Honeybees and nectar nicotine: deterrence and reduced survival versus potential health benefits

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Abstract

Secondary metabolites produced by plants for herbivore defence are often found in floral nectar, but their effect on the foraging behaviour and physiological performance of pollinators is largely unknown. Nicotine is highly toxic to most herbivores, and nicotine-based insecticides may contribute to current pollinator declines. We examined the effects of nectar nicotine on honeybee foraging choices and worker longevity. Free-flying honeybee (*Apis mellifera scutellata*) workers from six colonies were given a choice between multiple nicotine concentrations (0–1000 µM) in artificial nectar (0.15–0.63 M sucrose). The dose-dependent deterrent effect of nicotine was stronger in lower sugar concentrations, but even the highest nicotine concentrations did not completely repel honeybees, i.e. bees did not stop feeding on these diets. Nicotine in nectar acts as a partial repellent, which may keep pollinators moving between plants and enhance cross-pollination. In the second part of the study, newly emerged workers from 12 colonies were caged and fed one of four nicotine concentrations (0–300 µM) in 0.63 M sucrose for 21 days. Moderate (≤30 µM) nicotine concentrations had no significant detrimental effect, but high nicotine concentrations reduced the survival of caged workers and their nectar storage in the honey comb. In contrast, worker groups that survived poorly on sugar-only diets demonstrated increased survival on all nicotine diets. In the absence of alternative nectar sources, honeybees tolerate naturally occurring nectar nicotine concentrations; and low concentrations can even be beneficial to honeybees. However, high nicotine concentrations may have a detrimental effect on colony fitness.

Key-words: secondary metabolite, sucrose concentration, feeding preference, deterrence, nectar storage, *Apis mellifera scutellata*

Introduction

The presence of secondary metabolites (SM) in floral nectar seems paradoxical, considering the reward function of nectar, and little is known of their role in mediating interactions between plants and pollinators. Secondary compounds that have evolved as defences against herbivory may occur in nectar as an inevitable consequence of their presence in vegetative tissues and transport in phloem, but adaptive functions of nectar SM have also been proposed (Adler, 2000). Nectar SM may prevent microbial degradation of nectar (Herrera et al., 2010), act as a filter of flower visitors by deterring nectar robbers (Johnson et al., 2006), and encourage pollinators to move more quickly between flowers, thus possibly enhancing cross-pollination (Adler, 2000; Kessler and Baldwin, 2006).

Most nectar SM studied so far repel pollinators (Adler and Irwin, 2005; Detzel and Wink, 1993; Tadmor-Melamed et al., 2004). The feeding response to nectar SM is dose-dependent: low concentrations of some phenolics and alkaloids are preferred by honeybees to sugar-only controls, while high concentrations inhibit ingestion (Hagler and Buchmann, 1993; Singaravelan et al., 2005). The attractive or deterrent effect of SM may depend not only on the concentration of the compound in question but also on the sugar concentration of the nectar. Deterrence of bumblebees by natural levels of the alkaloid gelsemine in artificial nectar is offset by increasing the sugar concentration from 0.99 to 1.80 M (Gegear et al., 2007), and honeybee responses to phenolics also appear to depend on sugar concentration (Liu et al., 2007). Tests of pollinator responses to varying SM concentration in artificial nectar have commonly used a single sugar

concentration (often 0.63 M sucrose) (Adler and Irwin, 2005; Detzel and Wink, 1993; Hagler and Buchmann, 1993; Singaravelan et al., 2005; Tadmor-Melamed et al., 2004).

Whether SM-containing nectar is acceptable to pollinators also depends on the presence or absence of alternative nectar sources. Dilute or SM-containing nectars become more acceptable to honeybees in the absence of alternatives (London-Shafir et al., 2003; Nicolson and Human, 2008). During winter months, workers of the Asian or Eastern honeybee (*Apis cerana*) may forage heavily on the toxic nectar of *Tripterygium hypoglaucum*, containing the diterpenoid triptolide, or on the phenolic-containing nectar of *Elsholtzia rugulosa* (Liu and Fu, 2004). While nectar SM may be harmless when honeybees can forage on a variety of plants, detrimental effects are observed when the choice of flowering plant species is limited. Post-ingestive effects of nectar SM on honeybees include negative effects on foraging behaviour (Liu and Liu, 2010), interference with social communication (Barron et al., 2009; Gao et al., 2010), and an increase in mortality (Liu and Fu, 2004; Reinhard et al., 2009; Tan et al., 2007). However, the consumption of SM may also be beneficial, as it has been shown to reduce the pathogen and parasitoid load of tobacco hornworms, fall armyworms and bumble bees (Barbosa et al., 1986; Manson et al., 2010).

Several recent studies on nectar SM have involved alkaloids, well known as feeding deterrents due to their bitter taste (Gurevitch et al., 2006). Nicotine is a naturally occurring alkaloid which is widely distributed in the plant kingdom, but best known from the family Solanaceae, which includes many agricultural crops and tobacco (Leete, 1983; Siegmund et al., 1999). Nicotine is highly toxic to most herbivores through its action on acetylcholine receptors,

thus affecting various biological functions (Kleinsasser et al., 2005; Thany and Gauthier, 2005; Yildiz, 2004). Pollinators may encounter nicotine in both nectar and pollen (Detzel and Wink, 1993). Nectar nicotine concentrations of 3 and 31 µM have been measured in *Nicotiana glauca* and *N. attenuata* respectively (Kessler et al., 2010; Tadmor-Melamed et al., 2004). Nicotine in artificial nectar repels pollinating moths and hummingbirds of *N. attenuata* (Kessler and Baldwin, 2006). In an earlier study of honeybees in nucleus hives, nectar nicotine at naturally occurring concentrations did not affect hatching success and larval survival, but higher nicotine concentrations (300 µM) reduced larval survival and the foraging activity of workers (Singaravelan et al., 2006). In addition to its occurrence in plants, nicotine is used as a natural insecticide in organic farming (Casanova et al., 2002; Isman, 2006). Synthetic analogues of nicotine, namely neonicotinoids, are used worldwide as insecticides due to their high affinity for insect nicotinic acetylcholine receptors (Matsuda et al., 2001; Tomizawa and Casida, 2005), and have been suggested as contributing to the observed pollinator declines that are currently of great concern internationally (Maini et al., 2010; vanEngelsdorp and Meixner, 2010).

In the present study, we investigated the feeding response of honeybees to nectar nicotine, and the effect of dietary nicotine on survival of adult workers. Firstly, free-flying honeybees were given a choice between multiple feeders containing different nicotine concentrations in sucrose solution. We hypothesized that honeybee workers would be increasingly deterred by nicotine as nicotine concentration increased and sugar concentration decreased. Secondly, caged adult workers were fed different nicotine concentrations in sucrose solution for 21 days, and food uptake and survival were recorded. We predicted that honeybees

would not be affected by low and moderate nicotine concentrations (≤30 µM), while higher nicotine concentrations were expected to reduce survival.

Materials and methods

Nicotine preference test with free-flying honeybees

Honeybee (*Apis mellifera scutellata*) colonies used in the experiments were wild caught colonies situated at the University of Pretoria Experimental Farm; queens were naturally mated and no disease treatments were applied. Six colonies were trained to feed from gravity feeders (250 ml plastic jars inverted over Petri dishes), which allowed >50 workers to feed simultaneously. Experiments were carried out in late summer (February and March 2010), on sunny days with ambient temperatures above 25˚C. Each colony (placed >2 m apart) was presented with eight randomly positioned feeders placed in a circle, and feeder positions were switched every 15 min. Feeders were positioned 1 m from the hive entrance and all colonies were tested simultaneously to prevent honeybees from visiting feeders of other colonies. During preliminary trials it was observed that workers defended their nectar supply when it was placed this close to the hive entrance, chasing away visiting bees from neighbouring hives. After each hive in close proximity received its own feeding station, workers exiting the hives were seen to land on the feeding platform closest to the entrance, and no in-flight competition between workers from different hives was noted. On each feeding station, one feeder contained water; the others sucrose solutions with nicotine ((–)-nicotine, Ref. N3876, Sigma Aldrich, Munich, Germany) at concentrations of 0, 3, 6, 15, 30, 60 and 300 µM. Three sucrose concentrations (0.15, 0.32 and

0.63 M) were tested on separate days, and all feeders were weighed before and after each 2 h test period from 10h00–12h00 (Scout Pro™ SPU402, 0.01 g, Ohaus Cooperation, Pine Brook, NJ, USA). Small reductions in the mass of water feeders were caused by evaporation (honeybees were absent from these feeders), and uptake from the remaining feeders was corrected accordingly. Because workers were not fully deterred by the highest nicotine concentration in 0.63 M sucrose, this trial was repeated with three additional concentrations (150, 500 and 1000 µM), giving a choice between 11 feeders.

Effect of nicotine on sucrose and water uptake and survival of caged workers

One frame with capped worker brood was removed from each of 12 colonies at the University of Pretoria Experimental Farm. Experiments were carried out in winter and spring (June until October 2010). Frames were placed in an incubator and newly emerged workers were collected daily (a total of 400 workers per frame). Four hoarding cages with 100 freshly emerged (<24 h) honeybees each were prepared for each colony. The cages consisted of a wooden frame (11×8.5×7 cm) with a wire mesh bottom for ventilation and glass slides at the front and back for observation. Each cage contained a piece of honey comb (5×5 cm). Cages were kept in an incubator (34 ± 1˚C, 45% relative humidity (Back, 1956)) in darkness, to mimic conditions within the hive. Plastic feeding vials (15 ml) with a cut feeding hole (1×0.5 cm) were inserted horizontally into the cages, one with water and one with the experimental diet, both provided fresh daily.

The four experimental diets consisted of a 0.63 M sucrose solution without nicotine (control) and with low, moderate and high nicotine concentrations (3, 30 and 300 µM). No

protein was provided, as caged *A. m. scutellata* have recently been shown to survive longest on sugar-only diets (Pirk et al., 2010). The four cages of each colony were randomly assigned to one of the four diets and fed this diet for 21 days (i.e. 1200 workers per experimental diet; total of 4800 workers used in the experiment). Food and water uptake were recorded by weighing the vials (±0.1 mg, Mettler Toledo AG-64, Microsep Ltd, Johannesburg, South Africa) before and after 24 h of feeding time. Simultaneously, storage of the artificial nectar in the comb was quantified on a scale of 1 (no storage) to 5 (full comb), and dead bees were counted and removed from the cages. Food and water uptake were corrected for evaporation, determined by sporadically measuring the sucrose concentration of test diets before and after the 24 h experimental period using a refractometer (Eclipse Optical Hand Held Refractometer 45-81, Bellingham & Stanley Ltd, UK). Sucrose and free water uptake per bee were calculated, thus correcting for decreasing group size over time. Note that sucrose uptake from the feeders was not equivalent to sucrose consumption because the workers stored some of the diet removed from the feeders in the honey comb, and also consumed stored sucrose from the comb.

Data analysis

Data were tested for normality and homogeneity of variance. For the preference test, we used repeated measures (RM) ANOVA to compare food uptake between nicotine concentrations (N=7 or 10) in relation to sucrose concentration. To compare food uptake between sucrose concentrations, slopes of linear regressions between log nicotine concentrations and food uptake (for each colony and each sucrose concentration separately) were compared using RM-ANOVA. Sucrose uptake and nectar storage were compared between nicotine treatments (N=4) and test days (N=21) using RM-ANOVA. Sucrose uptake in the first 10 days (when the number of live

workers was similar in all cages) was compared between the worker groups from the 12 colonies for each treatment separately using one-way ANOVA. *Post-hoc* comparisons were conducted with Tukey's HSD test. Linear regression analysis was used to test for a relationship between supplementary water uptake and nicotine concentration. Kaplan-Meier survival regression analyses were performed to test for differences in survival between nicotine diets, followed by Gehan's Wilcoxon pair-wise comparisons. Level of significance was $\alpha<0.05$; data are presented as means ± SE.

Results

Nicotine preference test with free-flying honeybees

On all three sugar concentrations, food uptake differed significantly between nicotine concentrations ($F_{6,30}\geq15.61$, $P<0.001$), with honeybees decreasing their uptake with increasing nicotine content of the food source ($F_{1,4}\geq7.84$, $P\leq0.05$; $R^2\geq0.66$) (Fig. 1). Food uptake was not significantly different on the control diet and low nicotine concentrations (0–15 µM: $P\geq0.13$). Higher nicotine concentrations repelled honeybees, but higher sugar concentrations prolonged the onset of the adverse effects: deterrence started at 30 µM nicotine on the dilute diet (0.15 M; $P\leq0.02$), but only at 150 µM nicotine on the highest sugar concentration (0.63 M; $P\leq0.01$). Indeed, linear regression slopes (nicotine concentration vs. uptake) differed between sugar concentrations ($F_{2,10}=15.13$, $P<0.001$), being steeper on the dilute diet than on more concentrated diets ($P<0.01$; 0.15 M: slope m= -12.4 ± 1.4, $R^2=0.92$; 0.32 M: m= -6.3 ± 0.7, $R^2=0.89$; 0.63 M: m= -4.9 ± 0.5, $R^2=0.92$). Total food uptake per colony during the 2 h test

period increased with sugar concentration, averaging 154.5 ± 44.2 ml on the 0.15 M diet and 500.1 ± 67.0 ml on the 0.63 M diet. Consequently, nicotine uptake also increased with sugar concentration (0.15 M: 0.63 ± 0.21 mg; 0.63 M: 8.01 ± 1.15 mg).

Effect of nicotine on sucrose and water uptake by caged workers

In this no-choice experiment, dietary nicotine did not significantly affect sucrose uptake from the feeder by caged honeybees ($F_{3,33}$=2.26, P=0.10). On all diets, sucrose uptake per bee increased towards the end of the experiment ($F_{20,220}$=16.61, P<0.001), as the number of individuals per cage decreased (Fig. 2A). Sucrose uptake was compared between the worker groups from the 12 colonies for the first 10 days of the experiment, where >80% of the workers were alive in each treatment (we did not compare uptake during the second half of the experiment as sucrose uptake per bee appeared to depend on the number of individuals per cage; and survival differed between groups – see below). Sucrose uptake did not differ between the 12 worker groups on the control and 3 µM diets ($F_{11,84}$≤1.77, P≥0.07), but differed on the higher nicotine concentrations ($F_{11,84}$≥2.78, P<0.01). Uptake of supplementary water (range 3.5–14.6 mg bee^{-1} day^{-1}) was not related to dietary nicotine concentration ($F_{1,46}$=0.41, P=0.53; R^2=0.01).

Effect of nicotine on nectar storage

Nicotine affected nectar storage by caged workers in the honey combs ($F_{3,33}$=15.23, P<0.001), with storage being lower on the highest nicotine concentration (300 µM) than on control and lower nicotine concentrations (P<0.001; Fig. 2B). The comb was filled to a maximum of two thirds and then workers consumed the stored nectar from Day 6 onwards (which coincides with a decrease in sucrose uptake from the feeder; Fig. 2A). Thus, the amount of nectar stored differed

between days ($F_{20,220}$=14.15, P<0.001). All combs except one on the control diet were empty at the end of the experiment.

Effect of nicotine on worker survival

Survival was generally high, with 59.1 ± 5.1% of workers from nine groups surviving until Day 21 on the control treatment (Fig. 3A). However, the survival of groups originating from three colonies dropped below 50% before the last third of the experiment, and only 7.7% were alive at the end of the experiment (Fig. 3B). Cages from the different colonies are hereafter referred to as "strong" groups (N=9) and "weak" groups (N=3). Interestingly, dietary nicotine had a different effect on strong than on weak groups, and groups were therefore analyzed separately. In strong groups, survival was similar on control and low and moderate nicotine concentrations (Z≤-1.36, P≥0.17), but was significantly reduced on the highest nicotine concentration, compared to all other treatments (Z≥5.41, P≤0.001; Fig. 3A). In contrast to this, dietary nicotine drastically improved the survival of weak groups, with workers on all three nicotine concentrations surviving 56.6 ± 8.5% longer than their sugar-only diet counterparts (Z≥7.35, P≤0.001; Fig. 3B). Survival was higher on the low than on the moderate and high nicotine concentrations (Z≥-3.15, P≤0.01).

Discussion

Using preference tests on free-flying honeybees, we have shown that nectar nicotine is deterrent in high concentrations, but the workers are more tolerant of this alkaloid when the sugar

concentration is higher. Under no-choice conditions in the laboratory, adult workers tolerate naturally occurring nectar nicotine concentrations, but nectar storage in the honey comb and worker survival decrease on high dietary nicotine. Interestingly, weak worker groups that survive poorly on sugar-only diets demonstrate increased survival on nicotine diets. Below we discuss the effects of this secondary metabolite in nectar on the foraging choices and survival of this major pollinator, and possible implications for plant-pollinator interactions.

Feeding response to nectar nicotine

Honeybees decreased their uptake of artificial nectar as its nicotine concentration increased, indicating that they were deterred by the presence of nicotine. Nicotine gives nectar a bitter taste, but honeybees have been assumed to have poor taste perception, as they possess only ten gustatory receptors, compared to more than 60 gustatory receptors identified in other insects (Robertson and Wanner, 2006). Accumulating evidence, however, suggests that honeybees are indeed able to detect secondary metabolites (Liu et al., 2007; Liu et al., 2004; Singaravelan et al., 2005; Wright et al., 2010), and their taste perception is likely to be more complex than assumed from the number of gustatory receptors (de Brito Sanchez et al., 2007). While electrophysiological recordings did not detect antennal receptor cells for bitter substances (de Brito Sanchez et al., 2005), Wright et al. (2010) have recently shown that gustatory receptors on the proboscis do respond to such substances. Honeybees are less likely to drink sucrose-quinine solutions presented to the proboscis as the toxin concentration increases (Wright et al., 2010). Quinine, when added to a sucrose solution, was also found to inhibit the response of sugar receptors, indicating that bitter substances may interfere with sweetness perception (de Brito Sanchez et al., 2005).

Honeybees were remarkably tolerant to nectar nicotine, ingesting relatively large quantities of sugar solutions containing nicotine at much higher concentrations than those recorded in nectar to date (Kessler et al., 2010; Tadmor-Melamed et al., 2004). Nicotine, when injected in low concentrations (10 µM) into the antennal lobes of honeybees, did not interfere with olfactory learning, and even improved short-term memory (Thany and Gauthier, 2005). Injections of 100 and 1000 µM nicotine increased sucrose sensitivity within minutes after administration (Thany and Gauthier, 2005). Increased sucrose sensitivity may counterbalance possible effects of bitterness on sweetness perception, and may have caused the ingestion of fairly high nicotine concentrations in our study. It is, however, not known how quickly nicotine affects the honeybee brain after oral ingestion.

A rapid response to plant SM does not necessarily mean that the response is purely taste-mediated. When *Manduca sexta* caterpillars were fed a nicotine-containing diet, they initially fed rapidly but stopped feeding after 24–30 s (Glendinning, 1996). The authors concluded that this was a post-ingestive response, as nicotine failed to stimulate the caterpillars' gustatory receptors, and taste-mediated aversive responses would have been faster in this species (Glendinning, 1996). In honeybees, in addition to gustatory responses to toxins, a post-ingestive mechanism involving serotonin has been identified as playing a role in conditioned food aversions (Wright et al., 2010). It is therefore possible that honeybees may have used post-ingestive mechanisms to detect the nectar nicotine in our study. We used naïve workers and there was no indication that the response to nicotine depended on the sequence of tests. Longer-term nicotine effects, both positive and negative, are therefore unlikely, as bees would have either increased their nicotine

intake over time (if it was in any way beneficial or addictive after this short-term exposure), or would have been increasingly deterred (if nicotine had caused toxicity symptoms).

The acceptability of nicotine depended on the sugar concentration of the artificial nectar, with honeybees being more tolerant of nicotine when the sugar concentration was higher. The dose-response curve shifted to the left and became steeper with decreasing sugar concentration. Sweetness may likewise mask the bitter taste of alkaloids in caterpillars, in which simple carbohydrates such as *myo*-inositol can interfere with the response to caffeine (Glendinning, 2002), and the deterrent effect of a given quinine concentration on feeding in blowflies depends on the sucrose concentration in the mixture (Moss and Dethier, 1983). We observed an increase in uptake of concentrated sugar diets, which is expected for honeybees as these diets are more profitable (Roubik and Buchmann, 1984).

Even very high concentrations of nicotine in artificial nectars did not completely repel honeybees. This is a common finding in studies of the response of pollinators to nectar SM (Singaravelan et al., 2005; Tadmor-Melamed et al., 2004; Tan et al., 2007); and Singaravelan and colleagues (2005) found that low concentrations of nicotine and caffeine elicited a significant feeding preference in honeybees. Partial repellence by SM has implications for plant fitness: moth and hummingbird pollinators removed more nectar from nicotine-silenced *N. attenuata* plants than from control plants with nicotine-containing nectar (Kessler and Baldwin, 2006). Thus, nectar SM maximize the number of flower visits per unit nectar produced and keep pollinators moving between flowers, which may enhance outcrossing (Kessler and Baldwin, 2006).

Effect of nectar nicotine on honeybee survival

We were interested in the effect of longer-term exposure to dietary nicotine on the longevity of honeybees. Similar amounts of the artificial nectar were consumed on all treatments, indicating that the sucrose intake is defended irrespective of nectar nicotine concentration. Although honeybees were provided with water *ad libitum*, there was no indication that workers attempted to dilute the nicotine by drinking more water on the nicotine diets.

Differences in the quantity of artificial nectar stored in the honey comb were observed between treatments. While similar amounts were stored on the control and lower nicotine concentrations, storage was reduced on the highest nicotine concentration (300 µM), suggesting that high SM concentrations and a lack of alternative nectar sources may reduce honey production (see also Liu et al., 2004). Caged honeybees consumed the stored nectar after a few days, which coincided with increased activity (A. Köhler, pers. observation) and the development of endothermic ability (Stabentheiner et al., 2010). The evaporation of nectar in the cells led to a more concentrated sugar solution, which might have resulted in workers preferring it over the more dilute nectar provided *ad libitum* in the feeder. The preference for concentrated nectar might have been a result of the active heating tasks performed by workers, since these workers normally get “refilled” from the honey store (Basile et al., 2008). In addition, the consumption of smaller volumes of concentrated nectar reduces the amount of water that workers have to evaporate (workers do not defecate inside the hive).

Nicotine in sucrose solution is toxic to adult honeybees, tested 48 h after the start of oral exposure, at an LD_{50} concentration of 12 mM (Detzel and Wink, 1993). This is a much higher

concentration than honeybees would encounter under natural conditions. In our study, naturally occurring nicotine concentrations (31 µM in nectar of *N. attenuata*; Kessler et al., 2010; Tadmor-Melamed et al., 2004) did not affect the survival of caged workers from the strong groups (61% survived until Day 21), but mortality increased on the 300 µM nicotine treatment (47% alive on Day 21; Fig. 3A). This contradicts previous findings on workers that showed no increase in mortality after receiving the same nicotine concentration for 15 days (Singaravelan et al., 2006). The increased mortality on the highest nicotine concentration may have been caused by an interference of the alkaloid with food utilization. Dietary nicotine has been shown to reduce the efficiency of food conversion in tobacco hornworms (Bentz and Barbosa, 1990).

Surprisingly, 25% of the groups tested in our study showed lower survival in captivity, with less than 8% of the bees surviving until Day 21 on the sugar-only diet. The sucrose uptake on the control diet did not differ between weak and strong groups, so the high mortality was not caused by a deficit in sugar intake. *Varroa* mites, known to transmit various viral diseases (Tentcheva et al., 2004), were found in the test colonies. Workers from the three weak groups could have been infected with a viral disease transmitted by *Varroa*, or could have been weakened from excessive feeding of the mites during early honeybee development, but no noticeable anomalies, e.g. deformed wings (de Miranda and Genersch, 2010), were observed in freshly emerged workers. *Varroa* mites have also been shown to suppress honeybee immunity (Yang and Cox-Foster, 2005), which could have affected longevity in our experiment. Brood samples of the three colonies that produced weak groups showed characteristics of a bacterial brood disease, possibly European foulbrood, which is widespread in South Africa (Human and Pirk, 2010); whereas brood of the remaining colonies appeared healthy (the inspection was done

double-blind). We extracted the DNA of honeybee workers from our experiment (NucleoSpin® Tissue kit, Machery-Nagel GmbH & Co. KG, Düren, Germany) and tested for *Nosema apis* (a gut pathogen) and *Melissococcus plutonius* (the causal agent of European foulbrood) using PCR with primer sequences as in Chen et al. (2008) and Roetschi et al. (2008). Both bacterial diseases had earlier been found in our apiary (U. Strauss, unpublished data), but were not present in workers from the survival experiment and therefore did not cause the lower survival of the weak groups. However, other bacterial or viral diseases or excessive mite infestation could possibly have affected the physical condition of adult workers, thus might have caused the shorter lifespan (Kovac and Crailsheim, 1988).

Interestingly, nicotine increased the survival in these weak colonies. In contrast to the strong colonies, where survival was reduced on the 300 µM nicotine diet, survival of the weak colonies was higher on this highest nicotine concentration compared to the sugar-only control, suggesting that dietary nicotine may provide health benefits. Nicotine causes dose-dependent inhibition of the growth of various bacterial and fungal pathogens (Pavia et al., 2000), and has been shown to kill parasitoids in two caterpillar species (Barbosa et al., 1986). Nicotine also has antiviral effects, as shown for the hepatitis C virus, where the alkaloid inhibits viral replication (Yamashina et al., 2008). In a recent study, another nectar alkaloid, gelsemine, reduced infection by a protozoan pathogen (*Crithidia bombi*) in bumble bees (Manson et al., 2010). If survival of workers from the weak groups were drastically reduced by a pathogen, and dietary nicotine adversely affected this pathogen, then it is possible that the otherwise observed negative effect of ingesting high nicotine concentrations was offset.

Many plant SM have antimicrobial properties (Cowan, 1999), and animals exploit therapeutic SM to mitigate costs of parasitism, infection and other homeostatic challenges (Forbey et al., 2009). Such self-medication has also been demonstrated in various insects (Castella et al., 2008; Lefèvre et al., 2010; Singer et al., 2009); honeybees provide a further example, through their collection and use of propolis, a resin with high anti-pathogen properties (Simone-Finstrom and Spivak, 2010). There was no indication in our study, however, that workers from weak groups ingested more nicotine than those from strong groups. Preference experiments with multiple nicotine concentrations are needed to investigate whether challenged honeybees would actively seek nectar nicotine. Studies on foraging behaviour have often demonstrated how diet selection is influenced by avoidance of plant SM. A possible exploitation of nectar SM for therapeutic purposes would provide a different perspective on the feeding choices of nectarivorous animals, and may further help to explain the role of nectar SM in mediating interactions between plants and their pollinators.

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References

Adler, L.S., 2000. The ecological significance of toxic nectar. Oikos 91, 409-420.

Adler, L.S., Irwin, R.E., 2005. Ecological costs and benefits of defenses in nectar. Ecology 86, 2968-2978.

Back, E., 1956. Einfluss der im Pollen enthaltenen Vitamine auf Lebensdauer, Ausbildung der Pharynxdruesen und Brutfaehigkeit der Honigbiene. Insectes Sociaux 3, 285-292.

Barbosa, P., Saunders, J.A., Kemper, J., Trumbule, R., Olechno, J., Martinat, P., 1986. Plant allochemicals and insect parasitoids - effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae). Journal of Chemical Ecology 12, 1319-1328.

Barron, A.B., Maleszka, R., Helliwell, P.G., Robinson, G.E., 2009. Effects of cocaine on honey bee dance behaviour. Journal of Experimental Biology 212, 163-168.

Basile, R., Pirk, C.W.W., Tautz, J., 2008. Trophallactic activities in the honeybee brood nest - Heaters get supplied with high performance fuel. Zoology 111, 433-441.

Bentz, J.-A., Barbosa, P., 1990. Effects of dietary nicotine (0.1%) and parasitism by *Cotesia congregata* on the growth and food consumption and utilization of the tobacco hornworm, *Manduca sexta*. Entomologia Experimentalis et Applicata 57, 1-8.

Casanova, H., Ortiz, C., Peláez, C., Vallejo, A., Moreno, M.E., Acevedo, M., 2002. Insecticide formulations based on nicotine oleate stabilized by sodium caseinate. Journal of Agricultural and Food Chemistry 50, 6389-6394.

Castella, G., Chapuisat, M., Christe, P., 2008. Prophylaxis with resin in wood ants. Animal Behaviour 75, 1591-1596.

Chen, Y.P., Evans, J.D., Smith, I.B., Pettis, J.S., 2008. *Nosema ceranae* is a long-present and wide-spread microsporidean infection of the European honey bee (*Apis mellifera*) in the United States. Journal of Invertebrate Pathology 97, 186-188.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews 12, 564-582.

de Brito Sanchez, M.G., Giurfa, M., de Paula Mota, T.R., Gauthier, M., 2005. Electrophysiological and behavioural characterization of gustatory responses to antennal 'bitter' taste in honeybees. European Journal of Neuroscience 22, 3161-3170.

de Brito Sanchez, M.G., Ortigao-Farias, J.R., Gauthier, M., Liu, F., Giurfa, M., 2007. Taste perception in honeybees: just a taste of honey? Arthropod-Plant Interactions 1, 69-76.

de Miranda, J.R., Genersch, E., 2010. Deformed wing virus. Journal of Invertebrate Pathology 103, S48-S61.

Detzel, A., Wink, M., 1993. Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allochemicals. Chemoecology 4, 8-18.

Forbey, J.S., Harvey, A.L., Huffman, M.A., Provenza, F.D., Sullivan, R., Tasdemir, D., 2009. Exploitation of secondary metabolites by animals: a response to homeostatic challenges. Integrative and Comparative Biology 49, 314-328.

Gao, J., Zhao, G., Yu, Y., Liu, F., 2010. High concentration of nectar quercetin enhances worker resistance to queen's signals in bees. Journal of Chemical Ecology 36, 1241-1243.

Gegear, R.J., Manson, J.S., Thomson, J.D., 2007. Ecological context influences pollinator deterrence by alkaloids in floral nectar. Ecology Letters 10, 375-382.

Glendinning, J.I., 1996. Is chemosensory input essential for the rapid rejection of toxic foods? Journal of Experimental Biology 199, 1523-1534.

Glendinning, J.I., 2002. How do herbivorous insects cope with noxious secondary plant compounds in their diet? Entomologia Experimentalis et Applicata 104, 15-25.

Gurevitch, J., Scheiner, S.M., Fox, G.A., 2006. Chapter 11: Herbivory and plant-pathogen interactions, The ecology of plants, 2 ed. Sinauer Associates, Sunderland, Massachusetts, USA, pp. 213-228.

Hagler, J.R., Buchmann, S.L., 1993. Honey bee (Hymenoptera: Apidae) foraging responses to phenolic-rich nectars. Journal of the Kansas Entomological Society 66, 223-230.

Herrera, C.M., Canto, A., Pozo, M.I., Bazaga, P., 2010. Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities. Proceedings of the Royal Society B 277, 747-754.

Human, H., Pirk, C.W.W., 2010. Honey bee diseases, Part 1. Southern African Honey Council (SAHC) Newsletter 2, 12-15.

Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annual Review of Entomology 51, 45-66.

Johnson, S.D., Hargreaves, A.L., Brown, M., 2006. Dark, bitter-tasting nectar functions as a filter of flower visitors in a bird-pollinated plant. Ecology 87, 2709-2716.

Kessler, D., Baldwin, I.T., 2006. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. Plant Journal 49, 840-854.

Kessler, D., Diezel, C., Baldwin, I.T., 2010. Changing pollinators as a means of escaping herbivores. Current Biology 20, 237-242.

Kleinsasser, N.H., Sassen, A.W., Semmler, M.P., Harréus, U.A., Licht, A.-K., Richter, E., 2005. The tobacco alkaloid nicotine demonstrates genotoxicity in human tonsillar tissue and lymphocytes. Toxicological Sciences 86, 309-317.

Kovac, H., Crailsheim, K., 1988. Lifespan of *Apis mellifera carnica* Pollm. infested by *Varroa jacobsoni* Oud. in relation to season and extent of infestation. Journal of Apicultural Research 27, 230-238.

Leete, E., 1983. Biosynthesis and metabolism of the tobacco alkaloids, in: Pelletier, S.W. (Ed.), Alkaloids - Chemical and biological perspectives. John Wiley and Sons, New York, pp. 86-139.

Lefèvre, T., Oliver, L., Hunter, M.D., de Roode, J.C., 2010. Evidence for trans-generational medication in nature. Ecology Letters 13, 1485-1493.

Liu, F., Chen, J., Chai, J., Zhang, X., Bai, X., He, D., Roubik, D.W., 2007. Adaptive functions of defensive plant phenolics and a non-linear bee response to nectar components. Functional Ecology 21, 96-100.

Liu, F., Fu, W., 2004. Plant with toxic nectar kills native honey bees in SW China. American Bee Journal 144, 707-708.

Liu, F.L., Fu, W.J., Yang, D.R., Peng, Y.Q., Zhang, X.W., He, J.Z., 2004. Reinforcement of bee-plant interaction by phenolics in food. Journal of Apicultural Research 43, 155-157.

Liu, Y., Liu, F., 2010. Post-ingestive effect of plant phenolics on the feeding behaviour of the honeybee *Apis cerana*. Physiological Entomology 35, 175-178.

London-Shafir, I., Shafir, S., Eisikowitch, D., 2003. Amygdalin in almond nectar and pollen - facts and possible roles. Plant Systematics and Evolution 238, 87-95.

Maini, S., Medrzycki, P., Porrini, C., 2010. The puzzle of honey bee losses: a brief review. Bulletin of Insectology 63, 153-160.

Manson, J.S., Otterstatter, M.C., Thomson, J.D., 2010. Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162, 81-89.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M., Sattelle, D.B., 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. TRENDS in Pharmacological Sciences 22, 573-580.

Moss, C.F., Dethier, V.G., 1983. Central nervous system regulation of finicky feeding by the blowfly. Behavioral Neuroscience 97, 541-548.

Nicolson, S.W., Human, H., 2008. Bees get a head start on honey production. Biology Letters 4, 299-301.

Pavia, C.S., Pierre, A., Nowakowski, J., 2000. Antimicrobial activity of nicotine against a spectrum of bacterial and fungal pathogens. Journal of Medical Microbiology 49, 674-675.

Pirk, C.W.W., Boodhoo, C., Human, H., Nicolson, S.W., 2010. The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). Apidologie 41, 62-72.

Reinhard, A., Janke, M., von der Ohe, W., Kempf, M., Theuring, C., Hartmann, T., Schreier, P., Beuerle, T., 2009. Feeding deterrence and detrimental effects of pyrrolizidine alkaloids fed to honey bees (*Apis mellifera*). Journal of Chemical Ecology 35, 1086-1095.

Robertson, H.M., Wanner, K.W., 2006. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: Expansion of the odorant, but not gustatory, receptor family. Genome Research 16, 1395-1403.

Roetschi, A., Berthoud, H., Kuhn, R., Imdorf, A., 2008. Infection rate based on quantitative real-time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honeybee colonies before and after apiary sanitation Apidologie 39, 362-371.

Roubik, D.W., Buchmann, S.L., 1984. Nectar selection by *Melipona* and *Apis mellifera* (Hymenoptera: Apidae) and the ecology of nectar intake by bee colonies in a tropical forest. Oecologia 61, 1-10.

Siegmund, B., Leitner, E., Pfannhauser, W., 1999. Determination of the nicotine content of various edible nightshades (Solanaceae) and their products and estimation of the associated dietary nicotine intake. Journal of Agriculture and Food Chemistry 47, 3113-3120.

Simone-Finstrom, M., Spivak, M., 2010. Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie 41, 295-311.

Singaravelan, N., Inbar, M., Ne'eman, G., Distl, M., Wink, M., Izhaki, I., 2006. The effects of nectar-nicotine on colony fitness of caged honeybees. Journal of Chemical Ecology 32, 49-58.

Singaravelan, N., Ne'eman, G., Inbar, M., Izhaki, I., 2005. Feeding responses of free-flying honeybees to secondary compounds mimicking floral nectars. Journal of Chemical Ecology 31, 2791-2804.

Singer, M.S., Mace, K.C., Bernays, E.A., 2009. Self-medication as adaptive plasticity: increased ingestion of plant toxins by parasitized caterpillars. PLoS ONE 4, e4796.

Stabentheiner, A., Kovac, H., Brodschneider, R., 2010. Honeybee colony thermoregulation – regulatory mechanisms and contribution of individuals in dependence on age, location and thermal stress. PLoS ONE 5, e8967.

Tadmor-Melamed, H., Markman, S., Arieli, A., Distl, M., Wink, M., Izhaki, I., 2004. Limited ability of Palestine Sunbirds *Nectarinia osea* to cope with pyridine alkaloids in nectar of Tree Tobacco *Nicotiana glauca*. Functional Ecology 18, 844-850.

Tan, K., Guo, Y.H., Nicolson, S.W., Radloff, S.E., Song, Q.S., Hepburn, H.R., 2007. Honeybee (*Apis cerana*) foraging responses to the toxic honey of *Tripterygium hypoglaucum* (Celastraceae): changing threshold of nectar acceptability. Journal of Chemical Ecology 33, 2209-2217.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M., 2004. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Applied and Environmental Microbiology 70, 7185-7191.

Thany, S.H., Gauthier, M., 2005. Nicotine injected into the antennal lobes induces a rapid modulation of sucrose threshold and improves short-term memory in the honeybee. Brain Research 1039, 216-219.

Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: Mechanisms of selective action. Annual Review of Pharmacology and Toxicology 45, 247-268.

vanEngelsdorp, D., Meixner, M.D., 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103, S80-S95.

Wright, G.A., Mustard, J.A., Simcock, N.K., Ross-Taylor, A.A.R., McNicholas, L.D., Popescu, A., Marion-Poll, F., 2010. Parallel reinforcement pathways for conditioned food aversions in the honeybee. Current Biology 20, 2234-2240.

Yamashina, S., Mizui, T., Kon, K., Ikejima, K., Kitamura, T., Takei, Y., Watanabe, S., 2008. Effect of nicotine on innate antiviral pathways and HCV replication. Gastroenterology 134, A786-A787.

Yang, X., Cox-Foster, D.L., 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Sciences 102, 7470-7475.

Yildiz, D., 2004. Nicotine, its metabolism and an overview of its biological effects. Toxicon 43, 619-632.

Figure legends

Figure 1. Feeding preferences with nicotine-containing nectar. Uptake of artificial nectar (0.15–0.63 M sucrose) differing in nicotine concentration (0–1000 µM) by six honeybee (*Apis mellifera scutellata*) colonies (mean ± SE; SE partly omitted for clarity). Nicotine concentrations are plotted as log(x+1). Note the shift and change in slope of the dose-response curves with increasing sugar concentration. Different letters indicate significant differences between data series.

Figure 2. Sucrose uptake and nectar storage on nicotine diets. Sucrose uptake from the feeder (A) and artificial nectar storage in the honey comb (B) by caged workers (*A. m. scutellata*) on 0.63 M sucrose solutions without nicotine (control) and with three nicotine concentrations (N=12 groups from 12 colonies; mean ± SE; SE partly omitted for clarity). Sucrose uptake from the feeder was similar on all diets, but differed between test days. Nectar storage was quantified daily on a scale of 1 (no storage) to 5 (full comb). Nectar was stored during the first days of the experiment, but then consumed as the bees became more active. Different letters indicate significant differences between data series.

Figure 3. Survival on nicotine diets. Cumulative survival of honeybees (*A. m. scutellata*) on 0.63 M sucrose solutions without nicotine (control) and with three nicotine concentrations. (A) Survival of strong groups (N=9; mean ± SE; SE partly omitted for clarity) was significantly reduced on the highest nicotine concentration. (B) Survival of weak groups (N=3; mean) was

significantly improved by dietary nicotine. Different letters indicate significant differences between data series.

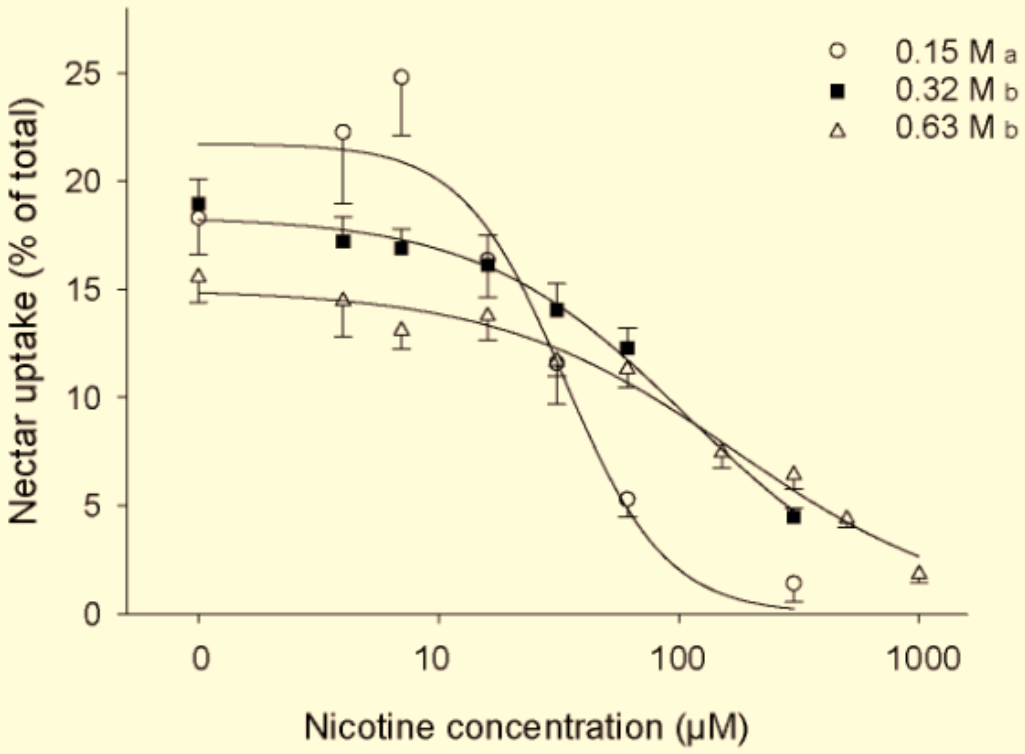
0.15 M a
0.32 M b
0.63 M b
Nectar uptake (% of total)
25
20
15
10
5
0
0
10
100
1000
Nicotine concentration (µM)

Figure 1.

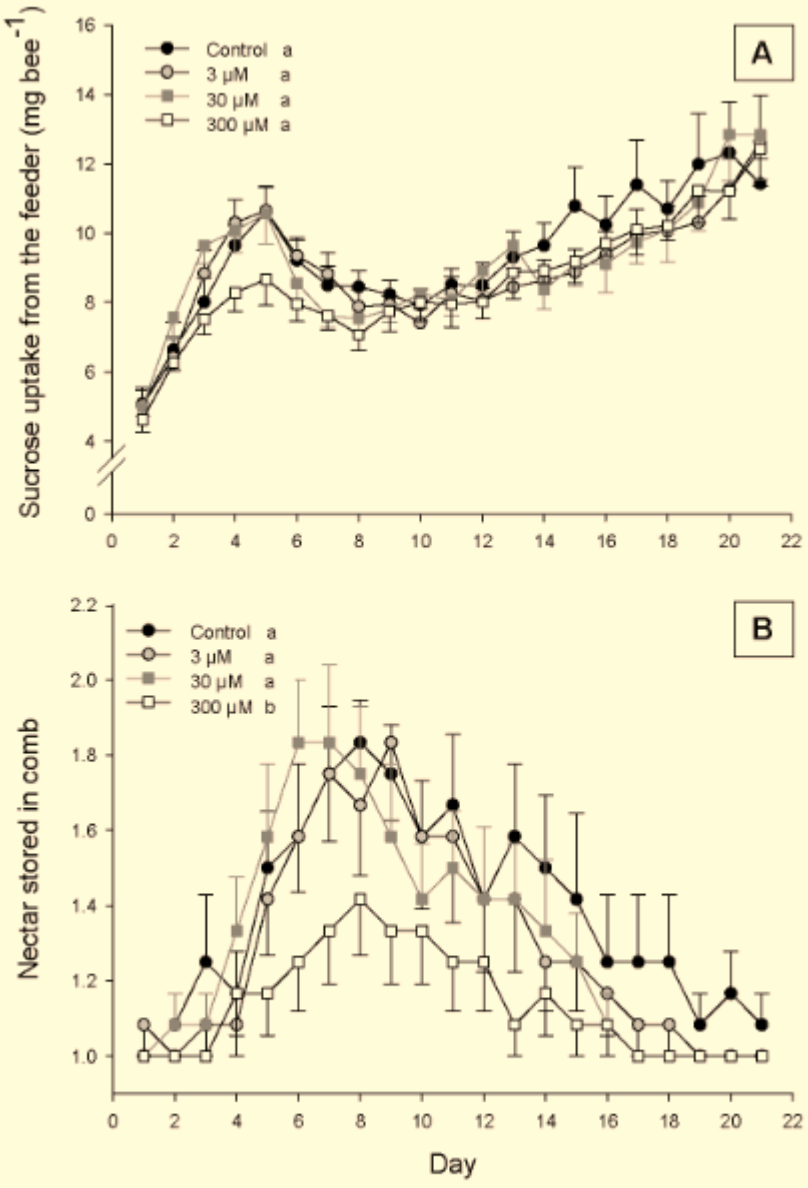
A
Control a
3 µM a
30 µM a
300 µM a
Sucrose uptake from the feeder (mg bee-1)
B
Control a
3 µM a
30 µM a
300 µM b
Nectar stored in comb
Day

Figure 2.

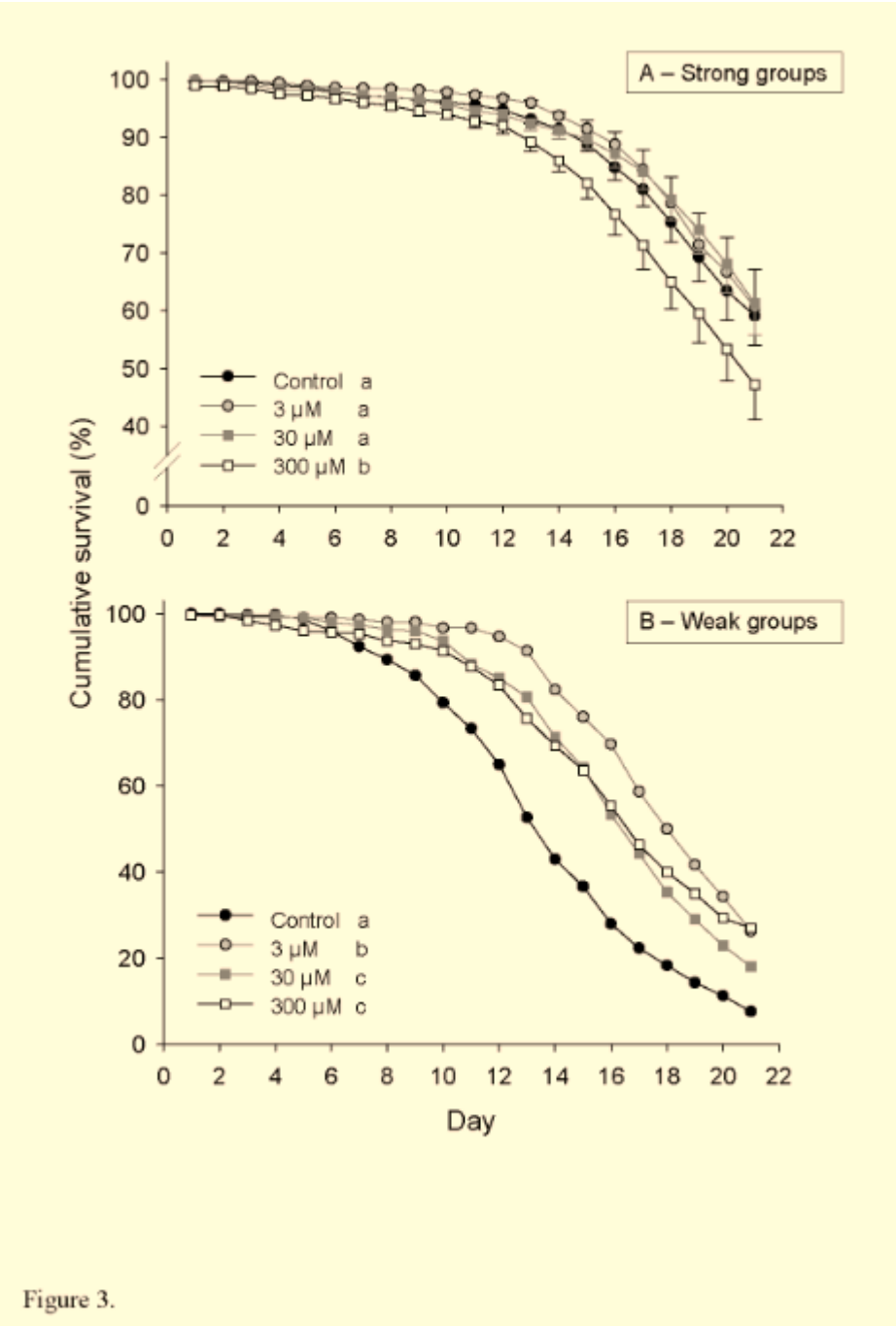
A – Strong groups
Control a
3 µM a
30 µM a
300 µM b
B – Weak groups
Control a
3 µM b
30 µM c
300 µM c
Cumulative survival (%)
Day
0
20
40
60
80
90
100
0 2 4 6 8 10 12 14 16 18 20 22

Figure 3.