toxins, if supplementary food sources are not available. Hence, we are interested in understanding whether compensatory feeding is subject to limitations imposed by alkaloids, especially for specialist nectar-feeding birds consuming nutrient-dilute diets.

Our interest in nicotine has an ecological basis because this alkaloid is present in nectar of many *Nicotiana* (Solanaceae) flowers at concentrations between 0-42 μM (Adler et al. 2012; Kessler et al. 2012). Despite the presence of nicotine in nectar, many *Nicotiana* species are

pollinated by hummingbirds and moths (Kaczorowski et al. 2005; Raguso et al. 2003) and sunbirds consume nicotine in the nectar of invasive *Nicotiana glauca* in South Africa and Israel (Geerts and Pauw 2009; Tadmor-Melamed et al. 2004). Assessment of the nicotine tolerance of nectar-feeding birds showed that generalist bulbuls tolerated much higher nicotine concentrations than sunbirds and white-eyes (Lerch-Henning and Nicolson 2013). There is a single study focusing on the physiological effect of alkaloids on a nectar consumer: nicotine and anabasine, both present in nectar of *N. glauca* flowers, reduced gut transit time of Palestine sunbirds *Nectarinia osea* by 30-42% and their sugar assimilation efficiency by 9-17%, compared with the control, alkaloid-free diet (Tadmor-Melamed et al. 2004).

The aim of this study was to investigate whether the presence of nicotine in artificial nectar influences compensatory feeding and digestive performance of nectar-feeding birds (one specialist, the white-bellied sunbird *Nectarinia talatala*, and two generalists, the Cape white-eye *Zosterops virens* and the dark-capped bulbul *Pycnonotus tricolor*). Although bulbuls and white-eyes are both considered generalist nectarivores, they respond differently to the presence of nicotine in nectar; white-eyes are deterred at low concentration while bulbuls tolerate this alkaloid (Lerch-Henning and Nicolson 2013). Therefore, we expect that the negative post-ingestive effects of nicotine will be less in bulbuls than in the other two species, reflecting their nicotine tolerance. We asked: (i) Does nicotine affect the ability of nectar-feeding birds to compensate for changes in nectar sugar concentrations? (ii) Does nicotine adversely affect their digestive performance, namely gut transit time and sugar assimilation efficiency? and (iii) Is the effect of nicotine less pronounced in bulbuls than in sunbirds and white-eyes?

Material and methods

Study species

White-bellied sunbirds were mist-netted in Jan Celliers Park, Pretoria, South Africa during the nonbreeding season of 2011 ($n = 9$) and 2012 ($n = 9$); mean body mass (\pm SE) was 8.07 ± 0.24 g. Cape white-eyes were captured with the same method at the National Botanical Gardens in Pretoria during the nonbreeding season of 2011 ($n = 7$) and 2012 ($n = 9$); mean

body mass (\pm SE) was 10.63 ± 0.13 g. Dark-capped bulbuls were caught with spring traps at the experimental farm of the University of Pretoria during the nonbreeding season of 2012 ($n = 6$) and 2013 ($n = 2$); mean body mass (\pm SE) was 37.43 ± 1.03 g. All birds were released at the place of capture after experiments were completed.

Birds were kept in outside aviaries covered with shade-cloth ($9 \times 5.5 \times 1.8$ m for sunbirds and white-eyes; $12 \times 6 \times 2$ m for bulbuls), during acclimation to captivity and between experiments. Two weeks before an experiment, birds were moved to individual cages ($30 \times 42 \times 46$ cm for sunbirds and white-eyes, and $36 \times 45 \times 90$ cm for bulbuls) in a climate-controlled room maintained at $20 \pm 2^\circ\text{C}$ on a 12:12 h light : dark cycle, where dawn and dusk were simulated with 0.5 h of dimmed light before and after the full light period that started at 08h00. The cages contained wooden perches and water baths. The maintenance diet, in both aviaries and cages, consisted of a 0.6 M sucrose solution with a nutritional supplement for protein, vitamins, and minerals (Ensure®, Abbott Laboratories, Johannesburg, South Africa). In addition to the artificial nectar, white-eyes and bulbuls received seasonal fruits such as papaya, apple and banana, as well as moistened ProNutro® cereal (Becketts CNR, Wadeville, South Africa). Sugar solution and water for the small birds and bulbuls were presented in 20 ml and 60 ml inverted stoppered syringes, respectively. Maintenance diet, water and fruits were renewed daily and presented *ad libitum*.

Experimental design

Trials were carried out with different test diets (sucrose solutions with or without nicotine), all prepared in advance and frozen until used. We mixed nicotine-containing solutions at 0.5, 5 and 50 μM (Sigma-Aldrich, (-)-nicotine, N3876). All birds were tested with all test diets (12 for compensatory feeding and 4 for gut transit time and sugar assimilation efficiency) and the sequence of the test diets for each individual bird was randomised. During trials white-eyes and bulbuls did not receive fruits or cereal and between trials at least one day of maintenance diet followed for all birds. Food intake (g) was recorded by weighing the feeders (± 0.1 mg, Mettler Toledo AG-64, Microsep Ltd, Johannesburg) before and after a trial. Plastic cups containing liquid paraffin (to avoid evaporative loss) were placed beneath feeders to correct food intake for possible spillage. The cups were weighed at the same time as feeders.

Compensatory feeding

Birds were presented with diets containing nicotine at different concentrations (0, 0.5, 5 and 50 μM) in three sucrose concentrations (0.25, 0.5 and 1 M); each nicotine concentration was presented in each sucrose concentration, hence birds were presented with a total of 12 test diets and a water feeder. Due to possible side bias (Franke et al. 1998) the position of the test diet and the water feeder was switched every 1.5 h. The duration of the experiment was 6 h, from 08h00 until 14h00. Food intake (g) was converted to sugar intake (g in 6 h) using the sucrose concentrations, molar mass of sucrose and density of sucrose solutions.

Gut transit time

Two or three birds were tested at the same time, in individual cages, watched through one-way mirrors. Birds were presented with a control diet (0.63 M sucrose) or one of three (0.5, 5 and 50 μM) nicotine-containing diets mixed in 0.63 M sucrose (in total 4 test diets). Diets were coloured with a red food colorant (Robertsons®, RED food colouring, Chloorkop, South Africa). Before the experiment, birds were food deprived; bulbuls from the late afternoon of the previous day and sunbirds and white-eyes for 1 h before the experiment. On the experiment day, birds were transferred into the smaller experimental cages (43 \times 27 \times 42 cm) where the coloured diet was presented. Each bird was tested twice on each test diet and the results averaged. Gut transit time was measured as the time (min and sec) from the first feeding event to the first appearance of red excreta on a white paper sheet placed on the bottom of the cage. This trial was conducted in the morning from 08h00 to 12h00.

Apparent assimilation efficiency

Test diets were as for measurements of transit time but without the red colorant. Before lights on at 07h30, birds were placed into experimental cages (43 \times 27 \times 42 cm), smaller than maintenance cages. At 08h00 the test diet (the nicotine-containing or control solution) was presented to each bird. The trial lasted until 14h00 and during these 6 h excreta were collected in plastic trays placed beneath the cages (excreta samples were allowed to evaporate). After 6 h, birds were returned to their holding cages. Dried excreta were collected from trays by adding a known volume of distilled water (15 to 20 ml), and frozen until analysis. The samples were assayed for sucrose, glucose and fructose concentration

using enzymatic kits (Sigma-Aldrich, Munich, Germany) and a spectrophotometer (Libra S12 Biochrom Ltd., Cambridge, UK). The amount of sugar excreted in 6 h was calculated as the product of the concentration of each sugar (sucrose, glucose and fructose) per ml of sample and the volume of the sample. The apparent assimilation efficiencies (AE*) were calculated for each bird as the proportion of ingested sugar that was not excreted:

$$AE^* = (\text{sugar in} - \text{sugar out}) / (\text{sugar in}) \times 100$$

where the sugar in (mg per 6 h) was calculated using sucrose concentration, molar mass of sucrose and density of sucrose solution. Sugar out (mg per 6 h) is the total amount of sugar excreted as the sum of sucrose, glucose and fructose.

Statistical analyses

Because not all data were heteroscedastic we performed non-parametric tests using StatSoft® *STATISTICA* (version 12) and IBM® *SPSS Statistics* (version 21). To test for an effect of nicotine on compensatory feeding (sugar intake in 6 h) we used generalized estimating equations that account for the repeated measures design. Models incorporated an exchangeable correlation matrix and significance was tested using Wald statistics. Post-hoc comparisons were determined by Bonferroni correction. We tested for the overall effect of sucrose concentration, test diet and two interactions sucrose concentration*test diet and test diet*species on sugar intake and within each sucrose concentration, we tested for an effect of test diets. We included bird species as a within subject variable to test whether species had an effect on sugar intake. Using a one-way ANOVA for each species separately, we tested if birds were able to compensate for changes in sucrose concentration. Gut transit time and apparent assimilation efficiency were analysed using Kruskal-Wallis ANOVA followed by a multiple comparison test. Because AE* is a proportion, this measure was arcsine-square root transformed for statistical analysis. The dependent variables were gut transit time or AE* and the categorical variable was test diet. Lastly, to test for an effect of species we included bird species as a categorical variable. All data are presented as mean values \pm SE and for all tests the alpha level was 0.05.

Results

Compensatory feeding

On the control diets, independent of sucrose concentration (0.25, 5 or 1 M), white-bellied sunbirds consumed on average 1.43 ± 0.07 g of sugar in 6 h, showing compensatory feeding ($F = 0.37$, $df = 2$, $p = 0.69$; Fig. 1a). For all three sucrose concentrations, the sugar intake on the highest nicotine diet was significantly smaller than on the other three diets (see Table 1 for statistical values). In addition, on the lowest sucrose concentration there was a significant difference in sugar intake between 5 μ M nicotine and the control diet ($p < 0.004$). Cape white-eyes showed a similar pattern to sunbirds and their average sugar consumption, over the three control diets, was 0.93 ± 0.05 g of sugar in 6 h ($F = 1.04$, $df = 2$, $p = 0.38$; Fig. 1b).

Table 1 Summary of statistical analyses (generalized estimating equations) testing for an effect of nicotine on compensatory feeding in white-bellied sunbirds *C. talatala* ($n = 9$), Cape white-eyes *Z. virens* ($n = 6$) and dark-capped bulbuls *P. tricolor* ($n = 8$)

Species	Variables	Wald χ^2	df	p	
Sunbirds	overall effect	sucrose concentration	16.63	2	< 0.001
		test diet	243.37	3	< 0.001
		sucrose conc*test diet	14.72	6	< 0.023
	within sucrose concentrations	0.25 M	675.14	3	< 0.001
		0.5 M	176.22	3	< 0.001
		1 M	270.48	3	< 0.001
White-eyes	overall effect	sucrose concentration	18.32	2	< 0.001
		test diet	796.68	3	< 0.001
		sucrose conc*test diet	1040.95	6	< 0.023
	within sucrose concentrations	0.25 M	471.57	3	< 0.001
		0.5 M	217.92	3	< 0.001
		1 M	1404.81	3	< 0.001
Bulbuls	overall effect	sucrose concentration	2.18	2	< 0.340
		test diet	1.51	3	< 0.680
		sucrose conc*test diet	13.54	6	< 0.040
	within sucrose concentrations	0.25 M	6.29	3	< 0.100
		0.5 M	0.41	3	< 0.820
		1 M	1.07	3	< 0.780

For all three sucrose concentrations, sugar intake on 50 μM nicotine was significantly smaller than on the other three diets (see Table 1 for statistical values). In addition, on the lowest sucrose concentration, sugar intake on 5 μM nicotine was significantly smaller than on 0.5 μM nicotine ($p < 0.006$) and the control diet ($p < 0.001$). Overall, for both bird species, sucrose concentration, test diets and their interaction had a significant effect on sugar intake (see Table 1 for statistical values). Dark-capped bulbuls also showed compensatory feeding, consuming on average 2.10 ± 0.06 g of sugar in 6 h, with no difference in sugar intake between sucrose concentrations ($F = 0.21$, $df = 2$, $p = 0.81$; Fig 1c). Nicotine did not affect their sugar intake: the effect of sucrose concentration, test diets and their interaction on sugar intake was not significant (see Table 1 for statistical values). Lastly, there was a significant difference in sugar intake between bird species (Wald $\chi^2 = 281.34$, $df = 2$, $p < 0.001$) where the sugar intake of bulbuls was significantly higher than that of sunbirds and white-eyes ($p < 0.001$). In addition, the interaction between test diet and bird species was significant (Wald $\chi^2 = 9569.38$, $df = 8$, $p < 0.001$).

Gut transit time

Nicotine did not affect gut transit time in sunbirds ($H_{3,36} = 3.45$, $p = 0.327$) or bulbuls ($H_{3,32} = 5.23$, $p = 0.156$). However, these two bird species showed an opposite trend in response to the highest nicotine concentration (50 μM): in sunbirds gut transit time was reduced and in bulbuls it increased (but not significantly, Fig. 2). Nicotine had a negative effect on gut transit time in white-eyes ($H_{3,28} = 8.27$, $p = 0.041$), where the highest nicotine concentration reduced gut transit time by about 20 min. A significant difference between bird species was found ($H_{2,96} = 36.23$, $p < 0.001$), with gut transit time of white-eyes being higher than that of sunbirds and bulbuls ($p < 0.001$).

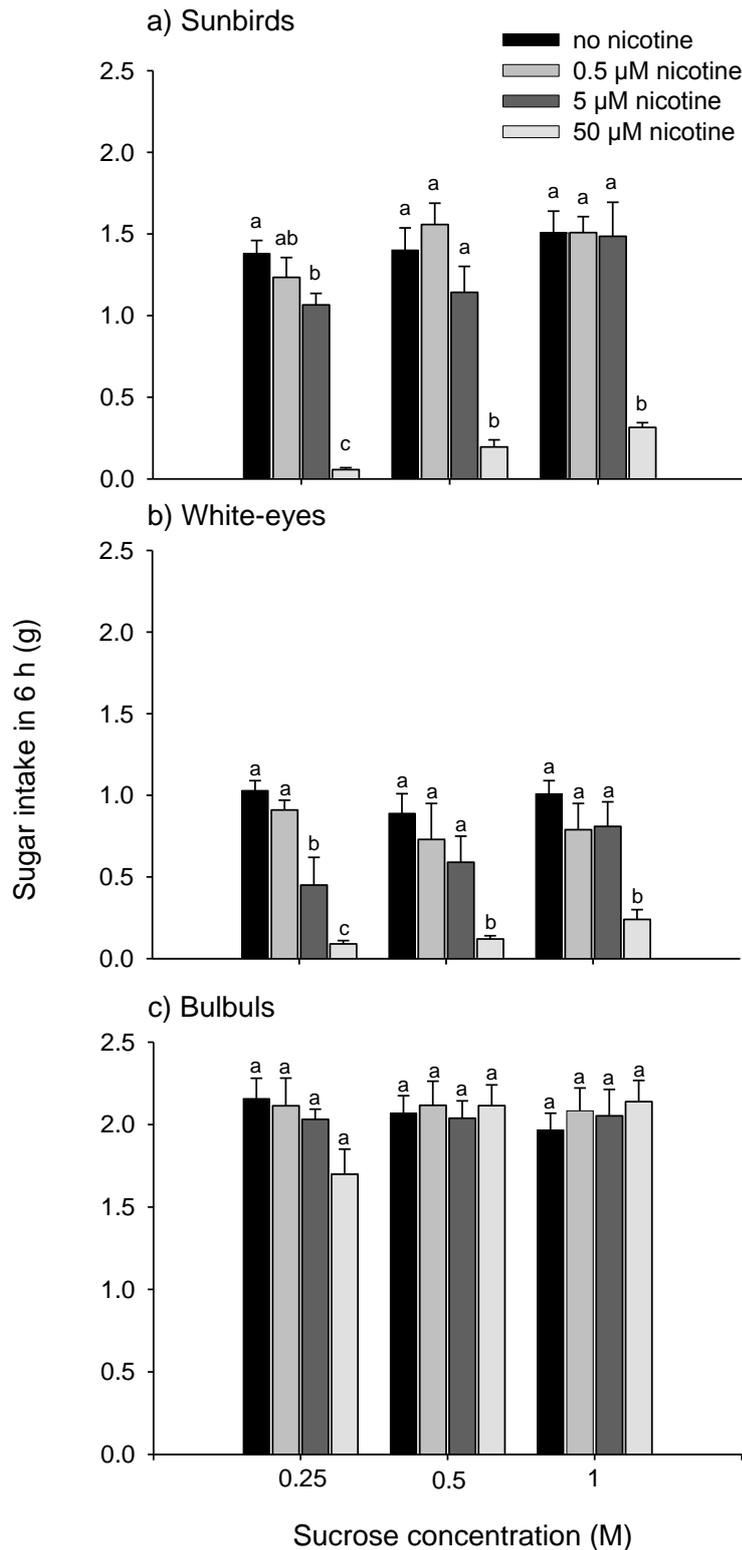


Fig. 1 Effect of nicotine on compensatory feeding measured as the sugar intake (g in 6 h) of (a) white-bellied sunbirds *C. talatala* ($n = 9$), (b) Cape white-eyes *Z. virens* ($n = 6$) and (c) dark-capped bulbuls *P. tricolor* ($n = 8$). Birds were fed different sucrose (0.25, 0.5 and 1 M) and nicotine (0, 0.5, 5 and 50 μM) concentrations for 6 h. Bars are mean values + SE. Significant differences within each sucrose concentration ($p \leq 0.05$) are indicated by different letters; correspondence of at least one letter indicates no significant difference

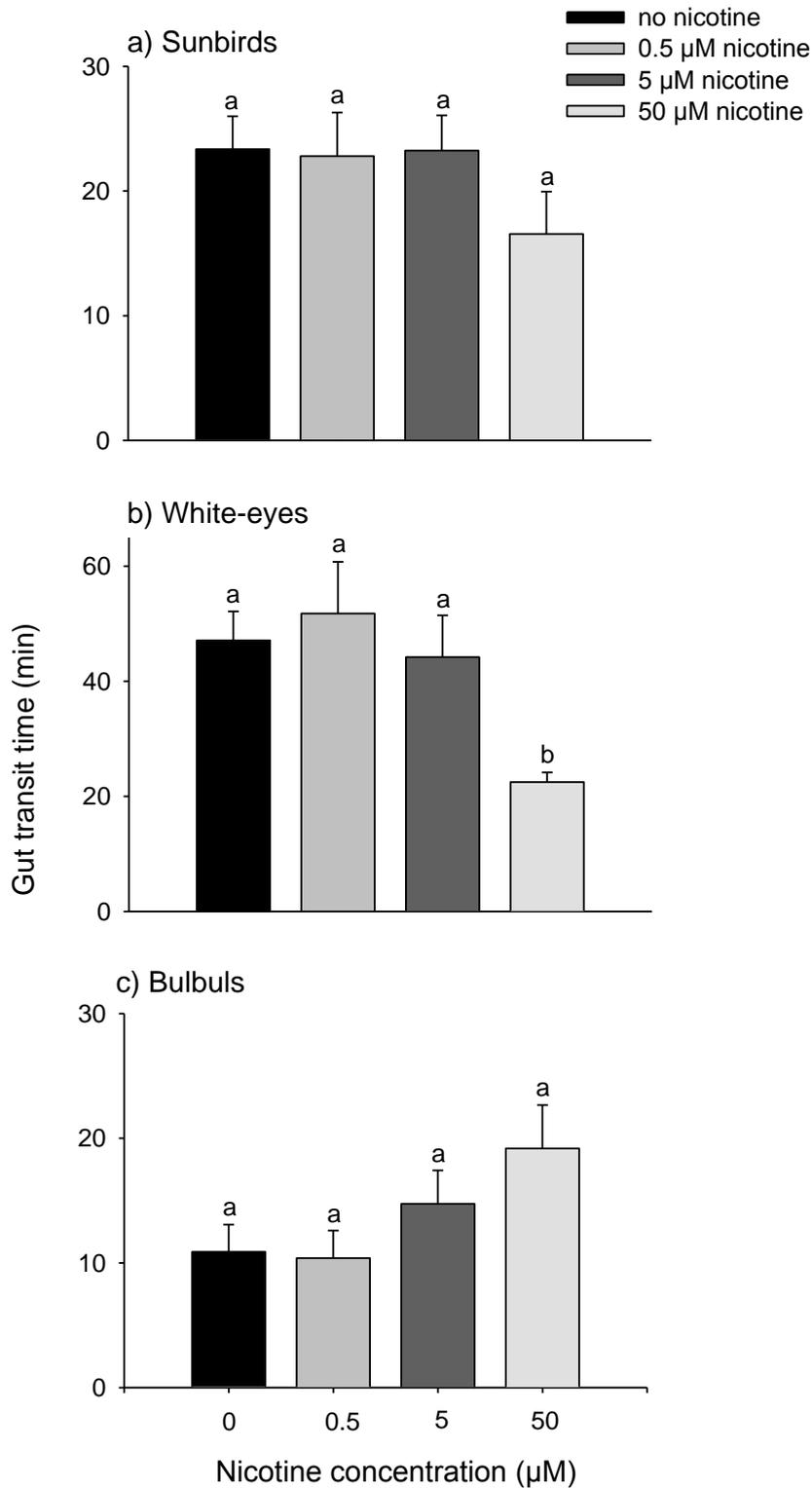


Fig. 2 Effect of nicotine on gut transit time (min) of (a) white-bellied sunbirds *C. talatala* ($n = 9$), (b) Cape white-eyes *Z. virens* ($n = 7$) and (c) dark-capped bulbuls *P. tricolor* ($n = 8$). Birds were fed three nicotine concentrations in 0.63 M sucrose and a control solution (0.63 M sucrose only) for 6 h. Bars are mean values + SE. Significant differences ($p \leq 0.05$) are indicated by different letters; correspondence of at least one letter indicates no significant difference

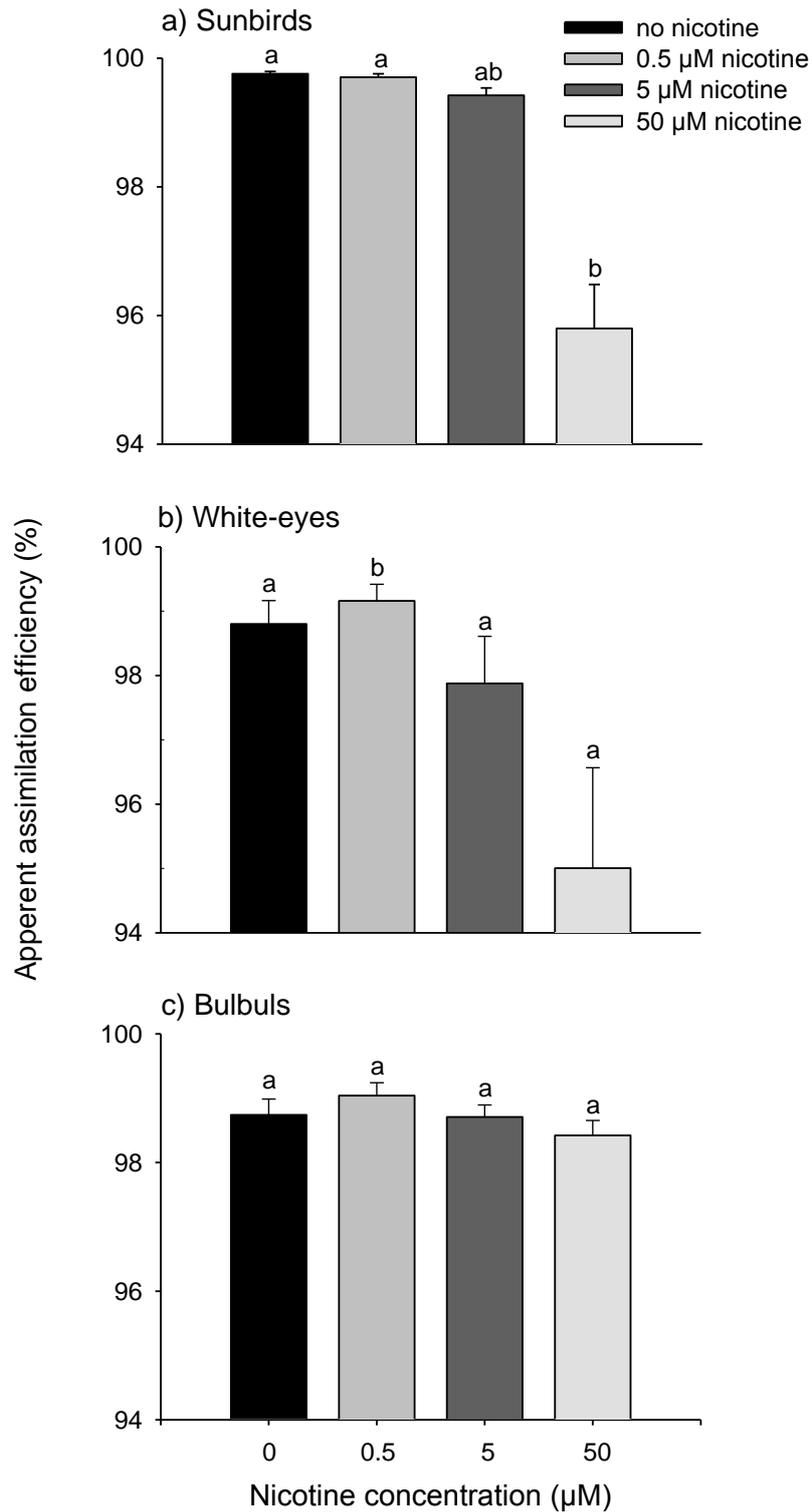


Fig. 3 Effect of nicotine on apparent assimilation efficiency (%) of (a) white-bellied sunbirds *C. talatala* ($n = 9$), (b) Cape white-eyes *Z. virens* ($n = 9$) and (c) dark-capped bulbuls *P. tricolor* ($n = 8$). Birds were fed three nicotine concentrations in 0.63 M sucrose and a control solution (0.63 M sucrose only) for 6 h. Bars are mean values + SE. Significant differences ($p \leq 0.05$) are indicated by different letters; correspondence of at least one letter indicates no significant difference

Apparent assimilation efficiency

In sunbirds, we found that nicotine reduced sugar assimilation efficiency ($H_{3,36} = 22.87$, $p < 0.001$; Fig 3). The AE* on the high nicotine diet (50 μM) was significantly smaller than on the control and 0.5 μM nicotine diets ($p < 0.001$). In white-eyes, nicotine also reduced sugar assimilation efficiency ($H_{3,36} = 12.51$, $p = 0.006$), with the AE* on the highest nicotine diet being significantly smaller than on 0.5 μM nicotine ($p < 0.005$), but not significantly different to the control diet ($p = 0.069$). Nicotine concentrations did not affect AE* in bulbuls ($H_{3,32} = 4.10$, $p = 0.251$). The AE* of the three bird species differed significantly ($H_{2,104} = 12.87$, $p = 0.002$), with values for sunbirds significantly higher than for white-eyes and bulbuls ($p = 0.004$ and $p = 0.011$, respectively).

Discussion

In line with our prediction, a high concentration of nicotine affected the ability of white-bellied sunbirds and Cape white-eyes to compensate for changes in sugar concentration, decreased their apparent assimilation efficiency and in the latter species also decreased gut transit time. However, dark-capped bulbuls showed compensatory feeding behaviour on all nicotine diets and their digestive performance was not affected by this alkaloid. First, we discuss whether this alkaloid affects the ability of nectar-feeding birds to adjust for low sugar concentration. Secondly, since absorption efficiency is directly related to retention time and absorption rate (Karasov and Levey 1990), we explain how nicotine may influence post-ingestive parameters. Thirdly, since the paracellular pathway is important in birds we discuss the implication secondary metabolites could have on foraging behaviour.

Effect of nicotine on compensatory feeding

The nicotine concentrations (0.5, 5 and 50 μM) used in this research are found naturally in *Nicotiana* flowers; nectar nicotine of 32 greenhouse-grown *Nicotiana* species ranged between 0-42 μM and nectar nicotine concentrations above 50 μM have been documented in wild *N. attenuata* (Adler et al. 2012; Kessler et al. 2012). The sugar concentration of nectar influences the deterrent effect of secondary metabolites, with increased sugar concentration

appearing to mask the bitter taste of alkaloids to bird and insect pollinators (Gegear et al. 2007; Köhler et al. 2012; Lerch-Henning and Nicolson 2013). Fruit and nectar consumers compensate for low sugar concentrations by increasing food intake to maintain constant energy intake (Martínez del Rio et al. 2001; Nicolson and Fleming 2003; Witmer 1998a, b). We found that all three bird species showed compensatory feeding over widely varying sucrose concentrations despite the presence of an average natural nicotine concentration (0.5 and 5 μM). As a consequence, they ingested more nicotine on the lower sugar concentrations during the 6 h test period (see also Lerch-Henning and Nicolson 2013). However, the highest nicotine concentration (50 μM) significantly reduced sugar intake in sunbirds and white-eyes, although not in bulbuls (Fig. 1). These results support our previous finding that bulbuls are less repelled by nicotine in artificial diet than sunbirds and white-eyes (Lerch-Henning and Nicolson 2013). Johnson and Nicolson (2008) reported that flowers visited by occasional nectarivores have nectars with a lower sugar concentration than those visited by specialist birds; this imposes an additional challenge to nectar feeding birds if nectar contains secondary metabolites, since increased nectar intake leads to a greater intake of these compounds. On the other hand, especially for specialist nectarivores, the higher water turnover on dilute nectar may help in excretion of secondary metabolites or their degradation products.

Effect of nicotine on digestive performance

The highest concentration of nicotine reduced transit time and apparent assimilation efficiency in white-eyes, but did not affect digestive parameters in bulbuls and only AE* in sunbirds (Figs 2 and 3). These results are in general agreement with the nicotine tolerance of these birds: sunbirds and white-eyes are repelled by low nicotine concentrations, whereas bulbuls tolerate much higher concentrations (Lerch-Henning and Nicolson 2013). Thus the effects of nicotine on the digestive performance helps to explain the differences in deterrent effects of this alkaloid on these three avian species.

The effect of nicotine on the digestive performance of two sunbird species also concurs with their relative tolerance of nicotine in artificial nectar (Lerch-Henning and Nicolson 2013; Tadmor-Melamed et al. 2004). Palestine sunbirds, when presented with 0.6 M sucrose solution containing 3 μM nicotine, showed reductions of 77% in food intake, 13 min in gut

transit time and 12% in sugar assimilation efficiency compared to a nicotine-free diet (Tadmor-Melamed et al. 2004). In contrast, 5 μM nicotine in the same sucrose diet had no effect on these parameters in white-bellied sunbirds. However, the methods used to measure sugar concentrations in bird excreta differed from ours: Tadmor-Melamed et al. (2004) used a refractometer and this method underestimates true assimilation efficiency because it also measures non-sugar solutes (Franke et al. 1998; Jackson et al. 1998). Little is known about how birds cope with nicotine in their diet and whether they excrete nicotine and/or nicotine metabolites. If nicotine metabolites are present in the excreta, using a refractometer to measure apparent sugar assimilation efficiency would raise refractometer readings and exaggerate the decrease in assimilation efficiency. In addition, Köhler et al. (2010) showed that AE^* in white-bellied sunbirds was $>99\%$, irrespective of diet sucrose concentration (0.25, 0.5 and 1 M); therefore apparent assimilation efficiency is not reduced at higher intake rates of pure sucrose solutions. However, we found a decrease in AE^* of $\sim 4\%$ when sunbirds had ingested very little of the diet with the highest nicotine concentration.

The physiological response of white-eyes to the high nicotine concentration was similar to that of sunbirds, even though the former are generalist feeders while sunbirds are nectar specialists. These results are in line with the nicotine tolerance of white-eyes, which was comparable to that of sunbirds at low sucrose concentrations (Lerch-Henning and Nicolson 2013). In addition, since assimilation efficiency is linked to retention time (Afik and Karasov 1995), it is not surprising that we found that high nicotine affected negatively both parameters. However, Cape white-eyes that are legitimate pollinators for *Aloe vryheidensis* are tolerant to phenolics in this nectar (Johnson et al. 2006). Interestingly, Australian silvereyes *Zosterops lateralis* did not avoid condensed tannins, which are widespread secondary metabolites in ripe fruit, when they were included in small artificial fruits; although white-eyes were repelled by these compounds when included in a cereal-based maintenance diet (Stanley and Lill 2001).

In our previous study on feeding behaviour, bulbuls were much more tolerant to high nicotine concentrations (up to 300 μM) than sunbirds and white-eyes (Lerch-Henning and Nicolson 2013): thus it is not surprising that 50 μM nicotine did not affect the physiological parameters measured in bulbuls. There was, however, a non-significant increase of 8 min in gut transit time at the highest nicotine concentration compared to the control diet. Two secondary metabolites, emodin and glycoalkaloids, both increase retention time in bird consumers

(Tsahar et al. 2003; Wahaj et al. 1998), although researchers measured defaecation rate, an indirect indicator of retention time. It has been suggested that the increase in retention time in yellow-vented bulbuls after consuming fruits containing emodin may involve a unique intestinal microflora (Tsahar et al. 2003). The gut retention time hypothesis of Cipollini and Levey (1997a) states that plant secondary metabolites could have either laxative or constipating effects on fruit consumers; laxative effects could facilitate seed passage to avoid negative impacts on seed viability, while constipating effects could increase seed dispersal distance since seeds are retained longer in the gut. The possible effects of secondary metabolites on retention time are complicated by the fact that complex food and nutrient dense diets are processed more slowly in the gut (Downs 1997; Levey and Martínez del Rio 1998; Markman et al. 2006; Witmer 1998b). For example, retention time was longer for dark-capped bulbuls fed on mealworms than those fed on apples, since mealworms are a more complex diet than apples (Downs 2008). Similarly, Palestine sunbirds showed a slower transit time when feeding on flies compared to nectar (Roxburgh and Pinshow 2002).

The apparent assimilation efficiency found for dark-capped bulbuls on a sucrose diet was high and is interesting considering the preference of this species for hexose sugars (Brown et al. 2010). Their nicotine tolerance, however, was unaffected by sugar type (sucrose vs. hexose) (Lerch-Henning and Nicolson 2013). Brown et al. (2010) reported a much lower AE* of 65% for dark-capped bulbuls fed 0.7 M sucrose, and we are unable to explain the discrepancy. Izhaki (1992) measured AE* values of 78-85% in yellow-vented bulbuls *P. xanthopygos* consuming different types of fruit. Other frugivores show very high apparent assimilation efficiencies for sugars, in spite of rapid passage rates (Witmer 1999; Witmer and Van Soest 1998). In addition, Australian generalist nectarivores also show high AE* for sucrose, fructose and glucose (all > 97.5%); those values are comparable to those for specialist nectar-feeding birds (Napier et al. 2013).

Secondary metabolites and the paracellular pathway

Frugivorous and nectarivorous birds rely heavily on a high rate of passive (non-mediated) paracellular absorption to assimilate small water-soluble molecules such as glucose (Caviedes-Vidal et al. 2007; Karasov and Cork 1994; Karasov and Levey 1990; Napier et al. 2008). The proportion of glucose absorbed through this pathway increases with more

concentrated diets (Levey and Martínez del Rio 1998). This enhancement of paracellular uptake on energetically more profitable diets that require lower ingestion rates has been demonstrated in silvereyes *Z. lateralis* and in three species of specialist nectarivorous birds, including white-bellied sunbirds (McWhorter et al. 2006; Napier et al. 2014; Napier et al. 2008). Generalist nectarivores visit flowers with lower nectar concentrations than do specialist nectarivores (Johnson and Nicolson 2008), and the absorption of any secondary metabolites via the paracellular pathway should be less than from the nectars consumed by specialist birds.

Nicotine, a water-soluble molecule, is absorbed via the paracellular pathway in cell culture (Nielsen and Rassing 2002). Karasov (2011), using a pharmacokinetic approach, showed that pigeons absorbed 44% of a dose of nicotine via the paracellular pathway. We found that a high nicotine concentration reduced AE* in sunbirds and white-eyes and one possible mechanism for this effect is that the decreased transit time reduced contact between solute and epithelium and thus paracellular absorption. Caviedes-Vidal et al. (2007) suggested that paracellular absorption is negatively correlated with body size and that the absorption of water-soluble molecules via the passive pathway is more important in small birds which have reduced small intestines. This was confirmed by Lavin et al. (2008); reduced paracellular absorption of nicotine might explain why nicotine affected the digestive performance of sunbirds and white-eyes but not of the much larger bulbuls. While nicotine is not known to actively reduce glucose transport (Karasov 2011), flavonoids and tannins have been shown to decrease mediated glucose absorption in rodents (Karasov et al. 1992; Skopec et al. 2010). While Skopec et al. (2010) showed that flavonoids inhibited *in vivo* glucose absorption in rats, which rely on mediated absorption of glucose, there was no such inhibition in robins. In this example, paracellular absorption confers tolerance to dietary flavonoids. However, because the passive pathway is less selective than a carrier-mediated system, birds may be more vulnerable to hydrophilic secondary metabolites (Diamond 1991). Karasov et al. (2012), using two bird species (including yellow-vented bulbuls) and two rodent species, showed that the absorption of radiolabelled water-soluble probes is greater in birds than in rodents. This higher paracellular permeability in birds, and the resulting absorption of toxins, could be an important ecological driving force constraining nectar and fruit selection and influencing foraging behaviour (Levey and Martínez del Rio 2001).

In conclusion, this research showed that tolerance to alkaloids present in nectar can be explained by constraints on the digestive performance of consumers (specialist and generalist nectar-feeding birds). However, the responses of two generalist nectar-feeding birds differ: dark-capped bulbuls were less repelled and physiologically more tolerant of nicotine than Cape white-eyes. Generalist consumers feed on a mixed diet including fruit, nectar and invertebrates depending on season, and are more likely to have a diet rich in secondary metabolites; thus, they may be more physiologically adapted to cope with such compounds than specialist nectar-feeding birds with a narrower diet. Even though white-eyes are categorized as generalist feeders, they were more sensitive to nicotine and responded similarly to the specialist sunbirds. Further research is needed to understand what mechanisms are involved in detoxification strategies and what makes generalist bird consumers more tolerant to secondary metabolites than specialist bird consumers.

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