Detoxification and elimination of nicotine by nectar-feeding birds

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Abstract

Many dilute nectars consumed by bird pollinators contain secondary metabolites, potentially toxic chemicals produced by plants as defences against herbivores. Consequently, nectar-feeding birds are challenged not only by frequent water excess, but also by the toxin content of their diet. High water turnover, however, could be advantageous to nectar consumers by enabling them to excrete secondary metabolites or their transformation products more easily. We investigated how the alkaloid nicotine, naturally present in nectar of *Nicotiana* species, influences osmoregulation in white-bellied sunbirds *Cinnyris talatala* and Cape white-eyes *Zosterops virens*. We also examined the metabolic fate of nicotine in these two species to shed more light on the post-ingestive mechanisms that allow nectar-feeding birds to tolerate nectar nicotine. A high concentration of nicotine (50 µM) decreased cloacal fluid output and increased its osmolality in both species, due to reduced food intake that led to dehydration. White-eyes excreted a higher proportion of the ingested nicotine-containing diet than sunbirds. However, sugar concentration did not affect nicotine detoxification and elimination. Both species metabolised nicotine, excreting very little unchanged nicotine. Cape white-eyes mainly metabolised nicotine through the cotinine metabolic pathway, with norcotinine being the most abundant metabolite in the excreta, while white-bellied sunbirds excreted mainly nornicotine. Both species also utilized phase II conjugation reactions to detoxify nicotine, with Cape white-eyes depending more on the mercapturic acid pathway to detoxify nicotine than white-bellied sunbirds. We found that sunbirds and white-eyes, despite having a similar nicotine tolerance, responded differently and used different nicotine-derived metabolites to excrete nicotine.

Introduction

Secondary metabolites are widespread in floral nectars (Adler 2000) including those consumed by bird pollinators. The paradox of toxins in a floral reward has led to a variety of studies examining different hypotheses for adaptive functions of toxins in nectar (Stevenson et al. 2017; Adler 2000). Possible beneficial effects include increasing specialization in plant-pollinator interactions and protection of nectar from microbes or unwanted consumers. In the case of nectars for birds, the dilute nature of these nectars (Johnson and Nicolson 2008) is relevant to the challenge of minimising any negative consequences of nectar toxins. Nectar sugar concentrations are highly variable and specialist nectar-feeding birds compensate for low sugar concentrations by consuming greater volumes to maintain a constant energy intake (Martínez del Rio et al. 2001; Nicolson and Fleming 2003). This compensatory feeding means that toxic secondary metabolites could potentially be consumed in large quantities if the nectar is very dilute (Lerch-Henning and Nicolson 2013). On the other hand, the high water turnover experienced by these birds (Nicolson and Fleming 2014) may also be advantageous in facilitating the excretion of secondary metabolites.

Specialist nectarivores such as sunbirds (Nectariniidae), honeyeaters (Meliphagidae) and hummingbirds (Trochilidae) consume several times their body mass in food per day when foraging on very dilute nectars (McWhorter and del Rio 1999; Nicolson and Fleming 2003; Purchase et al. 2013a). Sunbirds and honeyeaters are able to shunt excess water through the intestine, reducing the load on the kidneys (Beuchat et al. 1990; McWhorter et al. 2003; Purchase et al. 2013a). It is conceivable that they may excrete water-soluble secondary metabolites rapidly with the excess

water passing through the intestine, thus avoiding absorption and the subsequent requirement for post-absorptive strategies such as detoxification. Hummingbirds, however, do not modulate their intestinal water absorption (McWhorter and del Rio 1999; Hartman Bakken and Sabat 2006). All three lineages of specialist nectarivores produce dilute cloacal fluid with extremely low osmolality when feeding on dilute nectars, thus conserving vital electrolytes while excreting large volumes of excess water (Fleming and Nicolson 2003; Nicolson and Fleming 2014). They can also cope with temporary dehydration induced by restriction to concentrated nectars (Fleming et al. 2004a). Generalist or occasional nectar-feeding birds, such as white-eyes, bulbuls, orioles and weavers, consume even more dilute nectars than specialist nectarivores (Johnson and Nicolson 2008), but nectar forms a smaller portion of their diets, which also include fruit, seeds and insects. Consequently, these generalist or occasional nectar-feeding birds may have different mechanisms to cope with secondary metabolites in their diets compared to nectar specialists.

Consuming dilute nectars may also influence toxin absorption via the paracellular pathway. Paracellular absorption is an important route of glucose uptake in small birds (Caviedes-Vidal et al. 2007) and has been demonstrated in white-bellied sunbirds *Cinnyris talatala* and in silvereyes *Zosterops lateralis* (Napier et al. 2008; Napier et al. 2014). Paracellular absorption makes birds vulnerable to small water-soluble toxins such as nicotine (Karasov 2011; Karasov et al. 2012). Karasov (2011) demonstrated that pigeons absorbed 44% of a dose of nicotine via this route. However, dilution of the diet reduces the proportion of glucose absorbed by the paracellular route (Napier et al. 2008) which could reduce the amount of water-soluble toxins absorbed..

The most important mechanisms responsible for toxin tolerance in birds are likely to involve metabolism and elimination of ingested toxins. Detoxification is a post-ingestive metabolic tolerance mechanism involving the enzymatic biotransformation of xenobiotics into less toxic or non-toxic metabolites that can easily be eliminated. Animals use a cascade of enzymes to catalyse the oxidation, reduction, and hydrolysis of xenobiotics (phase I) and/or their conjugation to functional groups (phase II). Phase III detoxification encompasses the movement of unmodified toxins, unconjugated phase I or conjugated phase II products across lipid membranes out of cells by a range of transporter proteins and ATP-dependent pumps, followed by elimination through the kidneys (Liska 1998; Dearing et al. 2005, Sorensen and Dearing, 2006, Almeida et al. 2016). The action of detoxification enzymes, the synthesis of conjugation substrates and the active elimination of the by-products by the kidneys all require energy (Karasov and Martínez del Rio 2007; Au et al. 2013). Increased energy expenditure associated with detoxification processes has been observed in birds challenged with secondary metabolites in their diet (Guglielmo et al. 1996; Barceló et al. 2016). This suggests that there may be a tradeoff between the energy birds gain by passively absorbing water-soluble nutrients such as glucose through the paracellular route and the increased metabolic demand to elminate the water-soluble nectar toxins that are absorbed concurrently.

Nicotine is a broadly toxic secondary metabolite produced by *Nicotiana* species as a defence against herbivores. It mimics the neurotransmitter acetylcholine by binding to the nicotinic acetylcholine receptors found at neuromuscular junctions (Baldwin 2001; Steppuhn et al. 2004). Nicotine levels in the leaves and nectar of *Nicotiana* species are correlated with each other and inversely related to the extent of cross-pollination (Adler et al. 2012). Nectar nicotine concentrations range from 0 to 42 μM (Kessler et al. 2012; Adler et al. 2012). Kessler et al. (2012)

showed that variance in nectar nicotine concentration in *N. attenuata* strongly affected the foraging behaviour of hummingbird pollinators. The Argentinian species *N. glauca* is a widespread invasive plant and in South Africa, as in Israel, it is a valuable nectar resource for sunbirds in dry regions (Tadmor-Melamed et al. 2004; Geerts and Pauw 2009).

The repellent effect of nicotine on nectar-feeding birds depends on species, as well as the nicotine and nectar sugar concentrations. Specialist nectarivores such hummingbirds (*Archilochus alexandrei*) and sunbirds (*Cinnyris oseus*, *C. talatala*) and more generalist nectar-feeding birds such as white-eyes (*Zosterops virens*) and bulbuls (*Pycnonotus tricolor*) demonstrate dose-dependent deterrence and with higher nectar sugar concentrations lead to increased nicotine tolerance (Tadmor-Melamed et al. 2004; Kessler et al. 2010; Kessler et al. 2012; Lerch-Henning and Nicolson 2013). Overall, white-bellied sunbirds and Cape white-eyes exhibit an intermediate level of tolerance to nicotine, comparable to blackchinned hummingbirds (*Archilochus alexandrei*) but approx. 3-4 times higher than Palestine sunbirds (*C. oseus*) and 4-5 times lower than dark-capped bulbuls (*P. tricolor*). Remarkably little is known about the post-ingestive mechanisms utilized by nectar-feeding birds to tolerate dietary toxins. Besides detoxification mechanisms, it is also feasible that nectar-feeding species that can modulate intestinal water absorption will excrete water-soluble toxins such as nicotine rapidly with the excess water passing through the intestine. On a physiological level, high nicotine concentrations in artificial nectar negatively affect compensatory feeding, gut transit time and assimilation efficiency of sunbirds and white-eyes (Tadmor-Melamed et al. 2004; Lerch-Henning and Nicolson 2015). As yet, the effects of nicotine and other nectar secondary metabolites on osmoregulation are still unknown.

Here we investigated the effects of consuming nicotine on osmoregulation in two small nectarivores, *C. talatala*, and *Z. virens*, with a similar tolerance to nectar nicotine. We also examined the metabolic fate of nicotine in these two bird species to shed more light on the post-ingestive mechanisms that allow nectar-feeding birds to tolerate dietary nicotine. We predicted that (a) a high nicotine concentration would have a dehydration effect in both species due to the deterrent effect of nicotine; (b) nectar sugar concentration would influence the excretion of nicotine and its metabolites; and (c) the transformation products of nicotine oxidation and glucuronidation would be present in the excreta of birds exposed to dietary nicotine.

Material and Methods

Study species and their maintenance

During the non-breeding season (April-June) of 2011-2013, 12 white-bellied sunbirds (*C. talatala*, mean body mass ± SE = 8.24 ± 0.19 g) and 13 Cape white-eyes (*Z. virens*, mean body mass = 10.69 ± 0.18 g) were mist-netted in Jan Cilliers Park, Pretoria and at the Pretoria National Botanical Gardens. Birds were acclimated to captivity and to artificial diets in an outdoor aviary (8 × 2 × 5 m) at the experimental farm of the University of Pretoria. Two weeks prior to experiments, birds were moved to individual cages (30 × 42 × 46 cm) kept in a climate-controlled room. The climate-controlled room was maintained at 20 ± 2°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 08:00. After the study was concluded, birds were released at the place of capture.

The maintenance diet for both species consisted of an artificial nectar solution containing 0.6 M sucrose and the nutritional supplement Ensure® (Abbott Laboratories, Johannesburg, South Africa). In addition, for white-eyes, seasonal fruits such as papaya, apple and banana with moistened ProNutro® cereal (Becketts CNR, Wadeville, South Africa) were provided. The maintenance diet and water were presented *ad libitum* in 20 ml inverted stoppered syringes and renewed daily, together with fruits. Water baths were also provided.

Experimental procedure

In the first trial, sunbirds (n = 8) and white-eyes (n = 9) were exposed to four test diets: 0.63 M sucrose containing 0, 0.5, 5 or 50 μM nicotine (from Sigma, (-)-nicotine, N3876) and cloacal fluid (CF) was collected in liquid paraffin with the following protocol. Before 08h00, birds were moved from their maintenance cages to experimental cages (29 × 41 × 36 cm) with a bottom tray filled with liquid paraffin. A plastic mesh (vegetable bagging) stretched tightly over the tray prevented birds from making contact with the paraffin. After 6 h birds were moved back to their maintenance cages. As soon as birds were placed into experimental cages they were provided with a test diet and after 6 h CF was collected from each tray separately with a plastic pipette and stored in labeled plastic jars. The following day when the CF and the paraffin had separated, the exact volume of CF was measured in a graduated glass cylinder. The samples were frozen for later analysis of osmolality. Osmolality of CF was measured (in duplicate and in random order) using a freezing point depression osmometer (OSMOMAT® 030, Gonotec GmbH, Germany).

For the second trial (sunbirds and white-eyes, n = 4), two test diets were used: 0.25 M and 1 M sucrose, both containing 50 μM nicotine. Birds were exposed twice to the same diet, since it was

necessary to collect CF with two different methods. First, CF was collected in liquid paraffin (same protocol as above) to measure the total volume of excreta and secondly, CF was collected on a dry plastic tray to enable analysis of excreta for nicotine metabolites. To do so, birds were moved into small experimental cages (43 × 27 × 42 cm) while feeding on the test diets and excreta were allowed to evaporate at the bottom of the cage on a plastic tray. After 6 h, excreta were collected by adding a known volume of distilled water (10-15 ml) and scrubbing off the plastic tray with the water. Collected samples were frozen at – 20 ºC until further analysis.

Test diets were presented in random order to individuals for a duration of 6 h. After each experimental day, one day of maintenance diet followed. Feeders and birds were weighed before and after the experiment (± 0.1 mg, Mettler Toledo AG-64, Microsep Ltd, Johannesburg). A cup containing liquid paraffin (to avoid evaporative mass loss) was placed underneath each feeder to correct food intake for possible spills. Volumetric intake (ml in 6 h) was calculated using food intake (g) divided by the relative density of the sucrose solutions (g/L). Nicotine intake (µg) was calculated using volumetric intake of test diet (ml) × nicotine concentration (µM) divided by the molar mass of nicotine (g/mol). Sugar intake (g) was calculated using volumetric intake of test diet (ml) × sucrose concentration (M) divided by the molar mass of sucrose (g/mol).

Nicotine metabolite analysis

Chemicals and reagents. All reagents were of analytical grade unless otherwise indicated. Nicotine and methanol CHROMASOLV® for HPLC were purchased from Sigma-Aldrich (Louis, MO, USA). All solutions and dilutions were prepared with double distilled de-ionised water, produced

by an ELGA PURELAB Ultrawater purification system from Veolia Water Systems Ltd (High Wycombe, UK) unless otherwise stated.

Sample preparation. The excreta samples were lyophilised. Dried samples were powdered using a glass rod before adding 500 µl methanol. Samples were sonified using a Sonifier® Cell Disrupter B-30 fitted with a microtip (Branson Ultrasonics Corporation, Danbury, CT, USA) in 3 x 15 s pulses with 10 s cooling in between (settings: pulsed; Output control 3; % Duty cycle 60). Subsequently, the samples were centrifuged at 12 000 x *g* for 10 min and the supernatants were decanted while the pellets were discarded. The samples were dried using a Reacti-Vap™ III Evaporator (Thermo Scientific, IL, USA). Dried samples were stored at 4 °C until further analysis.

LC/MS/MS analysis. Dried samples were reconstituted in 300 µl 50% (v/v) methanol for LC/MS/MS analysis. The chromatographic analysis was performed on a Waters ACQUITY UPLC® system (Milford, MA, USA) coupled to a SYNAPT™ G2 mass spectrometer (Waters Corporation, Micromass UK Ltd, Manchester, UK), with hybrid quadrupole/ion mobility/orthogonal acceleration-time of flight geometry and an electrospray ionisation (ESI) source. Separations were carried out on an ACQUITY UPLC® BEH amide column (100 x 2.1 mm, 1.7 µm) obtained from Waters (Milford, MA, USA). Mobile phase A consisted of 0.1% (v/v) formic acid aqueous solution and mobile phase B consisted of 95% (v/v) acetonitrile and 0.2% (v/v) formic acid aqueous solution containing 10 mM ammonium acetate. The following gradient was used: 0 - 10 minutes, 100 - 50 % B; 10 - 13 minutes, 50 - 40% B; 13 - 13.3 minutes, 40 - 100% B; 13.3 - 20 minutes, 100% B. The flow rate of the mobile phase was 0.35 ml/min and the injection volume was 5 µl. Mass spectrometer conditions were optimised using a nicotine standard solution.

Optimal conditions included a capillary voltage of 3 kV and a cone voltage of 15 V. Mass spectra were acquired in positive ion mode ($[M + H]^+$). To ensure accurate mass acquisition, a lock-mass of leucine encephalin was used. Selective ion monitoring was used to detect specific fragment ions and the mass spectra were scanned for molecules with the following mass to charge ratios (*m/z*): nicotine (*m/z* 163), nicotine-*N*-oxide (*m/z* 179), *N*-methylnicotinium ion (*m/z* 177), nicotine *N*-glucuronide (*m/z* 340), nicotine *N*-oxide-glucuronide (*m/z* 356), 4-(methylamino)-1-(3-pyridyl)-1-butanone (*m/z* 179), nornicotine (*m/z* 149), 4-oxo-4-(3-pyridyl)-butanamide (*m/z* 178), nornicotine-glucuronide (*m/z* 326), nicotine-GSH (*m/z* 470), cotinine (*m/z* 177), cotinine methonium ion (*m/z* 191), norcotinine (*m/z* 163), *N*-hydroxymethly-norcotinine (*m/z* 192), norcotinine-glucuronide (*m/z* 276), cotinine-*N*-oxide (*m/z* 193), cotinine-*N*-oxide glucuronide (*m/z* 317), cotinine-*N*-glucuronide (*m/z* 354), cotinine-GSH (*m/z* 484), 3'-hydroxy-cotinine-GSH (*m/z* 369), 4-hydroxy-4-(3-pyridyl)-butanoic acid (*m/z* 181*),* 3-pyridylacetic acid (*m/z* 137), 4-(3-pyridyl)-butanoic acid (*m/z* 165), 3-pyridylacetic acid (*m/z* 137), glucuronide (*m/z* 178), glutathione (GSH, *m/z* 308) and nicotine+N-acetylcysteine (*m/z* 386). Possible nicotine related metabolites were identified in the bird excreta samples (based on the known molecular ions and the associated characteristic fragment ion patterns) and compared between dietary treatment groups. In addition, two standards were used (nicotine and cotinine) and retention times of all possible nicotine related identified compounds were related to the retention times of these standards based on their structures to further eliminate unrelated compounds. Nicotine and nicotine-derived metabolites detected in excreta samples were expressed as a percentage of the total amount of nicotine ingested.

Statistical analysis

We performed the statistical analysis using IBM® SPSS Statistics (version 23). The data is non-parametric and since the same individual was used for all four test treatments, we followed a dependent sample procedure. First, to analyse whether nicotine concentrations have a significant effect on the measured parameters, we performed for each parameter (intake, excretion, osmolality and the ratio of CF/intake) a Friedman-Test followed by Wilcoxon pairwise comparisons. Secondly, to analyse if species differ in the excreta osmolality and in the ratio of CF/intake, we performed a Friedman-Test followed by Mann-Whiney U-Test as post-*hoc* test. We used a *t*-test for dependent samples to test if sucrose concentration (low and high) had an effect on the excretion of nicotine (µg), in both species since we used the same individual for both treatments. In addition, to analyse whether species had an effect on the excretion of nicotine and nicotine-derived metabolites we performed a *t*-test for independent samples on the intensity values of each of the 8 identified compounds. All data are presented as mean values ± SE and for all tests the level of significance was $p < 0.05$.

Results

Both white-bellied sunbirds and Cape white-eyes showed a significant decrease in food intake (sunbirds: $\chi^2 = 15.45$, df = 3, p = 0.001 and white-eyes: $\chi^2 = 16.33$, df = 3, p = 0.001; Fig. 1) and in CF production (sunbirds: $\chi^2 = 16.35$, df = 3, p = 0.001 and white-eyes: $\chi^2 = 15.13$, df = 3, p = 0.002; Fig. 1). Intake and excretion of the highest nicotine concentration was significant lower than that of other test diets (sunbirds: p = 0.012; white-eyes: p = 0.008). The highest nicotine concentration caused a significant increase in CF osmolality in sunbirds ($\chi^2 = 15.12$, df = 3, p =

0.002; Fig 2) and white-eyes (χ^2= 11.80, df = 3, p = 0.008; Fig. 2). In birds feeding on the highest nicotine concentration, we measured CF osmolalities of 578 ± 97 mOsm/kg in sunbirds and 243 ± 41 mOsm/kg in white-eyes. We found a significant effect of bird species on osmolality (χ^2 = 26.38, d df =3, p < 0.001) and bird species differ significantly when feeding on the low (U_{13} = 9, Z = -2.60, p = 0.009) and high (U_{13} = 12, Z = -2.31, p = 0.021) nicotine concentration. The ratio of CF excreted to food intake in sunbirds and white-eyes decreased significantly at the highest nicotine concentration compared to the other diets (χ^2= 15.45, df = 3, p = 0.001 and χ^2= 18.20, df = 3, p < 0.001, respectively; Fig. 2) and species had a significant effect on this parameter (χ^2= 31.52, df = 3, p < 0.001). Sunbirds and white-eyes showed a significant difference in the ratio of CF/intake while feeding on the control (U_{13} = 8, Z = -2.69, p = 0.007), low (U_{13} = 1, Z = -3.37, p = 0.001) and high nicotine concentrations (U_{13} = 4, Z = -3.08, p = 0.002), although both species ingested similar amounts of nicotine (see Table 1 for values).

When low and high sucrose concentrations were compared, there were no significant difference in the amounts of nicotine excreted (see Table 2 for values) in either species (sunbirds: *t* = 0.215, df = 3, p = 0.844; white-eyes: *t* = 2.901, df = 3, p = 0.062). Hence, the results were averaged across both sucrose concentrations. Both species metabolised nicotine with very little unmetabolised nicotine recovered in the excreta: unchanged nicotine accounted for only 0.05 and 0.13% of the total amount of nicotine ingested for sunbirds and white-eyes, respectively. The major metabolites detected were norcotinine, cotinine methonium ion and nornicotine. Phase II metabolites were also detected: nornicotine-glucuronide, norcotinine-glucuronide and cotinine-glutathione (Fig 3). In addition to these nicotine-derived metabolites, birds also excreted an intermediate of the mercapturic acid pathway, nicotine-N-acetylcysteine (nicotine-NAC). We also detected

substantial levels of glutathione (GSH) in the excreta; however, this compound was probably split off from a nicotine-glutathione conjugation product.

Nicotine and nicotine-derived metabolites detected in excreta samples are expressed as a percentage of the total amount of nicotine ingested and differed between bird species (see Table 2). White-eyes excreted slightly higher amounts of unchanged nicotine than sunbirds (0.034 ± 0.002 and 0.024 ± 0.002 µg nicotine, respectively; $t = 6.74$, df = 14, p =0.014). In addition, white-eyes excreted significantly more norcotinine, GSH and nicotine-NAC than sunbirds (for statistical values see Table 3).

Discussion

Nectar-feeding birds may use their high water turnover rate to flush out secondary compounds and any biotransformation products. We analysed the excreta of sunbirds and white-eyes for nicotine and nicotine-derived metabolites and found that both species are able to metabolise nicotine. We predicted that if nicotine was presented in dilute nectar this would facilitate the excretion of the unchanged toxin due to the higher water turnover; however, this was not confirmed. A high concentration of dietary nicotine induced a physiological response similar to dehydration. We discuss these findings in terms of the possible osmoregulatory consequences of encountering nicotine in nectar and the detoxification mechanisms that nectarivores could use to cope with secondary compounds present in nectar.

Water balance and secondary metabolites

The drastic reduction in food intake observed at high nicotine concentrations could be due to deterrence or post-ingestive consequences (Lerch-Henning and Nicolson 2013; Lerch-Henning and Nicolson 2015). Largely due to the reduced food intake, we observed a significant reduction in CF volume and an increase in CF osmolality in both species at high nicotine concentrations. Sunbirds excreted three times less CF (0.15 ml/6 h) than white-eyes (0.44 ml/6 h), i.e. white-eyes excreted a higher proportion of the ingested diet than sunbirds. The CF osmolality of sunbirds increased 14 times while that of white eyes increased four times compared to birds receiving the nicotine-free diet. These observations mirror the physiological response to dehydration in birds. When feeding on concentrated nectars, birds conserve water by reducing production of cloacal fluid and increasing its osmolality (Fleming and Nicolson 2003; Fleming et al. 2004a). Sunbirds reduced CF output to 0.004 ml/h on a concentrated 2.5 M sucrose diet compared to 1.4 ml/h on a dilute 0.25 M sucrose diet (Fleming et al. 2004a). High CF osmolalities (~450 mOsm/kg) were recorded when birds were presented with either 2.5 M sucrose or 0.63 M sucrose containing 200 mM NaCl (Fleming and Nicolson 2003; Purchase et al. 2013b). White-bellied sunbirds are able to modulate their glomerular filtration rate, effectively shutting down urine production to avoid potential dehydration during the overnight fast period (Fleming et al. 2004b; Purchase et al. 2013a). Less is known about the water conservation abilities of white-eyes, but a field study of Australian silvereyes *Zosterops lateralis* showed considerable variation in water turnover (Rooke et al. 1983). Our data confirm that white-eyes are able to conserve water, although not as well as sunbirds.

It is also possible that the decreased CF output and increased CF osmolality we observed are not only due to reduced food intake, but also in part due to the antidiuretic effect of nicotine. Nicotine

can have an antidiuretic effect in humans and other mammals through its action on the antidiuretic hormone vasopressin (Burn et al. 1945; De Souza and Silva Jr 1977). It is possible that in birds nicotine also stimulates the pituitary gland to release arginine vasotocin, the avian analogue of the mammalian vasopressin, contributing to the dehydration-like response. Arginine vasotocin concentration increases in the plasma of white-bellied sunbirds fed concentrated sucrose diets (Gray et al. 2004). An antidiuretic compound is described as lowering water output even though water intake is unchanged. In nectar-feeding birds, food and water intake cannot be separated, so we cannot draw any conclusion regarding the antidiuretic effect of nicotine.

In contrast, some plant secondary metabolites such as phenolics and terpenes appear to have diuretic effects on herbivore consumers. These compounds cause an increase in water intake compared to a control diet, an increase in urine output in relation to ingested water and a decrease in urine osmolality (Dearing et al. 2001; Dearing et al. 2002). In these studies, the water balance of specialist herbivores was less affected by plant defence chemicals than that of generalist herbivores (Dearing et al. 2001; Dearing et al. 2002). This is confirmed by a recent study of seed-eating birds in which increased water intake on a diet supplemented with phenols was recorded in common diuca-finches *D. diuca* but not in rufous-collared sparrows *Zonotrichia capensis* which are more accustomed to consuming toxins (Barceló et al. 2016).

Detoxification of nicotine

Sunbirds and white-eyes, after consuming 50 µM nicotine in artificial nectar, excreted nicotine-derived metabolites similar to those found in humans, other mammals and insects. In humans, 70 – 80% of the absorbed nicotine is converted to cotinine before being metabolised further, and about

10-15% of the absorbed nicotine dose appears as unchanged cotinine in the urine (Hukkanen et al. 2005; Rangiah et al. 2011). Nectar-feeding birds mainly excreted norcotinine or nornicotine. In white-eye excreta, the major nicotine metabolite detected was norcotinine, which is the result of oxidative N-demethylation of nicotine (phase I detoxification reactions; see Fig. 3). In sunbirds, the major metabolite detected in excreta was nornicotine, the product of N-demethylation of nicotine (see Fig. 3). Cotinine methonium was also detected in both bird species at lower levels but no cotinine could be detected. All three of these metabolites are present in the urine of humans and other mammals exposed to nicotine, nornicotine as one of the major urinary metabolites and norcotinine and cotinine methonium being two minor metabolites (Hukkanen et al. 2005; Rangiah et al. 2011).

Birds also appear to utilise the major conjugation pathways when detoxifying nicotine including glucuronidation and glutathione conjugation, the first step of the mercapturic acid pathway. We detected norcotinine-glucuronide, nornicotine-glucuronide, cotinine-glutathione and nicotine-NAC. In addition, glutathione was detected in the excreta of both species. Conjugation of nicotine to glucuronic acid (glucuronidation) is an important reaction in the metabolism of nicotine (Hukkanen et al. 2005; Chen et al. 2010; Rangiah et al. 2011). In humans, up to 30% of urinary nicotine and cotinine are conjugated to glucuronides (Meger et al. 2002; Hukkanen et al. 2005; Chen et al. 2010).

Glucuronic acid is a derivative of endogenous glucose, and therefore expensive to excrete: its presence in urine is an indication that detoxification may impact animals energetically (Mangione et al. 2004; Sorensen et al. 2005). The glucuronidation pathway is a major route of phenolic

detoxification in birds (Jakubas et al. 1993; Guglielmo et al. 1996; Barceló et al. 2016). Glucuronic acid output is considered a biomarker of the capacity to detoxify phenolics (Ríos et al. 2012; Barceló et al. 2016) and may have high energetic costs (Guglielmo et al. 1996; Barceló et al. 2016) in birds. However, compensatory feeding may obviate any negative effects on the energy balance of birds. We did not find a significant difference in the amount of nicotine and its metabolites excreted when sunbirds and white-eyes fed on low and high sucrose concentrations; although this result might be a reflection of the small sample size.

In addition to glucuronic acid conjugation products, we also detected a glutathione conjugation product (cotinine-glutathione) and one mercapturic acid derivative, nicotine-NAC. In the mercapturic acid pathway, glutamate and glycine are split off from the glutathione molecule conjugated to the xenobiotic and the remaining cysteine from the glutathione molecule is acetylated to form mercapturic acids, which are excreted in urine (see Fig. 3) (Gloss et al. 2014). In general, higher levels of glutathione in excreta are associated with the detoxification and elimination of toxins from the body (Rinaldi et al. 2002; Lushchak 2012). Although glutathione conjugation products are not associated with nicotine detoxification in humans and other mammals, the glutathione conjugation pathway has been linked to the detoxification of nicotine in honeybees, *Apis mellifera* (Du Rand et al. 2015).

It is possible that fast gut transit rates help specialist nectar-feeding birds to eliminate secondary compounds and their transformation products from their bodies faster. Previously we found that high nectar nicotine did not affect gut transit time of white-bellied sunbirds (Lerch-Henning and Nicolson 2015). Sunbirds and honeyeaters are able to shut a portion of the excess water they ingest

on dilute nectar through the intestine (Purchase et al. 2013a). Nicotine, a water-soluble molecule, could be flushed out with the excess water, thereby avoiding absorption. However, we found very little unchanged nicotine in the excreta which suggests that nicotine was absorbed into the blood stream, catabolised in the liver and excreted as metabolites by the kidney. Nicotine can be absorbed across the intestinal epithelium via two possible routes. Nicotine in its unionized form is an amphipathic molecule that can easily cross intestinal cell membranes passively. However, at pH levels below 8, it exists in its water-soluble cationic forms that cannot easily pass through membranes. At physiological pH (7.4), 70% of the nicotine is ionized, but because of its small size (162 kDa) it could be absorbed across the intestinal epithelium through paracellular spaces by diffusion or solvent drag (Karasov et al. 2012). Karasov (2011) showed that pigeons absorbed 44% of a nicotine dose through the paracellular route.

The possibility that sunbirds might rapidly excrete substantial amounts of unchanged nicotine while shunting water through the gut was not excluded by our results. To ensure a functional response and that nicotine and any metabolites would be present at levels above the limits of detection, we chose a nicotine concentration at the high end of the natural concentration range in *Nicotiana* nectar (Kessler et al. 2012). The high concentration of nicotine used to investigate its metabolic fate reduced the food intake of the birds which led to them conserving water. On a diet of 50 µM nicotine sunbirds produced almost three times less cloacal fluid over a 6 h period than white-eyes. This in part could explain the lower recovery of ingested nicotine in sunbirds: almost half (~38%) of the percentage recovered in white eyes (~72%, see Table 3). One of the mechanisms used by sunbirds to conserve water is reducing glomerular filtration (Fleming et al. 2004b; Purchase et al. 2013a), which would also result in lower excretion of solutes, including nicotine

and its metabolites. In future studies, the use of radiolabeled nicotine and a lower concentration of nicotine in dilute artificial nectar will not only increase the limit of detection of nicotine and any nicotine-derived metabolites but will also ensure that the birds experience a high water turnover rate and avoid the potential trade-off between toxin elimination and water conservation.

Detoxification strategies of birds

Sunbirds and white-eyes appear to use similar oxidation and reduction reactions in combination with glucuronidation and glutathione conjugation to detoxify nicotine (Fig. 3). However, there were noticeable inter-species differences in the levels of certain nicotine-derived metabolites excreted in the CF (see Table 3). White-eyes excreted substantially more norcotinine, GSH and NAC-nicotine, which could indicate that white-eyes rely more heavily on metabolising nicotine via the cotinine pathway and mercapturic acid pathway than sunbirds. The glucuronic acid outputs of these two nectar-feeding species with a similar nicotine tolerance were not substantially different for the same nicotine intake. An exploratory study investigating secondary metabolite tolerance in granivorous birds, using glucuronic acid output as a biomarker of detoxification capacity, also found no interspecific differences in glucuronic acid excretion regressed against tannic acid intake (Ríos et al. 2012).. The authors suggested that the higher tannic acid tolerance of the generalist *Z. capensis* might be due to reliance on alternative detoxification routes to glucuronidation, such as ornithine conjugation or rapid excretion (Green et al., 2005; Jakubas et al., 1993). Glucuronic acid output might be a good biomarker for detoxification in birds, but not necessarily for detoxification capacity or the ability to detoxify and tolerate a particular secondary metabolite.

Differences in the xenobiotic detoxification capabilities of birds appears to be diet-related. Omnivorous species exhibit high detoxification capacity compared to species with a narrower diet (Fossi et al. 1995a; Fossi et al. 1995b; Liukkonen-Anttila et al. 2003; Rainio et al. 2012). Interspecific variation in detoxification capacity is usually related to differences in the mixed function oxidase or cytochrome P450 system, and mixed function oxidase activity is used as a biomarker to compare detoxification capacity among birds (Fossi et al. 1995b; Rainio et al. 2012).. In passerine birds, difference in mixed function oxidase activity is related to feeding habits and migratory status (Rainio et al. 2012) with, for example, insectivorous species having higher mixed function oxidase activity than granivorous species and migratory insectivores exhibiting the highest activity (Rainio et al. 2012). The detoxification capacity of nectar-feeding birds in terms of mixed function oxidase activity has not yet been explored.

Few studies have investigated the metabolic fate of ingested plant secondary metabolites in birds.. Birds either metabolise plant secondary metabolites through a combination of phase I and conjugation reactions or rapidly excrete the compounds unchanged. Ruffed grouse *Bonasa umbellus* fed high levels of the phenolic coniferyl-benzoate used hydrolysis, reduction and oxidation reactions to detoxify coniferyl-benzoate (Jakubas et al. 1993). In a related publication, Guglielmo et al. (1996) found that increased coniferyl-benzoate intake leads to an increase in excretion of glucuronic acid and ornithine (two major detoxification conjugates), associated with a 14% increase in energy requirements. Glucuronidation also plays a role in the detoxification of other phenolic compounds such as tannic acid in generalist (omnivorous) and specialist seed-eating birds (Ríos et al. 2012; Barceló et al. 2016). In contrast, cedar waxwings *Bombycilla cedrorum* excreted the cyanogenic glycoside, amygdalin, intact after ingestion (Struempf et al. 1999). If

cyanogenic glycosides are not hydrolysed, no cyanide is released and ingestion has minimal or no toxic consequences. Lessner et al. (2015) found that hydrolysis of plant glucosides was undetectable in yellow-vented bulbuls (*Pycnonotus xanthopygos)* and house sparrows (*Passer domesticus*), and suggested that a low capacity in passerine birds to hydrolyse plant glucosides compared to mammals could explain the tolerance of birds to these compounds. Another group of secondary metabolites that appears to be excreted unchanged by birds is monoterpenes. The greater sage-grouse (*Centrocercus urophasianus*), which feeds on monoterpene rich sagebrush (*Artemisia* spp.), shows reduced absorption of these compounds and excretes monoterpenes unchanged in the faeces (Kohl et al. 2015).

Conclusion

Our results demonstrate the ability of white-bellied sunbirds and Cape white-eyes to metabolise the nectar alkaloid nicotine. Cape white-eyes mainly metabolised nicotine through the cotinine metabolic pathway, with norcotinine being the main metabolite present in the excreta, while white-bellied sunbirds excreted mainly nornicotine. Both species also utilized phase II conjugation reactions to detoxify nicotine, with white-eyes depending more on the mercapturic acid pathway to detoxify nicotine than sunbirds. Taken together, our results support the notion that different birds use different detoxification strategies to deal with secondary metabolites (Barcelo et al. 2016). Recently there has been considerable interest in possible adaptive functions of secondary metabolites in nectar, usually in studies involving insects (honeybees and bumblebees) (Stevenson et al. 2017). However, a large number of plant species are pollinated by nectar-feeding birds and understanding the effect of secondary metabolites on bird pollinators as well as the mechanisms

used by nectar-feeding birds to escape the toxic effects of these secondary compounds is key in in understanding the adaptive functions of nectar secondary metabolites in plant-pollinator interactions. To our knowledge, this is the first study examining the metabolic fate of a plant secondary metabolite in nectar-feeding birds.

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Tables

Table 1 Sugar and nicotine intake of sunbirds and white-eyes. Sugar intake (g) and nicotine intake (µg) of sunbirds *C. talatala* (n = 8) and white-eyes *Z. virens* (n = 9) feeding on a sucrose diet of 0.63 M with different nicotine concentrations (0, 0.5, 5 and 50 µM of nicotine) for a duration of 6 h.

Species	**Sunbirds**		**White-eyes**	
Diet (0.63 M)	**Sugar intake (g)**	**Nicotine intake (µg)**	**Sugar intake (g)**	**Nicotine intake (µg)**
Control	1.38 ± 0.05	-	0.99 ± 0.14	-
0.5 µM nicotine	1.25 ± 0.08	0.47 ± 0.03	1.07 ± 0.12	0.40 ± 0.05
5 µM nicotine	1.19 ± 0.08	4.48 ± 0.30	1.11 ± 0.09	4.17 ± 0.32
50 µM nicotine	0.18 ± 0.02	6.81 ± 0.70	0.18 ± 0.02	6.76 ± 0.64

Table 2 Food and nicotine intake vs cloacal fluid output and nicotine excretion. Food intake (ml), nicotine intake (µg, amounts per individual and mean ± SE), cloacal fluid (CF) excretion (ml) and nicotine recovered in the cloacal fluid (µg, amounts per individual and mean ± SE) of sunbirds *C. talatala* (SB, n = 4) and white-eyes *Z. virens* (WE, n = 4) feeding on 0.25 M or 1 M sucrose containing 50 µM nicotine, for a duration of 6 h.

		INTAKE			OUTPUT		
Species	**Sucrose (M)**	**Food (ml)**	**Nicotine (µg)**		**CF (ml)**	**Nicotine (µg)**	
SB	0.25	10.47	88.724	94.92 ± 1.8	5.40	0.023	0.02 ± 0.0
SB	0.25	11.43	96.831		7.20	0.022	
SB	0.25	11.46	97.131		8.60	0.025	
SB	0.25	11.44	96.981		8.00	0.027	
SB	1	3.70	31.376	35.58 ± 3.6	2.00	0.021	0.02 ± 0.0
SB	1	4.20	35.580		1.80	0.021	
SB	1	3.32	28.148		1.60	0.031	
SB	1	5.57	47.214		2.60	0.022	
WE	0.25	10.05	77.990	66.13 ± 4.7	7.60	0.036	0.03 ± 0.0
WE	0.25	8.12	63.052		6.40	0.023	
WE	0.25	6.77	52.544		4.60	0.023	
WE	0.25	9.14	70.934		7.60	0.031	
WE	1	2.66	20.642	20.68 ± 3.9	1.60	0.043	0.04 ± 0.0
WE	1	2.86	22.218		1.80	0.043	
WE	1	1.15	8.932		1.80	0.026	
WE	1	3.98	30.926		2.60	0.044	

Table 3 Nicotine and nicotine-derived metabolites detected in the excreta of sunbirds and white-eyes. Sunbirds *C. talatala* (n = 8) and white-eyes *Z. virens* (n = 8) were exposed to sucrose solutions (0.25 and 1 M) containing 50 µM nicotine for 6 h before excreta were collected. Excreta samples were analysed for nicotine and nicotine-derived metabolites using UPLC/MS/MS. The molecular formula and the molecular ion $[M+H]^+$ of each compound are indicated. Nicotine and nicotine-derived metabolites detected in excreta samples are expressed as a percentage (%) of the total amount of nicotine ingested. The values reported in bold are significantly different between the two species (*t*-test for independent samples). GSH: Glutathione. Nicotine-NAC: Nicotine-N-acetylcysteine.

Name of Compound	Molecular formula	$[M+H]^+$	% of ingested nicotine dose		Statistical Results		Reference
			Sunbirds	White-eyes	t_{14}	P	
Nicotine	$C_{10}H_{14}N_2$	163.11	**0.05**	**0.13**	-	-	Miller et al. 2010
Norcotinine	$C_9H_{10}N_2O$	162.11	**8.65**	**39.57**	**-5.86**	**0.001**	Hukkanen et al.2005
Cotinine methonium ion	$C_{11}H_{15}N_2O$	189.16	9.07	7.57	2.06	0.058	Hukkanen et al. 2005
Nornicotine	$C_9H_{12}N_2$	148.06	0.88	1.72	-0.52	0.612	Rangiah 2011
Nornicotine-glucuronide[a]	$C_{15}H_{21}N_2O_6$	325.11	8.85	12.5	1.67	0.116	Meger et al. 2002 Rangiah 2011
Norcotinine-glucuronide	$C_{15}H_{19}N_2O_3$	277.09	3.38	1.86	-0.11	0.915	Meger et al. 2002 Rangiah 2011
GSH	$C_{10}H_{17}N_3O_6S$	308.17	**0.11**	**12.2**	**-3.66**	**0.003**	Dieckhaus et al. 2005)
Cotinine-GSH	$C_{20}H_{29}N_5O_6S$	482.29	5.44	4.22	1.46	0.167	Dieckhause et al. 2005
Nicotine-NAC	$C_{16}H_{24}N_4O_3S_2$	386.29	**1.19**	**4.27**	**-3.00**	**0.010**	Gloss et al. 2014

[a] Molecular ions corresponding to nornicotine-glucuronide were detected at three different retention times and further analysis is required to confirm the identity of the compounds eluting at these retention times

Figure legends

Fig. 1 Effect of dietary nicotine on food intake and cloacal fluid output

The volume of food consumed (ml) and cloacal fluid excreted (ml) in (a) white-bellied sunbirds *C. talatala* (n = 8) and (b) Cape white-eyes *Z. virens* (n = 9). Birds were fed four test diets (0, 0.5, 5 or 50 µM nicotine in 0.63 M sucrose) 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. 2 Effect of nicotine on the osmolality of cloacal fluid and the ratio of cloacal fluid to food intake

Effect of nicotine on (a) osmolality of cloacal fluid (mOsmol/kg) and (b) the proportion of cloacal fluid (ml) to food intake (ml) of white-bellied sunbirds *C. talatala* (n = 8) and Cape white-eyes *Z. virens* (n = 9). Birds were exposed to four test diets (0, 0.5, 5 or 50 µM nicotine in 0.63 M sucrose) for 6 h. Bars are mean values + SE and significant differences ($p \leq 0.05$) are indicated by different letters; correspondence of at least one letter indicates no significant difference.

Fig. 3 Conversion of nicotine to its major metabolites in nectar-feeding birds

Relative amounts (%) of metabolites found in the excreta of sunbirds *C. talatala* and white-eyes *Z. virens* are indicated in parentheses. Diagram adapted from Hukkanen et al. (2005) and Gloss et al. (2014). GSH: Glutathione. Nicotine-NAC: Nicotine-N-acetylcysteine.

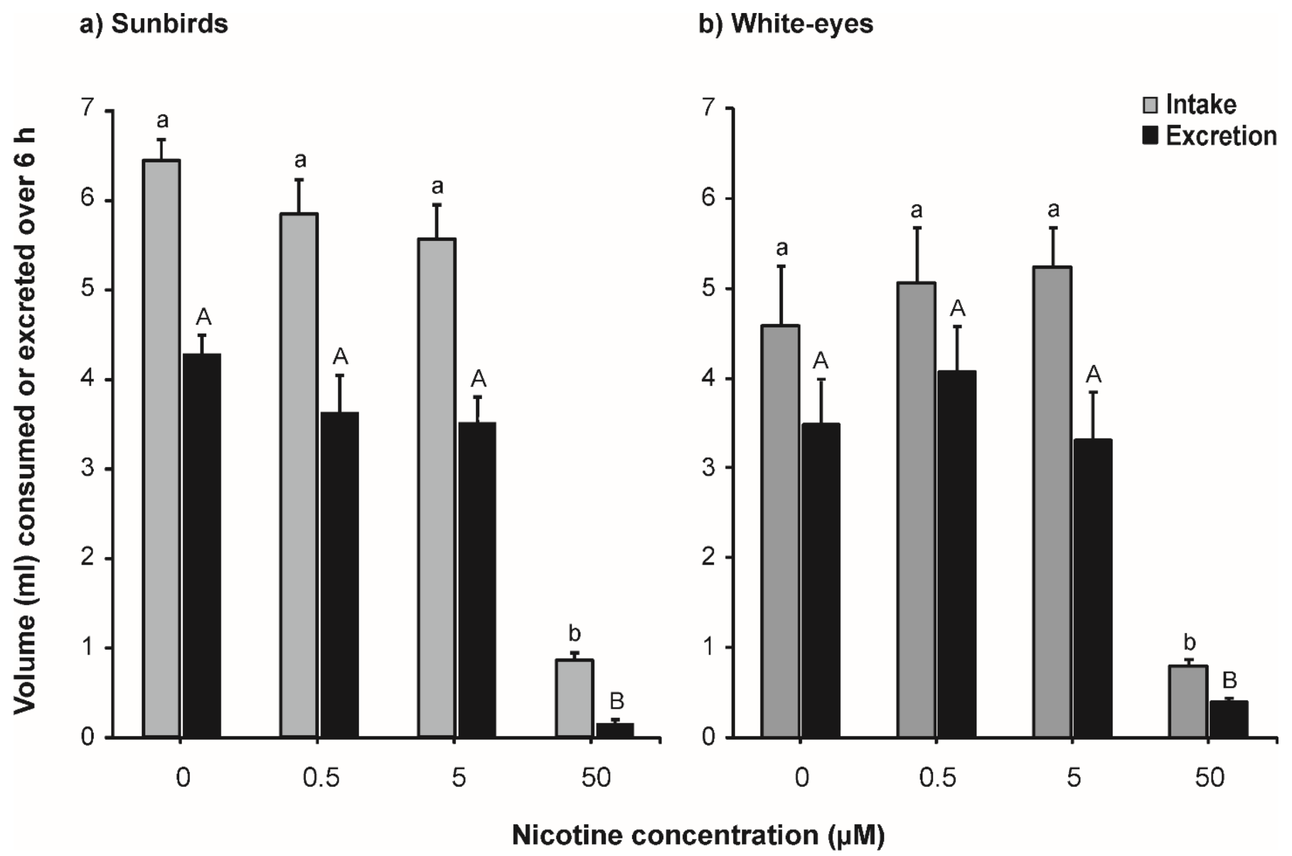

Figure 1.

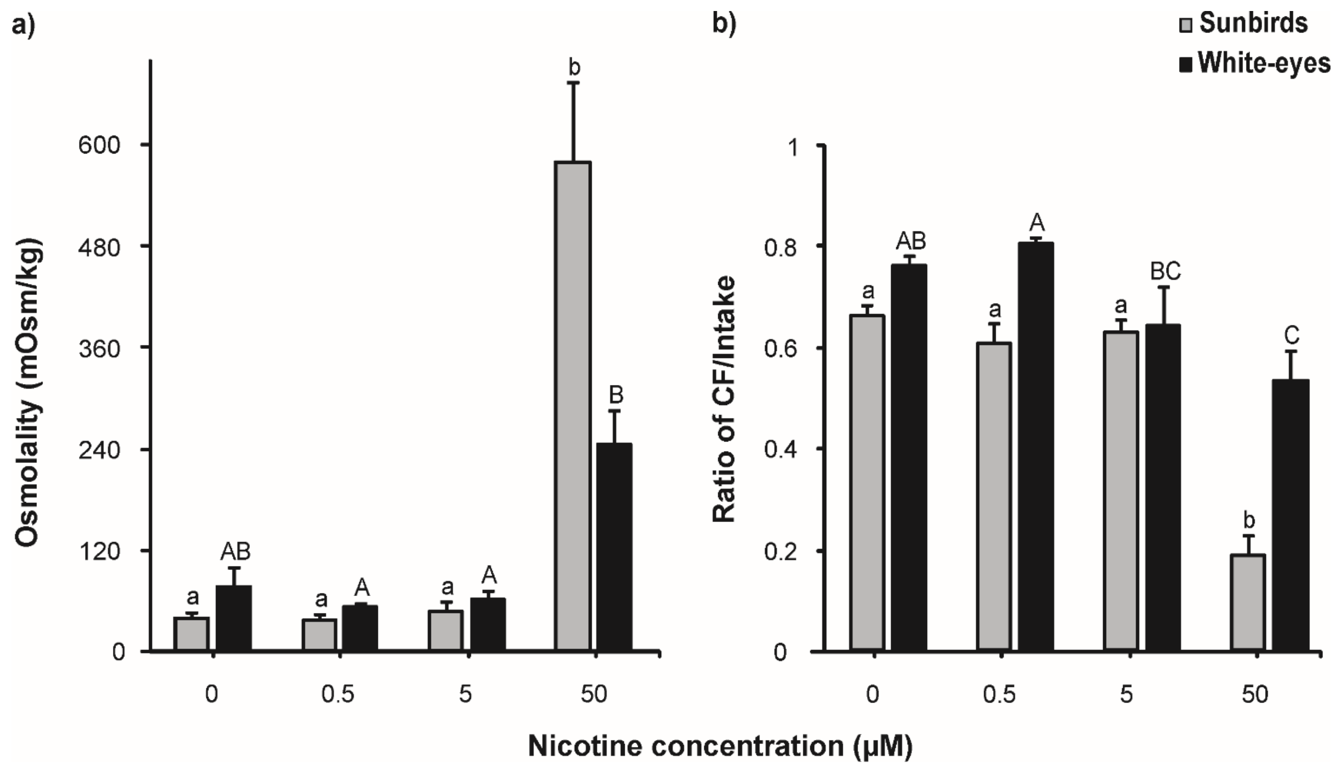

Figure 2.

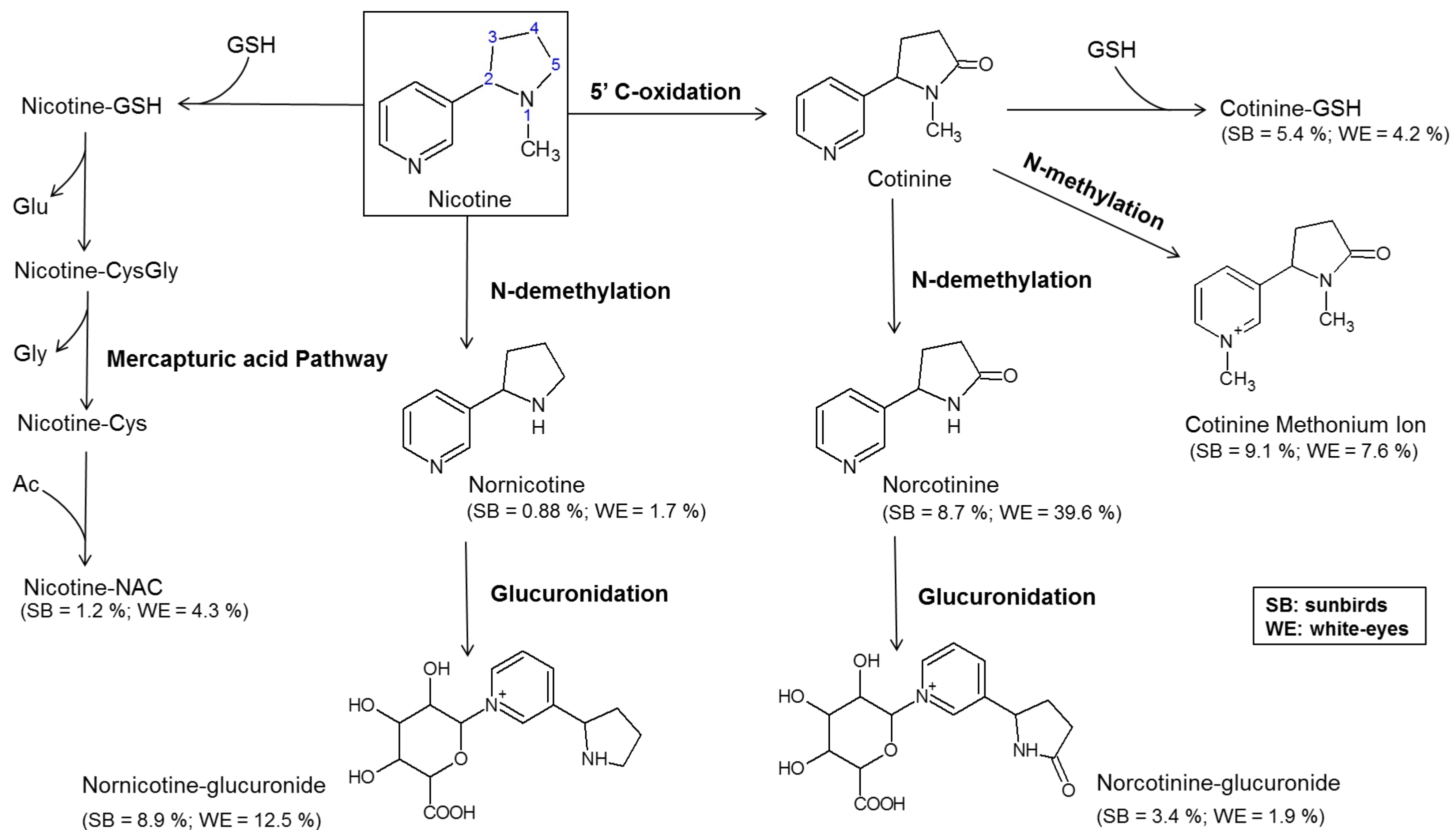

Figure 3.